

Investigation of the underlying physiological mechanisms regulating the vernal transition in
mares

by

Chelsea Drumwright Sinclair

B.S., Clemson University, 2013
M.S., Kansas State University, 2016

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Abstract

The ovarian and endocrine characteristics of mares related to seasonal changes in reproduction are well-documented. Further elucidation of the physiological mechanisms driving these seasonal changes is needed. Two experiments were conducted in an attempt to further our understanding of seasonality in mares. In other seasonal species, such as sheep and goats, simply exposing anestrus females to sexually active males increases gonadotropin secretion within minutes, followed by ovulation 2 to 8 d later. This “male effect” was investigated in anestrus mares. In late February, mares were stratified by parity and age and assigned randomly to 1 of 3 treatments (n=7 per treatment): (1) DXP (direct exposure; mares housed adjacent to stallion and allowed direct contact for 2 h on 3 d/wk by placing the stallion into a cage in the center of a tease pen); (2) IXP (indirect exposure; mares housed adjacent to stallion but not allowed direct contact); and (3) CON (control; mares housed away from stallion and not allowed direct or indirect exposure). Ovarian activity was monitored thrice weekly until ovulation. During the first, third, and fifth days of treatment, blood was collected hourly for 8 h beginning just before treatment initiation for later analysis of LH and PRL. Interval from treatment initiation to ovulation did not differ ($P > 0.05$) among treatments. No effect ($P > 0.05$) of treatment was detected for LH or PRL concentrations, but a significant effect of day ($P < 0.05$) was observed with LH concentrations being greater on the fifth exposure day compared with the first and third.

Endogenous opioid peptides have been shown to be involved in seasonal regulation in mares, both at the hypophysis and ovarian levels. The objective of this second study was to investigate the effects of an opioid antagonist, both alone and in combination with a GnRH agonist, on gonadotrophic hormone concentrations in transitional mares exposed to various progesterone environments. Seventeen stock-type, transitional mares were treated orally with

altrenogest for 15 days. Mares were stratified by age and parity and assigned randomly to 3 treatments: (1) NAL (0.5 mg/kg BW naloxone hydrochloride IV, $n = 5$); (2) DES (1.8 mg deslorelin acetate IM, $n = 6$); or (3) N&D (naloxone hydrochloride followed by deslorelin acetate after 45 min, $n = 6$). Assigned treatment was administered 3 times during the study: (1) phase 1 (P1) occurred on the last day of altrenogest; (2) phase 2 (P2) occurred upon detection of a follicle ≥ 35 mm; and (3) phase 3 (P3) occurred 7 d post-ovulation. Interval from P1 to P2 was shorter ($P < 0.05$) for N&D compared with NAL, but not for any other treatment combinations. Interval from P2 to ovulation was not different ($P > 0.05$). Significant effects of phase and time were identified, with treatment initiating a greater ($P < 0.05$) LH response during P3 compared with P1 and P2. The FSH response to treatment was greater ($P < 0.05$) during P1 and P3 compared with P2. Regardless of phase, deslorelin increased both gonadotropins whereas naloxone alone only resulted in a biologically significant increase in gonadotropins during P3.

Investigation of the underlying physiological mechanisms regulating the vernal transition in
mares

by

Chelsea Drumwright Sinclair

B.S., Clemson University, 2013
M.S., Kansas State University, 2016

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Approved by:

Major Professor
Dr. Joann Kouba

Approved by:

Co-Major Professor
Dr. Jeffrey Stevenson

Copyright

© Chelsea Sinclair 2019.

Abstract

The ovarian and endocrine characteristics of mares related to seasonal changes in reproduction are well-documented. Further elucidation of the physiological mechanisms driving these seasonal changes is needed. Two experiments were conducted in an attempt to further our understanding of seasonality in mares. In other seasonal species, such as sheep and goats, simply exposing anestrus females to sexually active males increases gonadotropin secretion within minutes, followed by ovulation 2 to 8 d later. This “male effect” was investigated in anestrus mares. In late February, mares were stratified by parity and age and assigned randomly to 1 of 3 treatments (n=7 per treatment): (1) DXP (direct exposure; mares housed adjacent to stallion and allowed direct contact for 2 h on 3 d/wk by placing the stallion into a cage in the center of a tease pen); (2) IXP (indirect exposure; mares housed adjacent to stallion but not allowed direct contact); and (3) CON (control; mares housed away from stallion and not allowed direct or indirect exposure). Ovarian activity was monitored thrice weekly until ovulation. During the first, third, and fifth days of treatment, blood was collected hourly for 8 h beginning just before treatment initiation for later analysis of LH and PRL. Interval from treatment initiation to ovulation did not differ ($P > 0.05$) among treatments. No effect ($P > 0.05$) of treatment was detected for LH or PRL concentrations, but a significant effect of day ($P < 0.05$) was observed with LH concentrations being greater on the fifth exposure day compared with the first and third.

Endogenous opioid peptides have been shown to be involved in seasonal regulation in mares, both at the hypophysis and ovarian levels. The objective of this second study was to investigate the effects of an opioid antagonist, both alone and in combination with a GnRH agonist, on gonadotrophic hormone concentrations in transitional mares exposed to various progesterone environments. Seventeen stock-type, transitional mares were treated orally with

altrenogest for 15 days. Mares were stratified by age and parity and assigned randomly to 3 treatments: (1) NAL (0.5 mg/kg BW naloxone hydrochloride IV, $n = 5$); (2) DES (1.8 mg deslorelin acetate IM, $n = 6$); or (3) N&D (naloxone hydrochloride followed by deslorelin acetate after 45 min, $n = 6$). Assigned treatment was administered 3 times during the study: (1) phase 1 (P1) occurred on the last day of altrenogest; (2) phase 2 (P2) occurred upon detection of a follicle ≥ 35 mm; and (3) phase 3 (P3) occurred 7 d post-ovulation. Interval from P1 to P2 was shorter ($P < 0.05$) for N&D compared with NAL, but not for any other treatment combinations. Interval from P2 to ovulation was not different ($P > 0.05$). Significant effects of phase and time were identified, with treatment initiating a greater ($P < 0.05$) LH response during P3 compared with P1 and P2. The FSH response to treatment was greater ($P < 0.05$) during P1 and P3 compared with P2. Regardless of phase, deslorelin increased both gonadotropins whereas naloxone alone only resulted in a biologically significant increase in gonadotropins during P3.

Table of Contents

List of Figures	x
List of Tables	xiii
Acknowledgements	xiv
Dedication	xv
Chapter 1 - Review of the Literature	1
Chronobiology of Reproduction	1
Breeding Season Proper	3
Luteal Phase	3
Follicular Phase	4
Follicular Development	5
Ovulation	8
Autumnal Transition	9
Anestrus	14
Vernal Transition	16
Mediation of Seasonality	19
Kisspeptin	19
Gonadotropin-inhibiting Hormone	24
Melatonin	25
Artificial Lighting	27
Gonadotropin-Releasing Hormone	28
Dopamine and Prolactin	31
Endogenous Opioid Peptides	34
Progestins	37
Summary	40
Chapter 2 - Stallion exposure fails to alter the onset of cyclicity in seasonally anestrus mares. 42	
Introduction	42
Materials and Methods	43
Animals and Housing	43
Experimental Design	44

Sample Collection and Analysis	45
Statistical Analysis	46
Results.....	47
Ovulatory Data	47
Hormonal Data	48
Discussion.....	51
Conclusions.....	55
Chapter 3 - An opioid antagonist, either alone or in combination with a GnRH agonist, alters gonadotropin and ovarian responses in transitional mares exposed to various progestin environments.....	
Introduction.....	57
Materials and Methods.....	58
Animals	59
Experimental Design.....	59
Sample Collection and Analysis	60
Statistical Analyses	61
Results.....	62
Ovarian Data	62
Steroid Hormones	64
Pituitary Hormones	65
Discussion.....	71
Conclusions.....	74
References.....	76

List of Figures

- Figure 1.1 – Follicular and hormonal changes associated with anestrus and the vernal transition period in mares. Data presented as mean $\pm$ S.E.M. daily values for a) number of follicles > 15 mm (closed circle) and diameter of the largest follicle (open circle), b) circulating FSH (closed circle) and LH (open circle), and c) circulating inhibin (closed circle) and estradiol (open circle). Data were normalized to the beginning of vernal transition (day 0). Data for the last 14 days of the vernal transition were normalized to the day of ovulation. Within endpoints, different letters (x and y) are different ($P < 0.05$). From Donadeu and Ginther (2007) [32]. 16
- Figure 2.1 - Concentrations of plasma LH for anestrus mares housed adjacent to, and allowed direct contact with, a stallion for 2 h thrice weekly (DXP; n = 7), housed adjacent to a stallion but not allowed direct contact (IXP; n = 6), or housed on opposite side of farm from the stallion and not allowed visual or direct contact (CON; n = 7) on the first (Feb. 23; a), third (Feb. 27; b), and fifth (Mar. 3; c) exposure d. The stallion and DXP mares were moved into the tease pen after the sample collection at 0800 and they were removed from the tease pen and returned to regular housing after the 1000 sample collection. *CON was greater than DXP and IXP ($P < 0.05$). 49
- Figure 2.2 - Concentrations of PRL for anestrus mares housed adjacent to, and allowed direct contact with, a stallion for 2 h thrice weekly (DXP; n = 7), housed adjacent to a stallion but not allowed direct contact (IXP; n = 6), or housed on opposite side of farm from the stallion and not allowed visual or direct contact (CON; n = 7) on the first (Feb. 23; a), third (Feb. 27; b), and fifth (Mar. 3; c) exposure d. The stallion and DXP mares were moved into the tease pen after the sample collection at 0800 and they were removed from the tease pen after the 1000 sample collection. *IXP greater than DXP and CON ($P < 0.05$); **IXP and CON were greater ($P < 0.05$) than DXP. 50
- Figure 3.1 – Pretreatment estradiol-17 β concentrations in transitional mares treated with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) on the final d of a 15-d altrenogest supplementation period (P1), upon first detection of a follicle $\geq$ 35 mm (P2), and

7 d post-ovulation (P3). Estradiol concentrations did not differ ($P > 0.05$) among treatments before P1 treatment. *Estradiol concentrations for mares receiving NAL and N&D were greater ($P < 0.05$) than DES before P2 treatment. **Estradiol concentrations tended ($P = 0.08$) to be greater in N&D mares compared with NAL, but not DES before P3 treatment. 65

Figure 3.2 – Concentrations of LH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) on the last d of 15-d altrenogest supplementation period (P1). Naloxone was administered to NAL and N&D mares at 0 min. Deslorelin was administered to DES and N&D at 45 min. Treatment with N&D increased ($P < 0.05$) and DES tended ($P < 0.1$) to increase LH concentrations, whereas NAL did not ($P > 0.1$). 66

Figure 3.3 - Concentrations of LH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) upon detection of a follicle ≥ 35 mm in diameter (P2). Naloxone was administered to NAL and N&D at 0 min. Deslorelin was administered to DES and N&D mares at 45 min. Treatment with N&D and DES increased ($P < 0.05$) LH, but not NAL ($P > 0.1$). 67

Figure 3.4 - Concentrations of LH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) 7 d post-ovulation (P3). Naloxone was administered to NAL and N&D at 0 min. Deslorelin was administered to DES and N&D mares at 45 min. All 3 treatments resulted in an increase ($P < 0.05$) in LH..... 68

Figure 3.5 - Concentrations of FSH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) on the last d of altrenogest administration (P1). Naloxone was administered to NAL and N&D at 0 min. Deslorelin was administered to DES and N&D mares at 45 min. FSH increased to a greater ($P < 0.05$) extent in both N&D and DES compared with NAL mares. 69

Figure 3.6 - Concentrations of FSH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) upon detection of a follicle ≥ 35 mm in diameter (P2). Naloxone was administered to NAL and N&D at 0 min. Deslorelin was administered to DES and N&D mares at 45 min. A greater ($P < 0.05$) FSH response was observed in DES compared with NAL, but not N&D. 70

Figure 3.7 - Concentrations of FSH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) 7 d post-ovulation (P3). Naloxone was

administered to NAL and N&D at 0 min. Deslorelin was administered to DES and N&D
mares at 45 min. A greater ($P < 0.05$) FSH response was observed in DES compared with
NAL, but not N&D. 71

List of Tables

Table 2.1 – Ovulatory data for anestrus mares housed on opposite side of farm from the stallion and not allowed visual or direct contact (CON), housed adjacent to a stallion but not allowed direct contact (IXP), or housed adjacent to, and allowed direct contact with, a stallion for 2 h thrice weekly (DXP).....	48
Table 3.1 - Transitional mare ovulatory and follicular traits after treatment with deslorelin acetate (DES), naloxone hydrochloride (NAL), or a combination of both (N&D) on the last day of altrenogest administration (Phase 1) and again upon detection of a 35 mm follicle (Phase 2).	63

Acknowledgements

I would like to acknowledge all of the individuals that helped me along the way to finish my degree. Firstly, I would like to thank all of the people that helped me with data collection, particularly Erika Stafford, Taylor Friedfeld, and Sarah Price. I would like to thank my colleagues at Clemson University for their encouragement and help, especially Dr. Kristine Vernon and Dr. Nathan Long. I cannot express adequately my gratitude for my committee members, Dr. Jeffrey Stevenson, Dr. Timothy Rozell, and Dr. Abigail Jager for the countless hours of help and feedback. Last, but certainly not least, I would like to thank Dr. Joann Kouba for her mentorship during the last 6+ years – I could not have done this without you.

Dedication

This dissertation is dedicated to Charles Armstrong Sinclair, the greatest man I ever knew. Rest In Peace 12/16/18.

Chapter 1 - Review of the Literature

Chronobiology of Reproduction

The science of biological timekeeping or the natural physiological rhythms and cyclical phenomena is known as chronobiology. Many organisms are dependent on chronobiological patterns as an adaptation for survival. Ability of organisms to respond to fluctuating light and dark cycles allowed them to predict cyclic changes in their environment. This capability to predict environmental conditions and make physiological adjustments gave them an advantage for survival, particularly regarding reproductive success. Seasonal rhythms in reproduction allow for species to give birth when the probability of offspring survival is at its peak, such as favorable weather conditions and greater availability of food resources [1].

Regulation of reproduction in mares, seasonally polyestrous long-day breeders, is dictated by various periodicities. There are circannual changes, which occur during the course of approximately 1 y, infradian changes, which have durations longer than 24 h but less than 1 y, and circadian rhythms that occur in an approximately 24-h period. The circannual reproductive rhythm in mares is broadly divided into two seasons, the breeding season and the non-breeding season. Furthermore, these circannual changes can be more specifically divided into 4 periods: breeding season proper, autumnal transition, winter anestrus, and vernal transition [2]. These 4 periods will be discussed in greater detail in the following sections. During the breeding season proper, infradian reproductive rhythms occur in the form of estrous cycles which are approximately 22 d in duration [3]. These cycles are regulated by hormonal and utero-ovarian events. Circadian rhythms of hormonal secretion, including hypothalamic, pituitary, and steroid hormones, affect reproductive functions, such as ovulation and parturition [1].

Light perception is the primary driver of seasonality in horses. Light is perceived through the retina where retinal ganglion cells capture photic stimuli. Retinal ganglion cells contain both melanopsin and pituitary adenylate cyclase activating polypeptide and form the retinohypothalamic tract, which monosynaptically innervates the suprachiasmatic nuclei (SCN) [4]. The SCN processes the photic input received through the retinohypothalamic tract and utilizes CLOCK genes for photoentrainment. The SCN has been termed a self-sustaining oscillator that acts as a “tuning fork” by which homeostatic needs can be adjusted [5]. Fibers from the SCN pass down through the superior cervical ganglia via a complex network to the pineal gland where terminal axons closely associate with pinealocytes [5]. Melatonin is then synthesized from serotonin in the pineal gland in the absence of light [6]. The rate-limiting enzyme for this conversion, N-acetyltransferase, reaches peak concentrations during dark periods and is in lowest concentration during light periods; therefore, melatonin concentrations are greatest during dark hours and lowest during light hours [7].

Availability of literature investigating the exact mechanisms by which melatonin interacts with the hypothalamic-pituitary-gonadal axis to regulate seasonal reproductive activity in horses is limited. Melatonin receptors have been identified in the pituitary of horses, to the greatest extent in the pars tuberalis, but also in the pars intermedia and pars distalis, whereas no binding occurred in the hypothalamus [8]. Because gonadotropin-releasing hormone (GnRH) neurons are not localized in the same areas as the putative melatonin binding sites and because other neurotransmitters interact with GnRH neurons, it is suspected that melatonin interaction with GnRH neurons is indirect and possibly mediated through other neuronal systems [9]. Investigation of the exact relationship between melatonin and GnRH is difficult to study because of the wide range of physiological effects elicited by melatonin. Furthermore, observations made

in other species, such as sheep, cannot be extrapolated to horses because of species-specific actions of melatonin. Further research is needed in this area to contribute to the existing body of knowledge of seasonal regulation of reproduction in horses.

Breeding Season Proper

The breeding season proper is the period of time in which mares undergo regularly occurring estrous cycles. Average date of first ovulation for mares in the Northern hemisphere is April 7 ± 9.1 d, after which mares will resume normal estrous cycles [10]. The breeding season proper extends from April through approximately September in the Northern hemisphere, with considerable variation in the cessation of regular estrous cycles [2]. The estrous cycle of the mare is 21 to 23 d in duration for horses [11,12]. The estrous cycle can be separated into two phases, the luteal phase and the follicular phase, both of which will be discussed hereafter in further detail.

Luteal Phase

The luteal phase of the estrous cycle lasts 15.8 ± 2.5 d and is generally characterized by a lack of ovulation or estrual behavior [11]. The luteal phase begins after ovulation has occurred, at which point both luteinizing hormone (LH) and estradiol (E2) concentrations are declining. In contrast to other species, theca cells of the ovulatory follicle begin to degenerate and do not serve as precursors for small luteal cells, whereas granulosa cells begin to luteinize 10 h after ovulation [13]. Synthesis of progesterone (P4) by the corpus luteum (CL) begins just after ovulation with maximal concentrations of P4 (8 to 16 ng/mL) reached by 8 d post-ovulation [14,15]. Eliciting a negative feedback at the level of the hypothalamus, P4 acts through higher brain areas to slow the pulsatile release of gonadotropin-releasing hormone (GnRH) to a pulse interval of approximately 120 min, which subsequently suppresses LH release [16]. Therefore, LH begins to

decline approximately 1 d post-ovulation when P4 secretion reaches a threshold of 2.0 ng/mL [17]. Secretion of follicle-stimulating hormone (FSH) is not suppressed by elevated P4 concentrations, likely because lesser frequency pulses of GnRH favor FSH secretion and feedback from other hormones, such as activin and inhibin [18]. A surge of FSH occurs just after ovulation, with the specific day of the cycle differing among individual mares. Although the FSH surge initiates with a follicular wave, diestrus ovulations rarely occur [19]. Basal concentrations of estrogen conjugates are reached by 1 to 2 d post-ovulation [20]. Estrogen conjugates, either from diestrus follicles or secretion from the CL, begin to increase again 3 d post-ovulation [21]. By 14 to 15 d post-ovulation, estrogen conjugate concentrations return to basal levels either because of regressing diestrus follicles or luteolysis [20].

Approximately 15 to 17 d post-ovulation, luteolysis begins to occur and is marked by a decrease in circulating P4 followed by structural regression of the CL [22]. Prostaglandin F_{2α} (PGF_{2α}) is released from the endometrium, and unlike ruminants, travels through the peripheral circulation to bind to receptors on the CL. It is postulated that the equine CL has a greater affinity for PGF_{2α}, thus eliminating the need for a countercurrent exchange system [23]. In ruminants, oxytocin is secreted from the CL to stimulate the release of PGF_{2α} from the endometrium. In contrast, unlike other species, luteal oxytocin synthesis does not occur in the mare, but rather there are specific cells within the endometrium that secrete oxytocin to stimulate PGF_{2α}. Therefore, a paracrine-autocrine relationship between PGF_{2α} and endometrial oxytocin controls luteolysis in mares [24].

Follicular Phase

Once the inhibitory effects of P4 are removed after luteolysis, continued follicular growth occurs, as well as increases in both LH and E2. The follicular phase lasts 7.7 ± 2.9 d in mares

and becomes shorter in duration as the summer solstice approaches [11]. A secondary surge in FSH occurs approximately 10 d before ovulation, during the mid-luteal phase, which triggers the growth of a cohort of follicles. Unlike the first FSH surge, this subsequent surge produces the future ovulatory follicle [18]. Estrogens secreted from the developing follicles begin to increase 6 to 8 d before ovulation and peak 2 d before ovulation [17,25]. Concentrations of LH begin to increase 2 d after the beginning of the decline in P4 associated with luteolysis. The increase in LH during the follicular phase is biphasic, as it increases more slowly (0.4 ng/mL/d) between 14 and 22 d post-ovulation, but at a faster rate (1.8 ng/mL/d) between d 22 post-ovulation and 1 d after the subsequent ovulation. The shift between the two phases of increased LH secretion coincides with the peak of the preovulatory surge in E2 [17]. It is suggested that the initial increase in LH following removal of negative feedback by P4 is moderated by the negative effects of E2, thus LH increases at a greater rate as E2 begins to decline just before ovulation. Alternatively, treatment of dominant follicles with human chorionic gonadotropin (hCG) in cattle is associated with a decrease in aromatase mRNA expression [26]. This indicates that E2 production may be downregulated once a threshold level of LH binding occurs within the dominant follicle.

Follicular Development

Follicular development occurs in major and minor waves in some monovular species, such as the cow and mare. Dominant follicles are only produced by major waves [27]. Folliculogenesis is characterized by 5 phases: recruitment, emergence, selection, deviation, and dominance [28]. Recruitment is defined as the phase in which a cohort of primordial follicles begins to grow and become increasingly dependent on gonadotropins [27,28]. Emergence is defined retrospectively as the last day before the future dominant follicle reached 6 mm in

diameter [27,28]. On average, the future dominant follicle in mares emerges approximately 1 d before the largest future subordinate follicle [29]. Emergence marks the beginning of a follicular wave; therefore, the duration of a follicular wave is defined as the period from emergence in the first follicular wave to emergence of the second follicular wave [30]. Selection is a common-growth phase lasting approximately 6 d, during which between 7 and 11 follicles continue to grow in response to FSH [27,28,31].

The beginning of deviation corresponds with the end of selection. Near the end of selection, if the largest follicle is ablated, the second largest follicle will establish dominance; therefore, all follicles in a cohort during selection are capable of establishing dominance [27]. The future dominant follicle is the one that reaches the greatest diameter by the end of the common-growth phase [27]. Peak FSH concentrations are generally observed when a follicle reaches approximately 13 mm in diameter [27]. There is typically a 3-d period between peak FSH concentrations and the beginning of deviation. During this pre-deviation period, all follicles in the cohort contribute to increasing concentrations of E2 and inhibin, which results in a decline in FSH concentrations. This leads to a “survival of the fittest” situation, whereby the largest follicle has the advantage because of its increased responsiveness to lesser concentrations of FSH during the decline and its responsiveness to LH via upregulated LH receptors in granulosa cells compared with smaller follicles, thus enabling its survival while the subordinate follicles are starved [27].

Beginning of deviation has been defined as a retrospective concept, being the observation period before an apparent change in diameter between the first and second largest follicle [27]. On average, this change in diameter occurs when the largest and second largest follicles are 22.5 and 19 mm in diameter, respectively. Ginther et al. (2003) noted that the beginning of deviation

may be difficult to define if all subordinate follicles in the cohort are small. Furthermore, defining deviation may be even more difficult in the mare because of overlapping non-ovulatory waves [27]. Periodic ablation of follicles that are not participating in the ovulatory wave makes it easier to define the occurrence of deviation; however, ablation is not a technique used by industry professionals and is generally only conducted in a research setting.

Approximately 1 d before deviation, the largest follicle begins to secrete increasing quantities of E2, which works both independently and synergistically with inhibin to reduce FSH secretion. Ablation of the largest follicle at the expected onset of deviation results in a reduction in E2 concentrations and a resultant increase in circulating FSH, whereas ablation of only the second largest follicle fails to produce this response [27]. Total inhibin concentrations increase just before the decline in FSH. Therefore, inhibin likely suppresses FSH secretion during the first 2 d of the FSH decline following a wave-inducing FSH surge, with E2 playing an increasingly greater role in FSH suppression starting 1 d before deviation [32]. Luteinizing hormone also plays an important role in deviation, as evidenced by the appearance of LH receptors on granulosa cells around the time of diameter deviation. Experimentally reduced concentrations of LH in mares near the time of deviation produced dominant follicles with smaller diameters and decreased circulating concentrations of E2 and inhibin compared with follicles under the influence of normal physiological LH concentrations [33]. Luteinizing hormone activates P450 side-chain cleavage enzyme (P-450scc), the enzyme responsible for conversion of cholesterol to pregnenolone. An increase in steroid acute regulatory (StAR) protein, P-450scc, and 3 β -HSD protein content of granulosa cells during and after deviation has been reported [34]. Intrafollicular factors such as E2, IGF-1, activin-A, and inhibin-A all play a role in increasing sensitivity of the dominant follicle to LH and FSH, facilitating deviation [27].

Dominance is the phase whereby the dominant follicle grows at a rate such that it creates an environment unfit for subordinate follicles to continue growing [35]. Dominance has occurred once all subordinate follicles in the wave have stopped growing and the dominant follicle is 1 to 2 mm larger in diameter than the next largest follicle [36]. Because mares are not suited for birthing more than one foal per pregnancy, ideally only one dominant follicle is produced per cycle to avoid the risk of a twin pregnancy.

Ovulation

Unique aspects of ovulation exist in mares because of the inverted structure of the ovary necessitating a common region for follicular rupture, known as the ovulation fossa. After deviation, the dominant follicle generally grows at a rate of approximately 3 mm/d until it reaches a plateau at approximately 41 mm in diameter 2 d before ovulation, which coincides with the peak of E2 [37]. As mentioned previously, it is at this point that LH rises more sharply and initiates a chain of events that ultimately results in ovulation of the dominant follicle.

The exact threshold of LH required for ovulation to occur is unknown and probably varies from mare to mare, but increased LH triggers a cascade of events via a cAMP-mediated protein kinase system that result in ovulation [38]. An increase in synthesis and secretion of prostaglandin E₂ (PGE₂) production causes hyperemia of the ovary and, more specifically, of the dominant follicle. This increase in PGE₂, along with an increase in histamines, bradykinins, and mast cell infiltration of the tissues surrounding the follicle causes significant edema, increasing intrafollicular pressure [38,39,40]. Although not to the magnitude observed during luteolysis, PGF_{2α} is synthesized and secreted to initiate ovarian smooth muscle contractions, thus contributing to increased intrafollicular pressure [38]. Concurrently, other prostaglandins (thromboxanes and leukotrienes), cause local vasoconstriction at the apex of the follicle and

contractions of the smooth muscle components of the ovary, both of which increase follicular pressure and decrease the tensile strength of the follicular wall [38,39,40]. The preovulatory LH surge causes a shift from E2 to P4 production by theca interna cells [38,39]. Increasing intrafollicular P4 concentrations increases the production of collagenase, which aids in degradation of the follicle wall [38]. Increasing LH concentrations also stimulate an increase in plasminogen activator in the follicular fluid, which converts plasminogen to plasmin. Plasmin, along with collagenase, proteases, and lysosomal enzymes cause the proteolysis of collagen, which decreases the tensile strength of the follicular wall [38,39,40]. Mechanical rupture involves evacuation of the majority of follicular fluid from the antrum of the follicle through the ovulation fossa. This process can take between 5 and 90 s, although some residual fluid may remain in the antrum [41]. The time required for complete apposition of the follicular wall ranges from 30 min to 5 h after the initial evacuation of follicular fluid [42,43].

Autumnal Transition

The autumnal transition period is one that is generally characterized by inconsistent estrous cycles and erratic estrual behavior. Its onset and duration is variable, but is most commonly considered to be the period of time between September through November in the Northern hemisphere [44]. The onset of anestrus is variable, however, and has been shown to be equally distributed across November, December, and January, thus indicating that the duration of the autumnal transition period is itself, highly variable [45]. In fact, based on the accepted definition of anestrus as having both hormonally and structurally quiescent ovaries with a lack of follicles growing beyond 20 mm, some mares do not become fully anestrus, but rather they just go through a period of persistent but anovulatory follicular growth until recrudescence of

ovulatory follicular growth occurs the following spring [46,47]. Factors that may be responsible for the differences observed in seasonality will be discussed in further detail later in this chapter.

The process of the autumnal transition is gradual during the course of at least 60 d and can be characterized by distinct ovarian and endocrine events, which are quite muted initially [44]. Beginning with the estrous cycle occurring after the summer solstice, average luteal P4 secretion begins to decline in each subsequent cycle leading up to anestrus. Furthermore, in one study mares lacking a declining pattern in P4 secretion continued to cycle through the winter months [48]. Despite this decline in P4 secretion, no changes in diameter, echogenic structure, or macroscopic appearance of the CLs have been observed, thus suggesting no changes in luteal cell proliferation, but rather changes in luteotropic factors as the likely precursor [44,45]. Although LH has been suggested as a contributor in luteal growth in mares, a decline in circulating LH concentrations is not observed until much later in the progression of the autumnal transition [49]. Indeed, it has been reported that pituitary LH content in mares declines between July and October; however, a temporally associated decline in circulating LH was not noted [50]. There is potential involvement of prolactin (PRL) in the decline in P4 secretion, being that it is luteotropic in other species and the seasonal decline in PRL is closely associated with the decline in P4 [51,52]. Furthermore, PRL receptors have been located in the CL in mares, but a direct association has not been investigated [53]. In conjunction with declining P4 secretion, the lifespan of CL is progressively shortened with each subsequent ovulation in autumn [54,55]. Furthermore, luteolysis is prolonged at a greater rate during autumn compared with summer [55]. Failure of sufficient $\text{PGF}_{2\alpha}$ secretion does not seem to be the cause for the increased incidence of these cycles with a spontaneously prolonged corpus luteum (SPCL), as $\text{PGF}_{2\alpha}$ metabolite concentrations were not different between SPCL cycles compared with normal cycles or summer

compared with fall cycles [56]. It seems that dopamine may play a role in the increased incidence of SPCL cycles during the autumnal transition, because mares administered the dopamine D₂ receptor antagonist, domperidone to mares in an attempt to prevent or delay the autumnal transition exhibited a decreased incidence of SPCL cycles, 4 to 6% incidence compared with 24% in control mares [56]. The typical incidence of SPCL cycles in mares during the breeding season is 8 to 10% [44]. Given that dopamine and PRL have an inverse relationship and dopamine D₁ and D₂ receptors have been located on equine CLs, it is plausible that dopamine may play a direct role in the luteal dysfunction seen during the autumnal transition [57, 58].

Near the autumnal equinox, steroidogenic capacity of follicles begins to decline [54]. Regardless of size, fall transitional follicles produce less E₂ per mm than follicles of comparable size in the summer [3]. The follicular growth pattern during the autumnal transition does not change much until cessation of ovulation before entering anestrus [59]. In fact, the diameter of the largest follicle in the first follicular wave that fails to produce an ovulation does not differ in size from previous waves. Afterwards there is a steady decline in the diameter of the largest follicle produced by each subsequent wave until ovarian quiescence occurs in anestrus [60].

There are 3 different states from which a mare can enter anestrus from the autumnal transition. The first of which is a case where there are waves of follicular growth and atresia following failure to ovulate the dominant follicle. This is how the majority of mares, approximately 40-60%, enter anestrus. This period of growth and atresia has been dubbed the receding phase. Alternatively, the mare may have a normal luteal phase followed by lysis of the CL but no follicular growth, which occurs in approximately 25-35% of mares. Lastly, the mare may move into ovarian quiescence following the gradual decline in P₄ from a SPCL which

occurs in approximately 20-30% of mares [45]. A decline in ovarian weight between July and October, followed by an increasing rate of decline from October through December, has been noted [49].

Despite ovarian follicular dysfunction and a decline in follicular steroidogenesis and LH secretion, hypothalamic GnRH content remains unaffected during the initial stages of the autumnal transition. In contrast, GnRH content declines later during the autumnal transition period, from October through December [49]. Further evidence indicating that ovarian activity is not altered by GnRH during the autumnal transition was provided by a study that found that continuous GnRH administration during the autumnal transition did not alter the timing of anestrus [61]. Because LH declines during the autumnal transition but it is not preceded by a decline in GnRH content, and pituitary GnRH receptor content remains unchanged, it is suspected that pituitary sensitivity to GnRH may be altered [49,62]. Given that changes in E2 precede alterations in LH secretion during both the autumnal transition and the vernal transition, it is plausible that alterations in pituitary sensitivity to GnRH may be altered by gonadal steroid feedback. Alternatively, production of gonadotropin-inhibiting hormone (GnIH) is mediated by melatonin and has been shown to inhibit LH response to GnRH [44]. In contrast, timing of the decline in LH during the autumnal transition was not different between ovariectomized pony mares exposed to long-day artificial lighting compared with those maintained under ambient photoperiod, thus indicating that photoperiod, and therefore melatonin, are not responsible for the suppression of LH secretion [63].

Pituitary LH content declines between July and October, followed by a more precipitous decline between October and December [49]. The decline in LH has been characterized by two profiles during the autumnal transition, the first of which is a successive decline during the

course of 2 to 3 cycles, eventually resulting in anovulation. The second profile was more abrupt in that LH concentrations during the preovulatory surge of the first anovulatory cycle was less than half of what was observed in the previous ovulatory cycle [50]. Pulse frequency of LH remains unchanged with the seasons however, pulse amplitude and mean LH is reduced during autumnal transition and anestrus [64].

Pituitary FSH content is not altered by season [49,62,65]. In contrast, the FSH surge pattern is altered from two peaks during the estrous cycle early in the autumnal transition to a single peak deeper into the transition [66]. Surges in FSH continue past anovulation in most mares and even continue throughout anestrus in some mares [66,67,68]. In mares that cease to have FSH surges during anestrus, the amplitude of the surges gradually declines with each successive wave as the anestrous period progresses [50]. Cessation of major and minor follicular waves occurs despite continued FSH secretion [67]. Lower quantities of FSH receptor mRNA have been found in ovaries during anestrus compared with ovaries harvested during summer; however, a decline in FSH receptor content during the autumnal transition has not been investigated [58]. Unlike LH, FSH does not seem to be affected by ovarian steroids during the fall transition or anestrus [44].

Relatively little attention has been paid to investigating the mechanisms involved in the autumnal transition because, from a practical standpoint, most breeders are concerned with the vernal transition period because early (January through March) foals are desired. However, with advances in oocyte and embryo freezing, breeding mares after the peak of the competition season to harvest oocytes and embryos is becoming more commonplace. In addition to expanding our knowledge regarding seasonality mechanisms in mares, further research to elucidate the autumnal transition is warranted as interest in fall breeding increases. Furthermore, having a

better understanding of the hormonal events in the autumn transition may help to explain the variability of the vernal transition and aid in the development of novel protocols to advance the onset of the season.

Anestrus

The period of ovarian quiescence that occurs during winter months is referred to as seasonal anestrus; however, this may be somewhat of a misnomer in the sense that some mares will display erratic, short-lived bouts of estrual behavior in the absence of corresponding follicular activity. In this sense, it may be more appropriate to describe this period as anovulatory, rather than anestrus. Other forms of anestrus beyond seasonal have been observed in mares, including nutritional and lactational. Lactational anestrus is relatively uncommon, occurring in only 3 to 4% of mares in adequate body condition and approximately 15% of mares in poor body condition [69,70]. The focus of this section, however, will be on seasonal anestrus.

In general, gonadotropin input is required for folliculogenesis to persist beyond a diameter of approximately 20 mm [71]. Without sufficient input of circulating FSH, steroidogenesis is hindered and further follicular development cannot occur [72]. As discussed in the previous section, follicular activity of mares beyond the last ovulation during the fall transition can be variable among individuals. Some mares continue to develop large follicles (≥ 25 mm) that fail to ovulate and subsequently regress whereas others display a more abrupt pattern with no follicular growth beyond 25 mm following the last ovulation [27,50]. Follicular waves still occur during anestrus but significant growth beyond the diameter associated with the beginning of deviation is uncommon [32]. Deep anestrus is considered to occur during the period

of time when the number of follicles > 15 mm is reduced and growth of the largest follicle does not exceed 21 mm [32].

Endocrine profiles of mares during anestrus are far less perplexing than profiles observed during the autumn and vernal transitions. Both hypothalamic GnRH content and GnRH secretion are reduced significantly during anestrus [73,74,75]. During anestrus, the pituitary is depleted of LH content [49]. In fact, the ability of the pituitary to synthesize LH is hindered during anestrus, as evidenced by the absence of mRNA for the LH β subunit [76]. In contrast, pituitary content of FSH is unchanged during anestrus [49]. Although peripheral FSH concentrations are reduced during anestrus, they are not reduced to the extent as observed with LH [63]. Despite continuation of follicular waves, E2 concentrations remain basal throughout anestrus. The authors noted that small statistical fluctuations in E2 concentrations occurred during anestrus but they were not necessarily associated temporally with increased follicular growth [32]. These small increases in E2 may be responsible for the unseasonal displays of estrus unaffiliated with follicular growth that are occasionally observed in anestrus mares [32,77,78]. Involvement of adrenal steroids have been suggested as a cause for these occurrences of estrus behavior, so it is possible that the slight increases in circulating E2 observed during anestrus may not be derived from the ovaries [79,80]. Figure 1.1 illustrates the relationships of these ovarian follicular and hormonal events during anestrus and the vernal transition period.

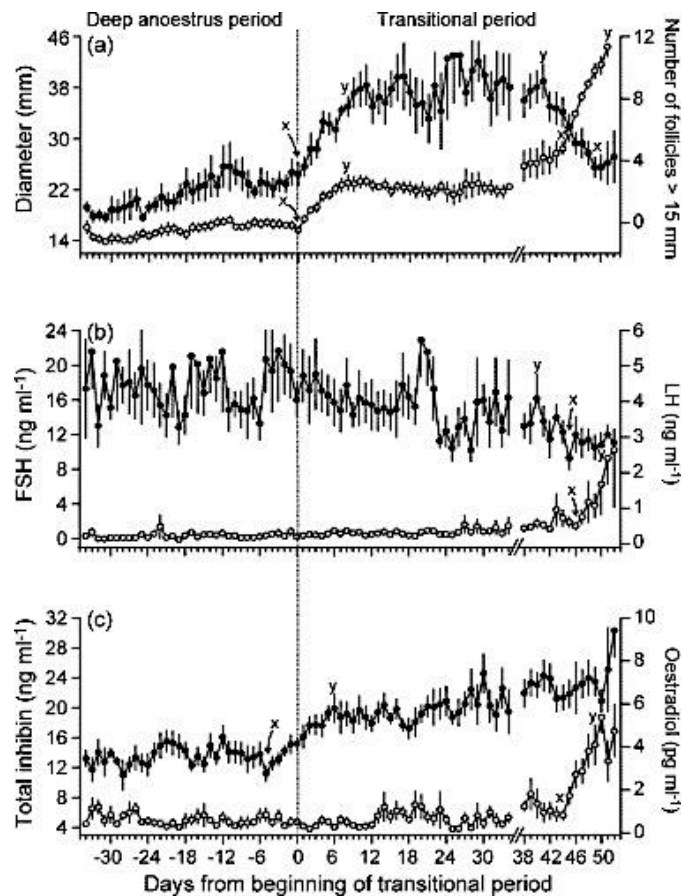


Figure 1.1 – Follicular and hormonal changes associated with anestrus and the vernal transition period in mares. Data presented as mean $\pm$ S.E.M. daily values for a) number of follicles > 15 mm (closed circle) and diameter of the largest follicle (open circle), b) circulating FSH (closed circle) and LH (open circle), and c) circulating inhibin (closed circle) and estradiol (open circle). Data were normalized to the beginning of vernal transition (day 0). Data for the last 14 days of the vernal transition were normalized to the day of ovulation. Within endpoints, different letters (x and y) are different ($P < 0.05$). From Donadeu and Ginther (2007) [32].

Vernal Transition

The vernal transition is best described as a series of events that lead to the return of reproductive competence in mares. This period can be a source of frustration for breeders because of erratic follicular activity and sexual behavior that are often not in agreement with one another. Attempts to breed during the vernal transition can be futile and costly as conception rates on transitional follicles are low and early embryonic losses are common because of failed

CL maintenance [81]. For these reasons, the bulk of research on seasonal manipulation in mares has been dedicated to the vernal transition period.

During the vernal transition, several successive follicular waves will yield a large (>30 mm) follicle, which subsequently regresses rather than ovulates. On average, a mare will have 3 to 4 anovulatory follicular waves separated by approximately 12 d before the first ovulation of the year [82,83,84]. Early anovulatory transitional follicles grow at a slower rate (2 mm/d) compared with late transition and breeding season proper follicles (6-8 mm/d), which can be a useful observation for those examining mares during this time of year [85]. Furthermore, early transition follicles show less vascularization and fewer mural granulosa cells compared with follicles produced later in transition [85].

It has been demonstrated previously that vascularity is positively related to the steroidogenic capacity of individual follicles [86,87]. Indeed, steroidogenic capacity of early transition follicles is reduced compared with those produced later in transition, as evidenced by greater circulating and follicular fluid E2 concentrations at the time of the third consecutive follicle >30 mm and the ovulatory follicle compared with the first and second consecutive follicles >30 mm [88]. Furthermore, intrafollicular P4 concentration is low in early transition follicles and steadily increases as the transition period progresses [89]. This can be explained by the fact that steroid acute regulatory (StAR) protein and steroidogenic enzymes are less abundant in early transition compared with later transition follicles, particularly 17α - hydroxylase and P450_{scc} enzymes [90]. Seemingly in disagreement with these findings, mares often display either erratic or continuous estrual behavior during the vernal transition period. The physiology behind this phenomenon has not yet been elucidated and warrants further investigation.

The exact neuroendocrine events that initiate recrudescence of the hypothalamic-pituitary-ovarian axis function have yet to be elucidated; however, there is evidence for opioidergic and dopaminergic involvement, in addition to evidence of upstream peptide hormone function. These will be discussed in detail in the next section, as this section will focus on the body of work downstream from GnRH. The first known endocrine event is an increase in both hypothalamic content and secretion of GnRH, which was observed just 2 wk after the winter solstice [91]. In support of this statement, GnRH secretion increases by 2 wk after anestrus mares are exposed to artificially lengthened photoperiod [74]. Presumably in response to the increased GnRH, circulating FSH begins to increase in early January [63,92]. Each of the anovulatory follicular waves during the vernal transition period is preceded by an increase in FSH secretion before follicular emergence when the follicle is between 15 and 23 mm in diameter [32,93]. Circulating FSH remains quite variable throughout the vernal transition and declines just before the first ovulation in response to increasing inhibin and E2 [32]. The negative association between FSH and inhibin is still demonstrated during these anovulatory follicular waves; however, concentrations of inhibin were greater during growth of the preovulatory follicle compared with the transitional anovulatory follicles [Fig. 1.1; 82].

On the contrary, LH significantly lags behind FSH and is the reason for ovulatory failure during transitional follicular waves. As such, it is typically the primary hormone of interest when investigating external manipulations during this time. Both LH subunit mRNA and LH content in the pituitary remains basal during early and mid-transition [49,76,93]. Consequently, LH secretion remains low throughout most of the vernal transition [63,94]. It is this LH-deficient environment that produces immature, steroidogenically incompetent, anovulatory follicles. Indeed, circulating E2 concentrations remain low throughout the majority of the vernal

transition. A nearly simultaneous increase in LH and E2 occurs approximately 5 d before the first ovulation [Figure 1.1;2]. It is tempting to assume there is a causal relationship, particularly considering that administration of E2 to ovariectomized mares during early transition results in increased mRNA encoding for the LH β subunit and LH secretion [92,93]. Circulating LH increases, however, in ovariectomized mares at the same time as intact mares in the absence of exogenous E2, thus challenging the notion of a causal relationship [63,91,95].

Despite increasing GnRH secretion 2 wk after the winter solstice, LH content and secretion does not increase until just before the first ovulation, which occurs approximately 2 to 4 mo after the winter solstice. The profile of LH secretion from anestrus through the vernal transition has been called the crux of seasonal recrudescence [2]. The next section will discuss areas of research that aim to both increase LH secretion and hasten the vernal transition period.

Mediation of Seasonality

Kisspeptin

Kisspeptins are a relatively “new” area of interest, as they were discovered less than 25 y ago. Kisspeptins are a family of peptides encoded by the *Kiss1* gene and derived from a 145-amino acid precursor. The longest kisspeptin (Kp54) is 54 amino acids in length. Although shorter variations are thought to be products of degradation, they are still biologically active. Highly conserved among mammals, Kp54 shows little variation in the last 10 amino acids [96]. Kisspeptin neurons are located in both the preoptic area and the median eminence, with a high density of cell bodies located in the arcuate nucleus in mares [97,98]. Furthermore, in these locations kisspeptin neurons were found in close proximity to approximately 34% of GnRH neurons, suggesting direct communication between the two [98]. Because of the location of kisspeptin neurons and evidence in sheep indicating that not only does content of *Kiss1* change

with season, but administration of kisspeptin causes ovulation in anestrus ewes, kisspeptin became a target of research attempting to elucidate seasonality mechanisms in mares [99,100].

When rodent Kp10 was administered to diestrous mares, an increase in LH occurred by 10 min after its administration, with peak concentrations between 20 and 30 min post-administration and concentrations returned to values within 2 standard deviations from baseline by 4 h post-treatment. Analysis of FSH was performed less frequently than LH, but concentrations of FSH also increased within 1 h post-treatment and returned to baseline by 4 h post-treatment [98]. These same authors administered the rodent Kp10 to estrual mares upon detection of an ovulatory follicle and uterine edema in an attempt to evaluate its efficacy as an ovulation-inducing agent; however, no differences in the interval from treatment to ovulation or in the number of mares ovulating by 48 h of treatment administration were observed between the Kp10 and saline-treated mares [98]. In an attempt to investigate whether repeated injections would sustain LH, mares in diestrus were given an equine Kp10 (eKP10) injection every 4 h for 48 h total. The initial two-fold acute increase in LH was not sustained by repeated injections and responsiveness to eKp10 decreased over time so the authors concluded that repeated supraphysiologic doses of eKp10 depleted GnRH content [101]. In contrast, both repeated injections (every 4 h for 48 h total) and continuous infusion of eKp10 for 3 d (6 h/d during the follicular phase) resulted in sustained increase in LH and no evidence of a decrease in responsiveness [101,102]. A further study investigated repeated (every 4 h) eKp10 injections at different times during both early and late follicular phase in an attempt to shorten the interovulatory interval. Although they found no differences in LH or FSH secretion, interovulatory interval, diameter of the ovulatory follicle, or P4 secretion by the CL after ovulation between either eKP10 treatments or saline-treated controls, an interesting change in

behavior was noted. A decrease in sexual receptivity scores of the mares was observed by 48 h after treatment with eKp10, with the decline being more pronounced in late follicular phase mares. At 72 h post-treatment, normal estrual behavior was resumed. The negative change in sexual receptivity observed in mares receiving eKp10 was correlated with a decrease in circulating E2 [103]. In multiple species, ovariectomy results in increased expression of *Kiss1* mRNA in the arcuate nucleus [104]. Chronic administration of E2 or P4 following ovariectomy abates this increase [105]. In mice, there is a corresponding increase in LH following ovariectomy, but not in *Kiss1* receptor knockout mice [106,107]. Furthermore, administration of a kisspeptin antagonist after gonadectomy inhibits the increase in LH [107]. Based on these data and the location of kisspeptin cells, it has been suggested that kisspeptin likely regulates, at least in part, ovarian steroid feedback on tonic and pulsatile GnRH secretion.

Decourt et al. (2014) examined the effects of eKp10 perfusion (6 mg/h for 6 h) during the early (follicle diameter < 17 mm), mid- (follicle diameter 20 to 25 mm), and late (follicle diameter 32 to 35 mm) follicular phase. Concentrations of LH were increased by eKp10 during all stages of the follicular phase but a greater response was observed when perfusion began in the early follicular phase. Concentrations of FSH increased during the early and mid- follicular phase only, with a greater response noted during the early follicular phase. Stage of follicular phase had no effect on the timing of ovulation and none of the eKp10-treated mares differed from saline-treated controls. The authors then sought to determine if elevation of LH and FSH would be sustained with perfusion of eKp10 for 72 h when initiated during the early follicular phase. Concentrations of LH returned to basal concentrations by the end of the 72-h period and FSH returned to basal concentrations by 36 h. Estradiol concentrations were greater in the eKp10-treated mares compared with vehicle-treated controls, but the timing of ovulation did not

differ. The authors then investigated whether depletion of gonadotropin content in the pituitary had been depleted by the perfusion of eKp10 by administering either a bolus of eKp10 or GnRH analog 20 h after the end of perfusion. The eKp10 bolus failed to increase LH but tended to increase FSH, whereas the GnRH analog elicited robust increases in both gonadotropins, thus indicating that a decrease in sensitivity to kisspeptin rather than depletion of pituitary stores was responsible for the lack of sustained gonadotropin secretion [108]. The inability of eKp10 to sustain elevated LH concentrations is a likely explanation for its failure to induce ovulation in mares, given that the ovulatory LH surge is more prolonged in mares compared with other species, such as ewes, that ovulate in response to Kp10.

To date, two studies investigating the role of kisspeptin in seasonality of mares have been reported. In anestrus mares, administration of two successive doses of eKp10 induced rapid increases in both LH and FSH by 5 min post-treatment [108]. Administration of GnRH analog 2 h after the second eKp10 also induced significant increases in LH and FSH; however, the AUC among the 3 injections was greater in response to the GnRH analog for FSH, but not different for LH. To test whether the increase in gonadotropins could be sustained, perfusion with 3 mg/h eKp10, 6 mg/h eKp10, or 20 µg/h GnRH was performed in anestrus mares. Peak LH concentrations were attained by 2 h after beginning the perfusion in all 3 treatments and peak concentrations did not differ among treatments. In contrast, FSH failed to increase in response to the lower dose of eKp10 but was significantly increased by both the 6mg/h dose of eKp10 and GnRH. Over the course of the 72-h perfusion, LH remained elevated in all 3 treatments compared with the vehicle control; however, after the peak was reached, LH declined to and remained at intermediate concentrations whereas LH increased steadily in the GnRH-treated mares. In contrast, FSH quickly returned to basal concentrations after the peak where they

remained for the duration of the perfusion [108]. The results of the former study were mirrored when anestrus mares were given a continuous rate infusion of eKp10 for 30 h at two dosages. Although an increase in LH was elicited by eKp10, an LH surge was not detected and subsequently the date of ovulation did not differ from saline-treated controls [109].

Although not yet demonstrated in the horse, continuous exposure to Kp10 in other species leads to desensitization or internalization of Kiss1 receptors [110,111]. The acute effect of eKp10, regardless of physiological state, coupled with the inability of a bolus of eKp10 to induce an increase in gonadotropins during eKp10 perfusion, supports the notion that the horse may be similar in this regard. The differential secretion patterns of LH and FSH following treatment with eKp10 during anestrus could potentially be explained by the fact that suppression of FSH during anestrus is far less pronounced than LH. Although not fully explained in horses, there is a temporal relationship between E2 and LH in seasonal recrudescence. Ovarian kisspeptin receptors have been identified in multiple species, including another seasonal, long-day breeder – the Siberian hamster. Furthermore, the kisspeptin receptors were found in steroidogenic cells and increased with exposure to increasing photoperiod [112]. This has not yet been investigated in mares but certainly warrants attention. Unfortunately, little information regarding the role of kisspeptin in seasonal regulation of mares has been produced. Although much clearer relationships have been defined in sheep (for review see [113]), this information cannot and should not be extrapolated to horses because of known differences in physiologic pathways, particularly regarding seasonality. Rather than whole-animal *in vivo* studies, it is the opinion of the author, that functional relationships at the cellular level, based on work in other species, should be the focus of future research investigating kisspeptin as a regulator of seasonality in horses.

Gonadotropin-inhibiting Hormone

Gonadotropin-inhibiting hormone (GnIH) is a RF amide-related peptide (RFRP) shown to have involvement with gonadotropin secretion and seasonality in birds. The mammalian homologue, RFRP3, has been shown to suppress LH and modulate gonadotrope sensitivity to GnRH [114]. Studies utilizing RFRP3 to inhibit LH secretion have produced ambiguous results. The investigation of this neuropeptide in horses is relatively new and at first, did not seem promising.

The first study investigating the ability of RFRP3 to suppress LH secretion was done *in vitro* [115]. Gonadotrope cells were harvested from anestrus mares that had been treated daily with GnRH to stimulate physiologic LH concentrations as observed during the breeding season. The cells were then cultured in either media alone, GnRH, or GnRH and RFRP3. No differences in LH content were noted between the positive control (GnRH) and RFRP3-treated media, thus indicating that RFRP3 does not reduce the responsiveness of gonadotrope cells to GnRH [115]. A concurrent series of experiments investigated RFRP3 *in vivo*. When administered to GnRH-treated mares by d 2 post-ovulation, RFRP3 failed to alter LH secretion. During the follicular phase, an intracavernous sinus (ICS) catheter was placed and mares received repeated injections of either RFRP3 or saline, followed by a GnRH challenge. No differences were noted in mean LH, pulse frequency, pulse amplitude, or GnRH-induced AUC for LH. And lastly, GnRH-treated anestrus mares were ICS catheterized and received a single injection of either RFRP3 or saline. Again, RFRP3 failed to alter the release pattern of LH [116,117]. The combination of these experiments indicated that RFRP3 did not regulate LH secretion in horses, as demonstrated in other species. Furthermore, adenohypophyseal RFRP3 receptor content in the mare does not change with season or with stage of the estrous cycle [117].

Infusion of RF9, a RFRP3-signaling antagonist, into anestrus mares induced a robust and sustained increase in LH, but did not alter pituitary responsiveness to GnRH compared with saline-treated controls [117]. Because of this, the authors postulated that RF9 may elicit its effects upstream of the pituitary by either stimulating or disinhibiting GnRH secretion. A subsequent study examined the effects of E2 pretreatment on the ability of RF9 to elicit an increase in gonadotropin concentration in anestrus mares and found that both the LH and FSH responses were greater in mares that had been pretreated with E2 [118]. It has since been recognized that the effects of RF9 on LH secretion are actually mediated through excitation of the *Kiss1* receptor, rather than working as an antagonist to the GnRH receptor [119,120]. The authors of the E2 pretreatment study postulated that E2 may mediate the ability of RF9 to work via GnRH release to regulate gonadotropin secretion by either increasing the sensitivity of the pituitary to GnRH or upregulating *Kiss1* receptors on GnRH neurons [118].

Clearly, as evidenced by the misconception upon which receptors RF9 takes action on, more work is needed to understand the exact mechanisms of action of RF9. The more recent understanding of RF9 interacting with *Kiss1* receptors rather than RFRP3 receptors at least provides clarity in the differential response on gonadotropin secretion between RFRP3 and RF9 in mares. Although it is unlikely that RF9 will have merit in a practical setting because of its short (40 min) half-life in the mare, continued research utilizing RF9 could be valuable in elucidating the physiological mechanisms involved in seasonality [118].

Melatonin

Because the physiology of melatonin is discussed in the first section of this chapter and the use of artificial lighting to manipulate endogenous melatonin secretion will be described in

the next section, the current section will focus on the use of exogenous melatonin in anestrus and transitional mares.

When anestrus mares are given melatonin orally, either before sunset or before sunrise, the interval from the start of treatment to ovulation was greater in mares receiving melatonin in the evening compared with morning melatonin or controls [121]. This indicates that exogenous melatonin is not able to override light perception, but if given in alignment with the dark phase, could delay recrudescence of cyclic ovarian activity [121]. This delay was later confirmed when melatonin implants inserted during anestrus delayed the first ovulation of the year when mares were exposed concurrently to short-day photoperiod, but failed to do so if mares were kept under long-day photoperiod [122]. The inability of melatonin to override photoperiod indicates that either photoperiod also elicits effects on reproductive rhythms independent of melatonin pathways or that other biological rhythms outside of photoperiod are also responsible for seasonality.

Continuous implantation of melatonin just before the summer solstice does not prevent or delay the onset of anestrus [123]. However, continuous melatonin beginning in June and continuing through mid-February advanced the date of first ovulation in the subsequent breeding season while failing to alter the onset of anestrus [122]. In contrast, implanting melatonin beginning at the summer solstice, followed by removal 4 wk later, tended to delay the timing of the last ovulation of the year and delayed the first ovulation of the subsequent breeding season [95]. These findings support the notion that light:dark (L:D) cycles cannot necessarily override endogenous biological rhythms, and that melatonin, as a proxy for day length, is not the overarching determining factor in seasonality, but rather a piece in a much larger puzzle.

Artificial Lighting

Experimentation with artificial lighting and ovarian recrudescence was first described more than 80 years ago [124]. Artificial lighting is and has been a popular option for breeders to prepare their mares for early breeding. Maintaining mares under lights, however, can be time-consuming and expensive. Although head masks utilizing artificial lighting are now available so that mares can be maintained on pasture, their cost is a limiting factor. Not understanding the physiological requirements of successful artificial lighting programs leads many mare owners to employ lighting protocols improperly. Many owners will keep mares under long-day photoperiod year-round, leave lights on all night, or start the lighting regimen at inappropriate times of the year, which leads to variable and inconsistent results. The ability of artificial lighting to advance the onset of the breeding season depends on a state of refractoriness, photoperiodic history, and a photosensitive phase during dark hours [125].

If mares are kept under constant stimulatory or inhibitory photoperiodic conditions, whether long-day or short-day, a condition known as refractoriness will occur in which mares can no longer respond to photic input and annual reproductive rhythms will be resumed regardless of photoperiod conditions [126,127,128]. In some species, photorefractoriness can be delayed or prevented by regularly alternating between light and dark cycles [129,130]. In ovariectomized mares, alternating between light and dark cycles prevented the decrease in LH associated with the anestrus period [131]. In contrast, frequent alteration of photoperiod failed to prevent anestrus in intact mares [125].

Anestrus mares exposed to a long-day photoperiod will ovulate on average 70 d after beginning exposure. The current recommendation is to provide between 14.5 to 16 h of light/d by supplementing natural photoperiod with artificial lighting either before sunset and into the

evening or in the early morning hours before sunrise. Photoperiodic history has been demonstrated as an important factor in response to artificial lighting in sheep. A 13 h photoperiod will induce stimulatory effects if utilized following a 16L:8D lighting scheme. In contrast, a 13-h photoperiod is inhibitory if it follows prolonged exposure to a 10 h photoperiod [132]. Unfortunately, this has not yet been investigated in the mare.

Mares, as in other species, have a photosensitive phase during the dark hours. This is evidenced by the fact that after 9.5 to 10 h of darkness, a short burst of light 1 to 2 h in duration can stimulate ovarian activity in anestrus mares [127,133,134]. Two studies compared interrupted lighting schemes (8L:9.5D:1L:5.5D and 4L:9.5D:1L:9.5D) with non-interrupted long-day schemes (16L:8D and 14.5L:9.5D) and found that, despite the interrupted schemes only providing 5 and 9 h total of light/d, all schemes yielded similar ovarian results [127,133]. Therefore, it seems that total duration of light versus dark is not necessarily what drives an ovarian response, but rather whether or not there is light after approximately 9.5 h of darkness. With all of these nuances determining how mares respond to light, it is important for owners to not be haphazard in its application.

Gonadotropin-Releasing Hormone

Supplementation of exogenous GnRH or GnRH agonists is a logical place to start in managing the anestrus mare considering it is the suppression of GnRH secretion that stifles LH secretion, resulting in an anovulation. Copious research has been conducted over the last 40 y investigating the ability of GnRH and its analogs/agonists to alter seasonality in mares. Although effective, GnRH comes at a high cost which prohibits most from utilizing it in their programs.

The first reports of GnRH administration to acyclic mares yielded differing results. Initially, GnRH administered twice daily to transitional mares stimulated ovulation [135]. In

contrast, when administered to deep anestrus mares, a high percentage ovulated but subsequently failed to develop a functional CL [136]. Hourly infusion of GnRH at doses as low as 2 µg to anestrus mares results in increased LH and FSH by 24 h, growth of a follicle > 25 mm by 1 wk and ovulation by 2 wks [137]. Although treatment was ceased 1 d post-ovulation, neither CL function nor duration of the luteal phase, as assessed by P4 secretion, was affected. Continuous infusion elicited the same effects as pulsatile infusion on FSH secretion, but increased LH secretion to a lesser extent compared with pulsatile infusion. Furthermore, mares receiving pulsatile GnRH ovulated 3 d sooner than mares receiving continuous GnRH [138]. Additional reports have shown that continuous GnRH administration is effective at inducing ovulation in transitional mares, but not as effective in deep anestrus mares [139,140]. In addition, one study noted a rapid decline in P4 after cessation of continuous infusion [140]. The hormonal milieu is different throughout the autumnal transition, anestrus, and into the vernal transition, thus necessitating accurate classification of mares when interpreting data. Perhaps the ability of the pituitary to respond to GnRH is decreased in deep anestrus mares relative to the vernal transition, as evidenced by the depletion of LHβ subunit mRNA. Furthermore, although mares may be induced to ovulate, other physiological events, such as luteal maintenance, must take place in order for pregnancy to be established. Given that the mare has a protracted and prolonged LH surge, not peaking until 1 d post-ovulation or reaching its nadir until approximately 3 d post-ovulation, cessation of GnRH immediately after ovulation, and thereby prematurely decreasing LH, takes for granted the luteotrophic effects of LH. In one study, however, timing of ovulation using human chorionic gonadotropin (hCG) after GnRH-induced development of a large follicle in anestrus mares may abate this decrease in luteal function [141].

Following successful use of native GnRH, the use of GnRH analogs and agonists became an attractive area of research because of their comparatively longer half-life. The GnRH agonists that have been investigated in this capacity include deslorelin, buserelin, and goserelin. One of the earlier reports utilizing twice-daily buserelin injections compared with a buserelin-releasing subcutaneous implant in transitional mares found that 50 and 64% of mares ovulated by 30 d of treatment initiation, respectively, compared with 0% of control mares [142]. Timing of treatment initiation is a determining factor for efficacy. One study found that twice daily administration of deslorelin was more effective at inducing ovulation in transitional mares with active ovaries (70%) compared with deep anestrus mares with inactive ovaries (20%). Furthermore, concentrations of LH, FSH, and P4 were lower in mares treated during deep anestrus compared with transitional mares. Although the pregnancy rate at 11 d post-ovulation was not different between the groups, the risk for embryo loss was 9 times greater in the deep anestrus mares (64%) compared with transitional mares (7%) [81]. Follicular activity at the time of treatment also will also affect outcomes, as demonstrated by an increasing percentage of mares ovulating in response to treatment twice daily with deslorelin as diameter of the largest follicle increased [143]. Another study compared native GnRH with buserelin administered at different times during the anovulatory season and found that the number of mares ovulating in response to native GnRH was similar among mares treated in January, February, and March, whereas the number of mares ovulating in response to buserelin was drastically lower in mares treated in January compared with February and March [144]. The authors also noted that LH concentration in response to treatment was greater in mares treated with native GnRH compared with buserelin across all time points. Furthermore, regardless of treatment, all mares induced to ovulate in January or February returned to anestrus after cessation of treatment.

Ability of GnRH or its analogs/antagonists to reliably induce ovulation in anestrus mares depends on the individual mare's ovarian status, thus requiring careful monitoring in order to employ its use at the appropriate time (i.e., transitional versus anestrus). As many mares in the Northern hemisphere are still in deep anestrus in January and February, despite investing a considerable amount of time and money to treat them with GnRH, the breeder still may not be able to produce a foal born in January or February. Therefore, the cost to benefit ratio in terms of getting the mare bred earlier in the year must be determined by each breeder based on their individual programs. Furthermore, if breeders attempt to induce ovulation in deep anestrus mares, based on the evidence of reduced CL function and embryonic loss, breeders might need to consider maintaining the mares on synthetic progestins at least until endometrial cup development, further adding to the financial commitment. In addition to being cost-prohibitive, using osmotic pumps or twice daily injections of GnRH for several weeks are not easily accessible or convenient. Unfortunately, unfavorable or inconsistent results have been produced from utilizing subcutaneous GnRH agonist implants, which would be more affordable and practical [142,145,146]. Because of these effects, other options have been investigated.

Dopamine and Prolactin

The anterior pituitary hormone, prolactin (PRL) is produced by lactotropes (Type I and II) as well as mammosomatotropes, which produce both prolactin and growth hormone [147]. In mares, PRL affects mammary development and lactation [148]. It is also associated with seasonality in mares. Circulating PRL is positively correlated with day length, being greatest in the summer and least in the winter months [149]. Secretion of PRL is primarily regulated by suppressive dopaminergic input from the hypothalamus [150]. For this reason, dopamine antagonists (DA) have been a popular subject in anestrus and transitional mare research.

Dopamine antagonists are commonly used to induce lactation in agalactic mares, particularly those that are pastured on fescue grass because they are susceptible to fescue toxicosis. Domperidone has a strong affinity for dopamine receptors, D2 and D3, and is one of the more commonly used DAs. Beginning on January 15 and continuing through the first ovulation, anestrus mares housed under natural photoperiod receiving 1.1 mg/kg BW daily of domperidone had a greater number of follicles > 20 mm by 14 d and ovulation occurred an average of 27 d after treatment initiation, 78 d earlier than untreated controls [151]. Furthermore, 75% of domperidone-treated mares maintained normal interovulatory intervals after the first ovulation. Concentrations of LH and estrogen conjugates were increased by 28 d after treatment began; however, FSH concentrations were not affected. Stimulation of follicular growth in the absence of increased FSH indicates that either PRL or dopamine may have effects directly at the ovarian level. To support this notion, dopamine receptors have been found in both theca and granulosa cells in addition to the observation of seasonal shifts in dopamine concentrations in the follicular fluid of follicles > 20 mm [152,153]. Furthermore, PRL receptors have recently been identified in follicles of all stages, CL, and oocytes in mares and content seems to change in accordance with season [154]. Although domperidone has been shown to be effective in hastening the vernal transition in some studies, other authors have reported no effect of domperidone employed at the same dose [155,156].

Sulpiride is a D2 receptor antagonist that has been commonly studied in anovulatory mares. Sulpiride has been shown to hasten the date of first ovulation in anestrus mares with a subsequent functional CL and luteal phase in some studies [157,158]. In contrast, a number of reports indicate that sulpiride is not effective in hastening the first ovulation or increasing circulating gonadotropins [159,160,161]. When dopamine and sulpiride were directly compared,

sulpiride, but not domperidone, increased follicular growth and hastened the date of first ovulation [162]. Regarding clinical efficacy, some practitioners recommend a combination of artificial lighting and DA to reduce the amount of variation in response [155].

Considering that E2 administration to ovariectomized mares results in increased PRL secretion, researchers hypothesized that insufficient E2 concentration in anestrus and transitional mares could be the reason for the inconsistency in response to DA [163]. Pretreatment of anestrus mares with E2 every other day beginning 11 days before initiation of sulpiride enhanced PRL secretion two-fold and increased the date of first ovulation compared with sulpiride alone [164]. The effects of E2 pretreatment were repeated when used in conjunction with domperidone [165]. A recent study indicated that mares with greater body condition score (BCS), body weight, and leptin concentrations responded to the E2 + sulpiride treatment scheme at a greater rate compared with less-nourished mares [166]. Although sulpiride alone increases PRL, the effect is short-lived and decreases with each subsequent treatment, whereas E2 pretreatment extended the PRL response beyond 24 hours [159,164].

Attempts to utilize PRL directly, rather than indirectly through DAs, have been made. Recombinant porcine PRL induces ovulation in anestrus mares 22 days after treatment initiation without an increase in LH secretion [167]. It has been hypothesized that DAs hasten the date of first ovulation, not by increasing LH, but rather by increasing PRL which then increases LH receptor content in the ovaries [168]. Although this has not yet been demonstrated in mares, supporting evidence in mice includes the report that PRL-deficient mice have complete reproductive failure because of reduced LH receptor content in CL [169]. Administration of a PRL antagonist or a dopamine agonist to mares and subsequently harvesting the ovaries to quantify LH receptor content would provide a better understanding of how PRL might affect

reproduction at the ovarian level. Further research is warranted to elucidate the exact physiological mechanisms by which PRL and dopamine influence seasonality in mares, particularly at the cellular level.

Endogenous Opioid Peptides

The family of endogenous opioid peptides (EOP) encompasses 3 families of peptides that are the result of cleavage of large protein precursors: β -endorphin (derived from proopiomelanocortin, POMC), enkephalins (derived from proenkephalin), and dynorphins (derived from prodynorphin). Given that morphine and other opiates were known to cause reproductive issues, investigation into the mechanism of action ensued. It has been demonstrated that EOP suppress LH secretion across multiple species, including humans [170], primates [171], cattle [172], sheep [173], pigs [174], and rats [175]. The EOP act at the level of the hypothalamus on GnRH neurons to suppress GnRH secretion, thereby suppressing LH secretion [176,177]. The influence of EOP on GnRH receptors is apparently moderated in part by gonadal steroids, as has been demonstrated in both males and females of various species [178]. The following section will focus on what is known about EOP involvement in the estrous cycle and regulation of seasonality in mares.

The initial investigation of opioid involvement in mare cyclicity involved the use of the opioid antagonist, naloxone, during the luteal and follicular phases in lactating and non-lactating mares. Although naloxone failed to elicit any effects on gonadotropins during the follicular phase, both LH and FSH were increased in response to naloxone administered during the luteal phase [179,180]. These results were in agreement with those observed in other species and indicated that EOPs may be at least partly responsible for mediating the suppressive effects of P4. Progesterone alone, however, is not sufficient to activate the suppression of gonadotropins

by the EOP system. This lack of suppression is evidenced by the effects of naloxone in ovariectomized mares that were pretreated with either P4 or estradiol benzoate for 8 d. Both LH and PRL were increased in response to naloxone in mares receiving estradiol benzoate, but naloxone failed to increase LH or PRL in mares pretreated with P4 alone or ovariectomized control mares not receiving any pretreatment [181]. Following the initial experiment, mares were pretreated with a combination of P4 and estradiol benzoate for 8 d and administered either naloxone or saline. An increase in LH was observed for all naloxone-treated mares, but the effects were greater in mares that had previously been pretreated with estradiol compared with those pretreated with P4 [181]. These results indicate that prolonged exposure to estradiol can activate the EOP-mediated inhibition of LH and that the alteration of P4 and estradiol dominance, similar to the innate fluctuations of the estrous cycle, mediate the EOP-suppression of LH. In support of this notion, estrogens upregulate P4 receptor expression in opiate neurons that synapse with GnRH neurons in rats, thus indicating that EOP neurons must be first exposed to estrogens before they can respond to P4 [182].

Seemingly in contrast to observations in cyclic mares, naloxone induces an increase in LH in mares during the anovulatory season despite the lack of steroid hormone input, given that the increase in LH was unaltered by degree of follicular activity [183]. This indicates that opioid inhibition of LH is active during the anovulatory season and this inhibition may be independent of steroid hormone influence. When comparing dose of naloxone with effects on gonadotropin secretion during anestrus and diestrus, a larger dose (0.1 mg/kg BW) failed to elicit a greater gonadotropin response than the smaller dose (0.04 mg/kg BW) during diestrus, which is in contrast to anestrus in which the larger dose elicited a greater gonadotropin response compared with the smaller dose [180,184]. These results indicate that opioidergic inhibition of LH

secretion may be more prominent during anestrus compared with the luteal phase during the breeding season. Because some mares maintain cyclicity throughout winter, the same group sought to determine if opioidergic tone was reduced among this subset compared with mares that are anestrus. During the non-breeding season in March, mares exhibiting anestrus and luteal phase mares that had continued to cycle throughout the non-breeding season were administered naloxone at the rate of 0.1, 0.05, or 0.25 mg/kg BW [185]. An increase in both LH and FSH was observed in winter-cycling mares at all dosages employed. In contrast, FSH increased only in response to the intermediate and larger dose of naloxone and LH failed to increase at any dose in anestrus mares. The authors concluded that opioidergic tone is greater during the non-breeding season compared with the breeding season and that this shift in tone does not occur in mares that continue to cycle throughout the winter.

Evidence of opioidergic involvement during seasonal anestrus at the level of the ovary also has been reported. Not only was the μ -opioid receptor identified in oocytes, cumulus cells, and granulosa cells, but receptor content was greater in ovaries harvested during anestrus compared with those harvested during the breeding season, with decreasing content noted as follicle size increased during the breeding season [186]. The authors then performed in vitro oocyte maturation of oocytes harvested from anestrus and transitional mares using media supplemented with either β -endorphin or naloxone. Oocyte maturation rate was decreased with the addition of β -endorphin and was increased with the addition of naloxone, thus indicating that EOP may be involved in seasonal regulation of meiotic competence of oocytes [186]. Unfortunately, no additional research of EOP involvement in seasonal regulation at the level of the ovary has been reported in mares since this finding.

Exploration of the shift in EOP tone during the vernal transition period has yet to be reported. Because of its effects on LH in anovulatory mares, perhaps the addition of an opioid antagonist could hasten the vernal transition; however, because of the relatively high cost of naloxone and limited accessibility, it is unlikely that repeated administration over a long period of time would become a commercially feasible option for breeders. In spite of this limitation, because of the inhibitory influence of EOPs extending beyond the hypophysis to include the ovary, addition of an opioid antagonist to existing treatments may prove to be beneficial. In addition to potentially improving transitional mare management, the investigation of interactions could further our understanding of the seasonal regulation of the hypothalamic-pituitary-ovarian axis, particularly with regard to the hierarchal order of events.

Progestins

The application of P4 or exogenous progestins during the vernal transition has been thoroughly investigated. Estrual behavior can last from 15 to 30 d during the vernal transition period in mares, which can present challenges for both those riding mares and those breeding mares. Prolonged periods of estrus are frustrating for those who ride and compete with mares because they tend to experience a decrease in performance resulting from increased irritability and distractedness of the mare. It can be frustrating for breeders because of confusion about whether or not the mare is ready to breed if breeders are not regularly monitoring mare ovarian activity.

One of the initial studies examining the effects of the synthetic progestin, allyl trenbolone, set out with the objectives of determining if estrus could be induced following treatment with allyl trenbolone in anestrus mares and whether estrus could be suppressed in transitional mares to avoid the extended displays of estrual behavior. Although the 12-d

treatment with progestin failed to induce estrus or ovulation in anestrous mares, treatment of transitional mares resulted not only in estrus suppression during treatment, but a decrease in the duration of post-treatment estrus, interval from treatment cessation to ovulation, and interval from estrus onset to ovulation were observed compared with untreated controls [187]. In a larger study utilizing allyl trenbolone in deep anestrous and transitional mares, ovulation occurred in 84% of mares by 18 d of treatment cessation, but the risk of ovulatory response rate was decreased in deep anestrous mares compared with transitional mares [188].

The putative mechanism by which progestins are able to hasten ovulation was known as the “rebound effect,” whereby stored LH content accrued during the progestin treatment period and was subsequently released in greater quantities following withdrawal of the progestin. Altrenogest is a common progestin used in equine reproduction for estrus suppression, pregnancy maintenance, and transitional mare management. Similar to the results using allyl trenbolone, daily oral altrenogest hastened estrus and ovulation in transitional mares, but not in deep anestrous mares [189]. Furthermore, LH and P4 concentrations were unaffected by treatment, but FSH concentrations were greater in treated mares compared with controls during the 15-d treatment period. To further refute the “rebound effect” hypothesis, another study detected no difference in LH response to administration of GnRH between progestin-treated and control mares [190]. Thus indicating that releasable LH content was not accrued in the pituitary in response to progestin treatment.

In addition to daily oral progestins, both a long-acting (7 to 10 d) intramuscular P4 injection and an intravaginal P4-releasing insert have been investigated regarding their efficacy in transitional mares. Similar to results using oral progestins, the long-acting P4 injection failed to hasten estrus or ovulation in deep anestrous mares [191]. In contrast, in transitional mares

there was a shorter treatment to ovulation interval compared with that of controls with a greater percentage ovulating by 24 d after treatment administration (83 vs 25% for control mares), respectively).

Intravaginal P4-releasing inserts have been used for decades in cattle and have been examined for use in horses. Use of the bovine controlled intravaginal drug releasing device (CIDR-B) inserts release P4 intravaginally in transitional mares and were effective in hastening the first ovulation in mares; however, a large proportion of mares presented with vaginitis after device insertion, discouraging the use of the CIDR-B in the horse industry [192,193]. More recently, an intravaginal P4-releasing insert was developed for mares and tested for efficacy. When this equine-specific P4-releasing device was inserted and kept in place for 10 d in transitional mares, the interval from removal to ovulation was shorter compared with controls [194,195,196]. Furthermore, use of this device caused little discomfort and a small incidence of vaginitis; however, it is not approved for use in the U.S. at this time.

Progestin treatment combined with artificial lighting in transitional mares can be quite effective. When exposing mares to a 16-h photoperiod for 60 d beginning December 1, followed by altrenogest administration for 12 d, the interval to estrus is 3.4 d on average with a greater number of mares ovulating by 12 d after progestin withdrawal [187]. Another study investigated the efficacy of an injection of sustained-release P4 to mares that had failed to respond to 60 d of artificial lighting and found that 57% of P4-treated mares ovulated by 4 wk of treatment compared with 7% of untreated mares [197].

The mechanisms whereby P4 or progestin administration hasten the onset of estrus and ovulation in transitional mares is not understood at this time. Because of the influence of gonadal steroids on several of the neurotransmitters mentioned previously in this section, it is the opinion

of the author that progestin treatment may alter the environment of the hypophysis in such a way that allows it to be more responsive to changes in the hormonal milieu. Considering that P4/progestin treatment is ineffective in deep anestrous mares, when inhibitive mechanisms would be exerting their strongest influence, but is relatively reliable (albeit variable in response among individuals) in transitional mares when estradiol is increasing, the above hypothesis may be plausible. Because of the variability in response to progestin in individual mares, combination therapies beyond artificial lighting are warranted. Such investigations could not only produce a more reliable method of transitional mare management, but could further our understanding of the seasonal changes in the hypothalamic-pituitary-ovarian axis.

Summary

Management of the transitional mare can be frustrating and expensive for breeders. Current methods to hasten the vernal transition come with a cost, in addition to the fact that many breeders attempt to breed after large follicles have been observed during this period that are not destined to ovulate, resulting in further financial losses associated with breeding and veterinary fees, shipping costs, labor, etc. For these reasons, research involving the transitional mare has been abundant during the last 40 y; however, it has decreased in intensity during the last decade. Although we have identified certain practices that aid in the management of transitional mares, responses are variable as a result of different levels of ovarian activity and also among individual mares. This variability is in part due to the fact that our understanding of the interactions among the physiological mechanisms involved in seasonality of the mare is poorly understood. Equine reproductive physiologists should push forward for the sake of both the advancement of knowledge and practical application by breeders. Conducting studies that

examine these interactions from basic and applied perspectives is needed and now is not the time to “leave well-enough alone.”

Chapter 2 - Stallion exposure fails to alter the onset of cyclicity in seasonally anestrous mares

Introduction

Ability of male presence to hasten the onset of estrous cycles during the non-breeding season (NBS) in females has been well-established in short-day, seasonally anovulatory species, such as sheep and goats [198,199]. Introduction of photo-stimulated rams to seasonally anovulatory ewes induced an increase in luteinizing hormone (LH) concentrations within minutes in addition to a simultaneous increase in follicle-stimulating hormone (FSH), leading to a preovulatory LH surge approximately 36 h after ram introduction [200,201]. Approximately 48 h after the onset of ram exposure, ovulation occurs in the absence of estrual behavior [202]. Similarly, exposure of seasonally anovulatory does to a photostimulated buck resulted in an immediate increase in plasma LH concentrations, followed by ovulation 2 to 8 d later [203]. It has been proposed that the presence of sexually active males induces an initial increase in gonadotropin-releasing hormone (GnRH), resulting in increased LH secretion, thereby stimulating follicular growth which then results in increased estradiol (E2) production by developing follicles. This presumed increase in estradiol is what leads to the preovulatory LH surge and thus ovulation [199].

To date, only 1 study has investigated this socio-sexual phenomenon in horses. During a 2-y study, Wespi et al. (2014) utilized two groups of anestrous mares housed in identical stables to investigate whether spatial proximity to stallions induced the onset of cyclicity. The authors found that mares exposed to stallions ovulated earlier in the breeding season, regardless of

individual contact time, stallion identity, or diameter of the largest follicle at the beginning of treatment [204].

The previous study [204] examined ovarian traits, but did not investigate the effect of socio-sexual stimuli on pituitary gonadotrophic hormone concentrations. The objectives of the current study were to determine if 2 different levels of socio-sexual stallion stimuli under natural photoperiod could hasten the vernal transition period and advance the date of first ovulation in mares. Furthermore, this study aimed to elucidate the effects of stallion exposure on LH and prolactin (PRL) concentrations. We hypothesized that the date of first ovulation would be hastened for mares directly exposed to the stallion, compared with mares that were indirectly exposed or with mares that had no stallion exposure. Furthermore, we hypothesized that LH and PRL would increase shortly after the onset of stallion exposure.

Materials and Methods

This experiment was conducted from February through May of 2016 at Kansas State University's Horse Teaching and Research Unit (39.225705° N, 96.600015° W). The experimental procedures described herein were approved by the KSU Institutional Animal Care and Use Committee.

Animals and Housing

Non-lactating, stock-type, anestrus mares ($n = 21$) were enrolled in the experiment, in addition to 1 Quarter Horse stallion. Age of mares used in this experiment ranged from 3 to 17 y, with an average of 4.4 ± 0.9 y. The average weight and BCS of mares used in this experiment were 490.9 ± 8.7 kg and 5.1 ± 0.1 , respectively. Mares were housed in dry lots without covering and allowed *ad libitum* access to brome grass hay and water. A commercial concentrate (Purina

Strategy, Purina Mills, St. Louis, MO) was provided to mares once daily to meet NRC requirements. Natural lighting was the only light source for the dry lot pens in which the mares were housed. Average duration of daylight at this location was 10.8, 12.0, and 13.3 h for February, March, and April, respectively. Mean daily temperature for each month was 39.2, 50.0, and 57.4°F for February, March, and April, respectively.

Experimental Design

Beginning February 15th and continuing through the first ovulation of the year for each mare, follicular activity was evaluated thrice weekly (M, W, F) via transrectal ultrasonography (Medison Sonovet SV- 600, Universal Medical Systems, Inc., Bedford Hills, NY) to confirm anestrus status and monitor follicular development. Anestrus was defined as the absence of follicles ≥ 20 mm in diameter coupled with the absence of a corpus luteum. Upon confirmation of anestrus, mares were stratified by parity and age and assigned to 1 of 3 treatments (n = 7/treatment): (1) DXP (direct exposure; mares housed adjacent to stallion and allowed direct contact for 2 h thrice weekly); (2) IXP (indirect exposure; mares housed adjacent to stallion but not allowed direct contact); and (3) CON (control; mares housed on opposite side of facility from stallion with no stallion contact). Mares in the DXP and IXP treatments were kept in dry lot pens adjacent to the stallion with a 3-m gap between fence lines. The DXP mares did not have direct contact with IXP mares. One IXP mare was not in anestrus so she was removed from the study.

Beginning February 23rd (d 0), the stallion was placed thrice weekly (T, Th, Sa) in a 3.1 m x 3.1 m stallion cage in the center of a 21.6 m x 22.6 m dry lot tease pen and the DXP group was moved into the tease pen for 2 h beginning at 0800 h. Mares could move freely in the tease pen and interact with the caged stallion during the 2 h period. The design of the stallion cage within the larger tease pen allowed the stallion to make direct contact (sniffing, licking, or biting)

with mares, but not mount the mares. Control and IXP mares remained in their pens during the treatment sessions. During the direct exposure sessions, the activity and behavior of the DXP mares and stallion was monitored constantly by the same observer to ensure safety of the animals and observe estrual behaviors. Once DXP mares ovulated, the individual mare was no longer moved into the tease pen during direct exposure sessions but remained housed in the DXP pen. Ovulation, defined as the disappearance of the dominant follicle and subsequent confirmation of a CL, served as the study end-point for each mare.

Sample Collection and Analysis

Blood samples were collected from all mares during the first, third, and fifth DXP exposure sessions (Feb. 23, Feb. 27, and Mar. 3). Beginning at 0800 h, just before DXP mares were moved into the tease pen, and continuing until 1500 h, 10 mL blood samples were collected once per h from each mare into evacuated, heparinized tubes (Vacutainer, Beckton, Dickson, and Company, Franklin Lakes, NJ) via jugular venipuncture. Samples were refrigerated immediately after collection at 4 °C until centrifugation. Whole blood was centrifuged at 1400 x *g* at room temperature for 15 min to separate plasma. Plasma was pipetted into 1.5 mL microcentrifuge tubes and frozen at -18°C for later analysis of LH and PRL.

Quantification of LH and PRL concentrations were performed at Louisiana State University using established radioimmunoassay techniques [205,206]. Intra-assay and inter-assay coefficients of variation and assay sensitivity were 6%, 9%, and 0.2 ng/mL, respectively, for LH; 7%, 12%, and 0.2 ng/mL, respectively, for PRL. To begin the 3-d protocol, 100 µL of sample plasma is added to 100 µL of a PBS gel solution with 0.05% Triton X 100. A set of systems tubes also was added to the beginning and the end of the assay. These tubes include: a total count tube (radiolabeled hormone only), non-specific binding (radiolabeled hormone added to normal

rabbit serum), buffer control (radiolabeled hormone and antibody added to 0 standard control), 8 sets of standards tubes (eight-point curve generated from an equine pituitary preparation), and high and low internal controls of known hormone concentration. All tubes (samples and systems) were duplicated in order to obtain the intra-assay coefficient of variation. Once all tubes were prepared, 200 μ L of specific primary antibody was added. The primary antibody for LH was a rabbit anti-ovine LH and the PRL antibody was rabbit anti-porcine PRL. The tubes were then incubated at room temperature overnight. The following day, 200 μ L of radiolabeled hormone was added and incubated at room temperature overnight. On the final day, 200 μ L of the precipitating second antibody (anti-rabbit gamma globulin) was added. Samples were then incubated for 90 min at room temperature. After incubation, cold phosphate-buffered saline (PBS) with polyethylene glycol (PEG) was added and samples were centrifuged for 30 min and the supernatant was decanted. Addition of PBS with PEG, centrifugation, and decanting was performed a second time, then the radioactivity of the remaining pellet was measured in a gamma counter.

Statistical Analysis

All statistical analyses were performed using SAS v 9.3 (SAS Inst., Cary, NC). Data were considered significant when $P \leq 0.05$ and a trend was noted if $0.05 < P \leq 0.10$. Continuous numeric response outcomes (interval from the first day of stallion exposure to ovulation, number of follicular waves, and diameter of the ovulatory follicle) were analyzed via ANOVA as a one-way treatment structure in a completely randomized design. Results are reported as least squares means $\pm$ SEM and *P*-values derived from ANOVA *F*-tests.

Concentrations of LH and PRL were analyzed as a one-way treatment structure in a completely randomized design with repeated measures using the MIXED procedure.

Autoregressive of order 1 (AR-1) was chosen as the repeated measures analysis for LH and PRL concentrations because it yielded the lowest AIC. The model included treatment ($n = 3$), exposure d ($n = 3$), hour nested within d, and mare ($n = 20$) as a random effect. Treatments were tested by the split-plot error term (mare within treatment) and all interactions were tested by the whole-plot error. Results from the MIXED procedure include least squares means $\pm$ SEM for treatment and treatment by time interactions, and P -values derived from ANOVA F -tests.

Results

Ovulatory Data

Diameter of the ovulatory follicle assessed during the last observation before ovulation did not differ ($P > 0.05$) among treatments (Table 2.1). The interval from the first d of stallion exposure (d 0) to ovulation did not differ ($P > 0.05$; Table 2.1) among treatments. The average date of first ovulation was March 30th for DXP and IXP mares and April 12th for CON mares. The number of follicular waves, defined as emergence of a follicle ≥ 25 mm that subsequently regressed, from the onset of treatments to ovulation did not differ ($P > 0.05$; Table 2.1) among treatments. During each direct exposure session, mares were constantly observed to determine degree of interaction with the stallion. When analyzed separately, the 4 individuals that monopolized the majority ($> 85\%$) of direct exposure time with the stallion, termed Alpha DXP, had a shorter ($P < 0.05$) intervals (23.8 ± 3.2 d) from initiation of treatment to ovulation compared with the remaining DXP mares (53.0 ± 6.8 d) that got little, if any, direct contact with the stallion during direct exposure sessions, termed Beta DXP. Furthermore, Alpha DXP mares had fewer ($P < 0.05$) follicular waves from the start of treatment to ovulation compared with Beta DXP mares, having 1.75 ± 0.5 and 4.0 ± 0.6 waves, respectively.

Table 2.1 – Ovulatory data for anestrus mares housed on opposite side of farm from the stallion and not allowed visual or direct contact (CON), housed adjacent to a stallion but not allowed direct contact (IXP), or housed adjacent to, and allowed direct contact with, a stallion for 2 h thrice weekly (DXP).

Parameters (units)	Treatments ¹		
	CON	IXP	DXP
OV Interval (d) ²	48.3±4.2	42.8±4.2	36.3±6.7
FW (#) ³	3.7±0.4	2.7±0.4	2.7±0.6
OFD (mm) ⁴	44.8±2.3	46.5±2.9	42.1±3.3

¹DXP and CON (n = 7), IXP (n = 6)

²Interval from the first d of stallion exposure (Feb. 23) to ovulation.

³Number of follicular waves from the first d of stallion exposure until ovulation.

⁴Ovulatory Follicular Diameter of the first ovulation.

Hormonal Data

No significant effects of treatment or h on LH concentrations were identified. A significant exposure d effect was identified, with LH concentrations being greater ($P < 0.05$) on the fifth exposure d compared with the first and third (Fig. 2.1). Concentrations of LH averaged 0.36 ± 0.18 , 0.38 ± 0.18 , and 0.87 ± 0.17 ng/mL for the first, third, and fifth exposure d, respectively.

A significant interaction between treatment, day, and h was identified for PRL. Average concentrations of PRL across all 3 exposure days were greater ($P < 0.05$) in IXP compared with DXP and CON, being 3.1 ± 0.4 , 5.3 ± 0.5 , and 3.6 ± 0.4 ng/mL for DXP, IXP, and CON, respectively (Fig. 2.2). Greater ($P < 0.05$) PRL concentrations were observed on the third exposure day (5.6 ± 0.4 ng/mL) compared with the first (3.1 ± 0.4 ng/mL) and fifth days (3.4 ± 0.4 ng/mL).

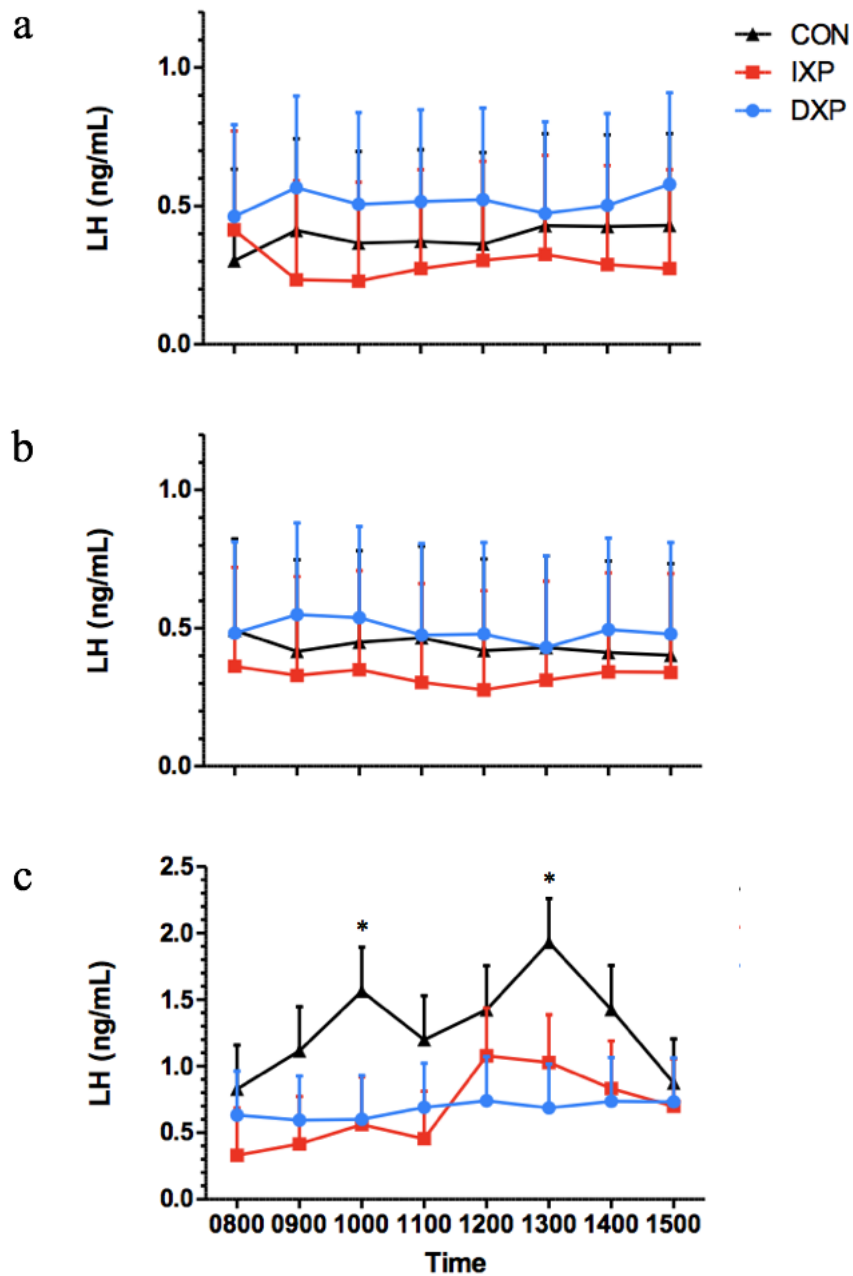


Figure 2.1 - Concentrations of plasma LH for anestrus mares housed adjacent to, and allowed direct contact with, a stallion for 2 h thrice weekly (DXP; $n = 7$), housed adjacent to a stallion but not allowed direct contact (IXP; $n = 6$), or housed on opposite side of farm from the stallion and not allowed visual or direct contact (CON; $n = 7$) on the first (Feb. 23; a), third (Feb. 27; b), and fifth (Mar. 3; c) exposure d. The stallion and DXP mares were moved into the tease pen after the sample collection at 0800 and they were removed from the tease pen and returned to regular housing after the 1000 sample collection. *CON was greater than DXP and IXP ($P < 0.05$).

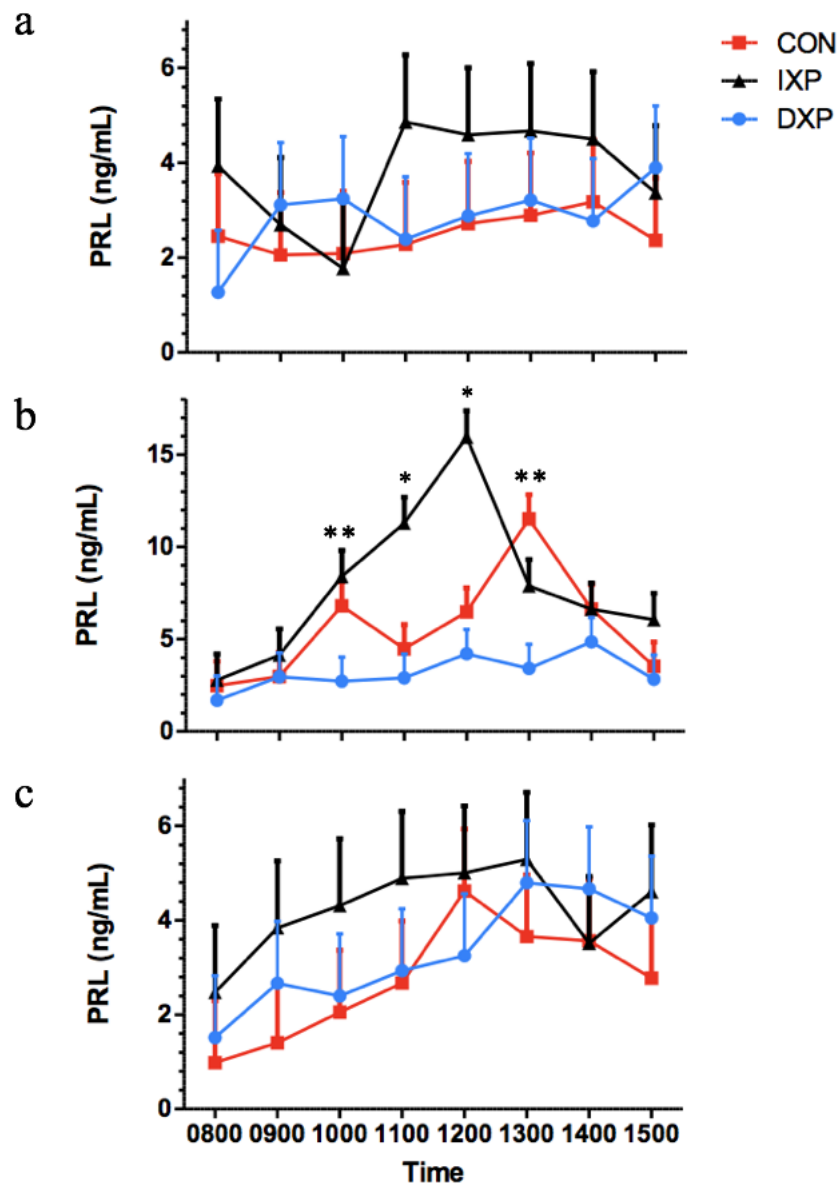


Figure 2.2 - Concentrations of PRL for anestrus mares housed adjacent to, and allowed direct contact with, a stallion for 2 h thrice weekly (DXP; $n = 7$), housed adjacent to a stallion but not allowed direct contact (IXP; $n = 6$), or housed on opposite side of farm from the stallion and not allowed visual or direct contact (CON; $n = 7$) on the first (Feb. 23; a), third (Feb. 27; b), and fifth (Mar. 3; c) exposure d. The stallion and DXP mares were moved into the tease pen after the sample collection at 0800 and they were removed from the tease pen after the 1000 sample collection. *IXP greater than DXP and CON ($P < 0.05$); **IXP and CON were greater ($P < 0.05$) than DXP.

Discussion

The effects of socio-sexual stimuli on anestrus females has been well-documented in short-day breeders, such as sheep and goats. The first report of male introduction stimulating ovulation in anestrus females, or the “ram effect,” was documented nearly 75 years ago [207]. The effects of socio-sexual stimuli in anestrus mares, however, has not been studied until recently, as most socio-sexual research in horses has focused on hormonal changes and ejaculate quality of stallions in response to intact mares or mare urine collected during different phases of the estrous cycle.

The first ovulation following ram introduction to anestrus ewes usually occurs by 30 to 72 h with an average of 50 to 65 h [208,209,210]. Given that mares have a much larger preovulatory follicle diameter compared with ewes, we anticipated a longer interval from the start of exposure to ovulation. With the exception of 2 DXP mares that had but one follicular wave before ovulation, all mares had 2 to 5 follicular waves between the start of treatment to ovulation. The average number of follicular waves that occur in transitional mares preceding the first ovulation is 3 to 4 waves [77]. In this study, the average number of follicular waves was slightly lower in both the DXP and IXP mares, whereas the average for CON mares corroborated with previous reports. Ewes that are within 2 to 4 wk of spontaneous ovulation respond more efficiently to male exposure compared with ewes in deep anestrus [210,211]. In the current study, stallion exposure began 6 wk from the average date of first ovulation for mares in the Northern hemisphere (Apr 7) and 2 mo after the winter solstice; therefore, it is unlikely that treatment was initiated while mares were considered to be in deep anestrus [212].

Exposure to stallions significantly advanced the date of first ovulation by approximately 20 d in anestrus mares and consequently resulted in a greater number of ovulations during the

observational period compared with unexposed controls [204]. Several noteworthy differences in experimental design existed between the aforementioned and current study [204]. The first major difference was in housing scheme. Mares in the previous study [204] were housed indoors in two identical barns for 21 h per day and were exposed to both natural and artificial lighting, whereas the mares in the current study were housed outdoors under natural lighting only. The exact artificial lighting program used in the previous study was not mentioned. Evidence exists that environmental temperature influences the date of first ovulation and that mares housed indoors may have an earlier onset of estrus compared with mares housed outdoors, presumably because of the difference in ambient temperature [213,214]. Differences in housing between the two studies could explain why a significant difference in date of first ovulation was only observed in the previous study [204].

The exposure protocol also differed notably between the two studies. The earlier study [204] utilized a teasing protocol that allowed the stallion to move freely down the corridor of the barn, whereas the current study utilized a stallion cage and mares were allowed to freely move toward and interact with the stallion. Furthermore, the previous study limited direct contact with a metal grate in between the mares and stallion, whereas the design of the stallion cage in the present study allowed for the stallion to lick, sniff, or bite the mares. Another major difference between the two studies was that the prior study was conducted during 2 different years, resulting in a greater number of replicates, whereas the current study was conducted during a single year. Had the present study utilized a greater number of mares, it is possible that a significant difference in the date of first ovulation between exposed mares and controls could have been detected.

In goats, the risk for ovulation was increased and time to ovulation was decreased after buck exposure among does that were more dominant in the social hierarchy. Does that ranked higher on the displacement index spent more time with and interacted sooner with bucks than those that ranked lower [215]. In the current study, among the 7 DXP mares, the 4 mares classified as Alpha DXP monopolized the majority (>85%) of the stallion's time during the direct exposure sessions by remaining at the stallion cage throughout the session and discouraging the Beta DXP mares from approaching. Of the 3 mares classified as Beta DXP, two were never observed spending time in direct contact with the stallion. The dominance status of the mares observed during the direct exposure sessions was maintained during times when the mares were not allowed direct contact with the stallion. For example, the Alpha DXP mares displayed more agonistic behavior towards their pen-mates, such as displacing, kicking, and biting, than the Beta DXP mares in the group housing scenario. The differential amount of time spent in direct contact with the stallion between the two subgroups might provide an explanation for the decreased interval from the start of treatment to ovulation observed in the Alpha DXP mares. The observation that Alpha DXP mares had fewer follicular waves during the interval from treatment initiation to ovulation compared with Beta DXP mares coincides logically. This observation may indicate that direct (i.e., tactile and olfactory inputs) rather than indirect contact (i.e., auditory and visual) may be more critical to hastening the first ovulation.

In anestrus ewes [216,217] and goat does [218,219], basal LH concentration and LH pulse frequency increases within minutes following exposure to sexually active males. The preovulatory LH surge occurs in anovulatory ewes approximately 22 to 26 h after ram introduction [211]. This phenomenon was not observed in the present study with mares. Frequency of sampling in the present study was not designed to detect changes in LH pulse

frequency. Despite mares, ewes, and does all being seasonally polyestrous, there seems to be physiological differences between mares and small seasonal ruminants with regard to strength of stimuli provided by male presence. Several studies have shown that olfactory cues alone are sufficient to stimulate anestrus ewes and that deprivation of smell significantly impairs the effects of rams on anestrus ewes [220,221]. Wax and wool, but not urine, from the rams were sufficient to stimulate anestrus ewes [222]. To the author's knowledge, research regarding reproductive traits and the effect of olfactory communication between stallions and mares has been limited to feces and urine, rather than any potential communication via coat, hair, or sweat glands, to which mares had access to during this study. It is possible that pheromone detection in equids is mostly limited to excreta, which the mares did not have direct access to during the study.

Considering the lack of a treatment by day interaction, the greater LH concentrations observed on the fifth exposure day, which was 9 d after the beginning of treatment, most likely resulted from the slight progressive increase in LH as the transition period advanced. In mares, slight increases (≤ 2.0 ng/mL) in LH occur as the mare moves into mid- to late transition [32]. On the fifth exposure day, the elevated LH observed in CON mares compared with IXP and DXP at 1000 and 1300 h may be random or because of the infrequent sampling. Concentrations of PRL did not differ between the treatments at any time during the sampling periods. In contrast, greater PRL concentrations were observed during the third exposure session in IXP and CON mares compared with the first and the fifth. Given that there was only an effect of day and not a treatment by day interaction, some external factor outside of the study design is the likely explanation. Several environmental factors have been shown to alter PRL concentrations in horses, including season, feeding, exercise, sexual excitement, disease, stall confinement,

trailing, and various other stressful stimuli [65]. No notable differences in the management routine of the mares were detected during any of the 3 sampling periods. Two IXP mares were observed continuously pacing the fence line while displaying signs of estrus during the direct exposure sessions and had greater PRL concentrations at the 1100 and 1200 time points compared with other mares, which could provide an explanation for the elevated PRL concentrations observed in that treatment on the third exposure day. The stressful stimuli of blood collection could be another explanatory factor, as certain mares in both the CON and IXP groups become more refractory to being restrained for blood collection as the experiment progressed. If refractoriness to sample collection, however, was the cause of the elevated PRL on the third exposure d, we would expect PRL to also be elevated on the fifth exposure d. On the other hand, a noteworthy difference between the 3 sampling periods was ambient temperature. The average daily temperature was 11, 22, and 14 °C on the first, third, and fifth exposure d. Reports in heifers have demonstrated that plasma PRL concentration increases as the temperature increases [223,224]. Therefore, it would be plausible that the difference in PRL concentrations observed during the third exposure session could be explained by the increased ambient temperature, but the same degree of elevation was not observed in all 3 treatments.

Conclusions

Direct or indirect exposure of anestrus mares to a stallion, directly or indirectly, failed to elicit an increase in basal LH or PRL concentrations. Further research with more frequent sampling is warranted to determine if LH pulse frequency is altered by exposure, as demonstrated in other seasonally polyestrous livestock species. The limited research investigating the male effect in anestrus mares is conflicting with regard to advancing the date of first ovulation, although the present study may have been in agreement with the previous

report had a higher statistical power been obtained. Based on the differences observed between the Alpha DXP and Beta DXP mares, additional research investigating the physiological differences between species with regard to socio-sexual stimuli is warranted. Group teasing might not be effective if subordinate mares get little, if any, direct contact time with the stallion. Individual teasing may be more beneficial; however, more research must be conducted to investigate the duration and frequency of contact, as well as timing of treatment initiation, necessary to elicit a hastening of ovulation.

Chapter 3 - An opioid antagonist, either alone or in combination with a GnRH agonist, alters gonadotropin and ovarian responses in transitional mares exposed to various progestin environments

Introduction

The physiological mechanisms controlling seasonality in horses, particularly mares, are poorly understood. Several management and pharmaceutical techniques have been investigated during the last 40 years, not only to improve our understanding of the seasonal changes in the secretion patterns of the hypothalamic-pituitary-gonadal axis, but also to help breeders become more efficient in producing foals that are born earlier than the natural foaling season so that they may have a competitive advantage when competing against their age-matched cohorts.

The use of progesterone and progestins in transitional mares for durations of 7 to 15 d has resulted in estrus within 3 to 10 d and subsequent ovulation by 12 to 21 d after withdrawal; however, the response rate was variable [187,188,191]. For this reason, P4/progestin therapy is usually combined with other therapies, such as artificial lighting or GnRH agonists, in a clinical setting. Although relatively effective in transitional mares, progestin therapy does not result in a return to estrus or ovulation in deep anestrus mares [187,191]. Given that progestin treatment is only effective in transitional mares, it seems that changes in inhibitory tone on the hypophysis may be requisite.

Endogenous opioid peptides (EOP) have been shown to be inhibitory to gonadotropin secretion at the level of the hypophysis and potentially at the ovarian level [179,180]. Furthermore, opioidergic tone changes throughout the estrous cycle and differs among reproductive seasons of the mare [179,180,184]. Changes occurring in opioidergic tone during

the estrous cycle seem to be dependent on gonadal steroids [180,181]. In contrast, inhibition of gonadotropins by EOP is greatest during deep anestrus and seems to be independent of steroid hormone input from the ovaries [183,184,185]. Evidence of involvement of EOP in seasonality at the ovarian level also has been indicated by the seasonal fluctuation of μ -opioid receptor content in oocytes, cumulus cells, and granulosa cells [186]. Furthermore, it has been demonstrated that addition of β -endorphin to media containing oocytes harvested from anestrus and transitional mares retards their rate of maturation rate of oocytes harvested from anestrus and transitional mares *in vitro* [186]. Although more is known about opioidergic inhibition during anestrus and the breeding season, little is known about the shift in opioidergic tone associated with the vernal transition period.

Because EOP antagonists elicit a gonadotropin response during diestrus, which is characterized by a high progesterone and low E2 environment, 1 objective of this study was to determine if the gonadotropin response to an opioid antagonist would occur in transitional mares administered an exogenous progestin. Furthermore, we aimed to compare that response to an opioid antagonist administered to transitional mares during a low progesterone/high E2 milieu, just before the first ovulation of the year, and under a high endogenous progesterone milieu during the first luteal phase. In order to assess pituitary responsiveness, a second objective of this study was to determine if gonadotropin and ovarian responses differed between mares administered the opioid antagonist, a GnRH agonist, or a combination of both under the 3 different steroid hormone milieus.

Materials and Methods

This experiment was conducted during March through May of 2017 at Kansas State University's Horse Teaching and Research Unit (39.225705° N, 96.600015° W). The

experimental procedures described herein were approved by the Institutional Animal Care and Use Committee of Kansas State University.

Animals

Seventeen non-lactating, stock-type mares between the ages of 4 and 15 y were utilized in this study, with an average age of 8.8 ± 3.9 y. Seven of the mares were nulliparous. At the beginning of the experiment, average body weight was 533.4 ± 27.7 kg with an average body condition score (BCS) of 5.4 ± 0.5 on a scale of 1 to 9 as described by Henneke et al. (1983). Mares were group-housed in 2 adjacent dry lots and allowed *ad libitum* access to brome grass hay and water. Mares were fed a commercial concentrate (Purina Strategy, Purina Mills, St. Louis, MO) once daily to meet NRC requirements.

Experimental Design

At the start of the trial, follicular activity was monitored for all mares thrice weekly via transrectal ultrasonography using a Chison ECO 5 ultrasound machine (Chison Medical Imaging Co., Jiangsu, China) with a Chison 7.5 MHz probe (Chison Medical Imaging Co., Jiangsu, China). A mare was considered to be transitional when at least 3 follicles ≥ 20 mm were observed. Upon attainment of transitional status, mares received 0.044 mg/kg oral altrenogest (Regu-Mate, Merck Animal Health, Madison, NJ) per os daily between 0700 and 0900 h for 15 consecutive d. Average start date of altrenogest treatment was March 20 ± 9.3 d.

At the conclusion of altrenogest treatment, mares were stratified by age and parity and assigned to 3 treatments: (1) DES (1.8 mg of desorelin acetate IM; Sucromate[®], Thorn Bioscience, LLC, Louisville, KY; n = 6); (2) NAL (0.5 mg/kg BW naloxone hydrochloride dihydrate IV; Sigma, St. Louis, MO; n = 5); or (3) N&D (0.5 mg/kg BW naloxone hydrochloride dihydrate IV and 1.8 mg deslorelin acetate IM; n = 6). Naloxone was administered at 0 min to

NAL and N&D mares. Deslorelin was administered at 45 min to DES and N&D mares. Mares received their assigned treatment 3 times during the experimental period. Phase 1 (P1) treatment occurred on the last d of altrenogest administration (3 h after altrenogest was given). Phase 2 (P2) treatment occurred upon first detection of a follicle ≥ 35 mm in diameter after concluding the 15-d altrenogest treatment. Phase 3 (P3) treatment occurred 7 d post-ovulation. During the experimental period, follicular activity was monitored every other d until P2 treatment administration, at which point follicular activity was monitored every 12 h until ovulation. Presence of a corpus luteum was confirmed 48 h post-ovulation. Two mares, 1 DES and 1 N&D, ovulated by 48 h after P1 treatment, and therefore did not receive P2 treatment; however, P3 treatment still occurred for those mares.

Beginning 24 h after P2 treatment administration, mares were bred via artificial insemination using fresh semen from one stallion of known fertility. Semen was extended at a 1:1 ratio using INRA 96 (IMV Technologies, Normandy, France) at a dose of 500 million progressively motile sperm. Mares were bred every 48 h until ovulation occurred. Pregnancy diagnosis was performed 14 d post-ovulation, marking the conclusion of the experimental period for each mare.

Sample Collection and Analysis

During all phases of treatment, indwelling jugular catheters were placed in mares using a lidocaine bleb for local analgesia at least 1 h before sample collection began. For mares in all treatment groups, 10 mL of whole blood were collected from the catheters every 15 min beginning 60 min (-60 min) prior to naloxone administration (0 min) to NAL and N&D mares and continuing through 300 min post-treatment, for a total of 6 h. Whole blood was collected into heparinized, evacuated blood tubes (Vacutainer, Becton, Dickson, and Company, Franklin

Lakes, NJ) containing 142 USP units of freeze-dried sodium heparin. Samples were refrigerated immediately after collection at 4 °C until centrifugation. Whole blood was centrifuged at 1400 x *g* at room temperature for 15 min to harvest plasma. Plasma was pipetted into 1.5 mL microcentrifuge tubes and frozen at -18 °C for later analysis of LH and FSH concentrations. Furthermore, progesterone concentrations were quantified in all -60 min samples from each phase and the 0 min samples were analyzed for E2 concentrations. Whole blood was collected via jugular venipuncture prior to the start of altrenogest to assess progesterone and confirm the absence of luteal structures.

Quantification of LH and FSH concentrations were performed at Louisiana State University using established radioimmunoassay (RIA) techniques [205]. Intra-assay and inter-assay coefficients of variation and sensitivity were 6%, 9%, and 0.2 ng/mL, respectively, for LH; 7%, 11%, and 1.4 ng/mL, respectively, for FSH. Quantification of progesterone concentrations in pre-altrenogest samples, P1 (-60 min), P2 (-60 min) and P3 (-60 min) were analyzed using a commercially available kit (ImmunoChem Double Antibody I¹²⁵ RIA Kit, MP Biomedicals, LLC, Orangeburg, NY). Quantification of E2 concentrations was performed on ether-extracted samples using a commercially available kit (17 β -Estradiol Double Antibody RIA Kit, MP Biomedicals, LLC, Orangeburg, NY). The intra-assay CV was 8.4% for E2.

Statistical Analyses

All data were analyzed using SAS v. 9.3 (SAS Inst. Inc., Cary, NC). Data were considered significant when $P \leq 0.05$ and a trend was noted if $0.05 \leq P \leq 0.10$. Continuous numeric response variables (interval from P1 to P2 and interval from P2 to ovulation) were analyzed via ANOVA as a one-way treatment structure in a completely randomized design. Results reported are least squares means $\pm$ SEM and *P*-values for treatment contrasts were

derived from ANOVA *F*-tests. Count responses, number of mares ovulating by 48 h of P2 treatment administration and number of mares with a positive pregnancy diagnosis were analyzed as a chi-square test for homogeneity by the FREQ procedure using the exact option to account for small sample size. Results reported are counts and percentages with *P*-values for chi-square tests.

Concentrations of LH and FSH were analyzed as a one-way treatment structure in a completely randomized design with repeated measures using the MIXED procedure. Autoregressive of order 1 (AR-1) was chosen as the repeated measures analysis for LH and FSH concentrations because it yielded the lowest AIC. The model included treatment (*n* = 3), phase (*n* = 3), sampling time (min) nested within phase, and mare (random effect). Additional analysis of P2 LH and FSH concentrations included pretreatment E2 concentrations as a covariable in the model. Additional analysis of P3 LH and FSH concentrations included progesterone concentrations as a covariable in the model. Results from the mixed procedure include least squares means $\pm$ SEM for treatment by phase by time interactions and *P*-values derived from ANOVA *F*-tests.

Results

Ovarian Data

One N&D and one DES mare developed large follicles during altrenogest treatment and ovulated 48 h after P1 treatment administration. These mares did not receive P2 treatment to avoid any potential carryover effect from P1 treatment, but did receive P3 treatment 7 d post-ovulation. These mares were not included in the analysis for interval from P1 to P2 or interval from P2 treatment to ovulation.

The interval from P1 to P2 treatment was shorter ($P < 0.05$) for N&D mares compared with NAL mares, but was not different ($P > 0.05$) between N&D and DES or DES and NAL mares (Table 3.1). The interval from P2 treatment to ovulation did not differ ($P > 0.05$) between the treatment groups (Table 3.1). The percentage of mares ovulating by 48 h of P2 treatment did not differ ($P > 0.10$; Table 3.1). Across all treatments, a greater ($P < 0.001$) number of mares ovulated by 48 h of treatment when pretreatment E2 concentrations were greater than the mean (6 of 7 mares) compared with mares that had pretreatment estradiol concentrations below the mean (1 of 8 mares). Diameter of the dominant follicle at the last observation before ovulation after P2 treatment did not differ ($P > 0.05$) between the groups (Table 3.1). Pregnancy rate was not different ($P > 0.10$) among treatments (Table 3.1).

Table 3.1 - Transitional mare ovulatory and follicular traits after treatment with deslorelin acetate (DES), naloxone hydrochloride (NAL), or a combination of both (N&D) on the last day of altrenogest administration (Phase 1) and again upon detection of a 35 mm follicle (Phase 2).

Parameters (units)	Treatments ¹		
	DES	NAL	N&D
P1 to P2 interval (d)	10.0±3.5 ^{ab}	12.0±3.9 ^b	7.0±1.0 ^a
P2 to OV interval (h)	184.8±81.6	105.6±24.7	112.8±40.6
OV 48 (%) ²	60	20	60
OFD (mm) ³	42.2±5.9	44.6±2.6	48.6±5.9
Pregnant (%) ⁴	80	80	60

Values presented as mean ± SEM.

¹N&D 0.5 mg/kg BW naloxone hydrochloride IV at 0 min followed by 1.8 mg deslorelin acetate IM at 45 min; DES 1.8 mg deslorelin acetate IM at 45 min; NAL 0.5 mg/kg BW naloxone hydrochloride IV at 0 min.

²Percentage of mares ovulating within 48 h of Phase 2 treatment administration.

³Ovulatory Follicular Diameter.

⁴Percentage of mares pregnant 14 d post-ovulation.

^{a,b} Values in a row lacking a common superscript differ ($P < 0.05$).

Steroid Hormones

Progesterone concentrations for all mares before P1 and P2 treatment were either below detectable limits (< 0.1 ng/mL) or biologically insignificant (< 0.14 mg/mL, $n = 2$). Progesterone concentrations 7 d after ovulation before P3 treatment did not differ between groups ($P > 0.05$).

As expected, there was a significant effect of phase on pretreatment E2 concentrations, with P2 mares having greater ($P < 0.01$) concentrations of E2 compared with P1 and P3.

Treatment tended ($P = 0.09$) to affect E2 concentrations. Pretreatment E2 concentrations did not differ ($P > 0.1$) between treatments before P1 treatment (Fig. 3.1). Mares in the N&D and NAL groups had greater ($P < 0.01$) E2 concentrations before treatment in P2 compared with DES mares (Fig. 3.1). Mares in the N&D group tended ($P = 0.08$) to have greater E2 concentrations compared with DES and NAL mares before P3 treatment (Fig. 3.1).

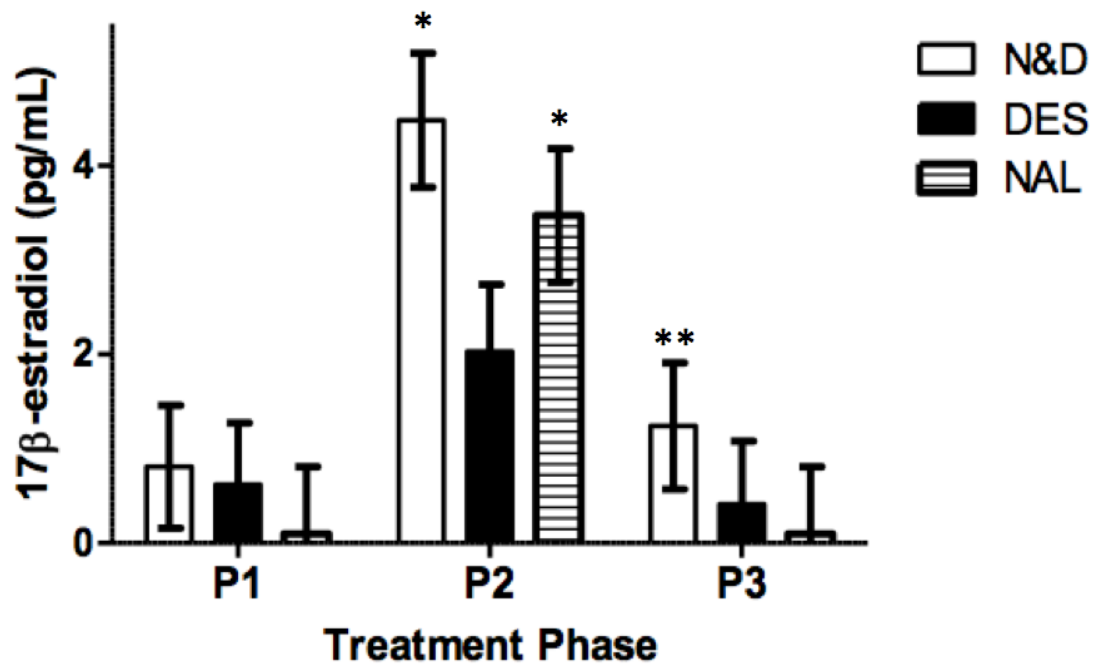


Figure 3.1 – Pretreatment estradiol-17 β concentrations in transitional mares treated with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) on the final d of a 15-d altrenogest supplementation period (P1), upon first detection of a follicle ≥ 35 mm (P2), and 7 d post-ovulation (P3). Estradiol concentrations did not differ ($P > 0.05$) among treatments before P1 treatment. *Estradiol concentrations for mares receiving NAL and N&D were greater ($P < 0.05$) than DES before P2 treatment. **Estradiol concentrations tended ($P = 0.08$) to be greater in N&D mares compared with NAL, but not DES before P3 treatment.

Pituitary Hormones

Significant ($P < 0.05$) effects of phase and time on LH concentrations were identified, with treatment eliciting a greater response in P3 compared with P1 and P2. During P1, treatment with N&D increased ($P < 0.05$) and DES tended ($P < 0.1$) to increase LH concentrations, whereas NAL did not ($P > 0.1$; Fig. 3.2). During P2, LH was increased ($P < 0.05$) by treatment with N&D and DES, but not NAL ($P > 0.1$; Fig. 3.3). All 3 treatments resulted in an increase ($P < 0.05$) in LH during P3 treatment (Fig. 3.4).

Significant effects of treatment ($P < 0.05$), phase ($P < 0.01$), and time ($P < 0.01$) on FSH were identified. A greater ($P < 0.05$) FSH response to treatment was observed during P1 and P3 compared with P2. During P1, FSH increased to a greater ($P < 0.05$) extent in both N&D and DES compared with NAL mares (Fig. 3.5). During P2 and P3, a greater ($P < 0.05$) FSH response was observed in DES compared with NAL, but not N&D (Figs. 3.6, 3.7).

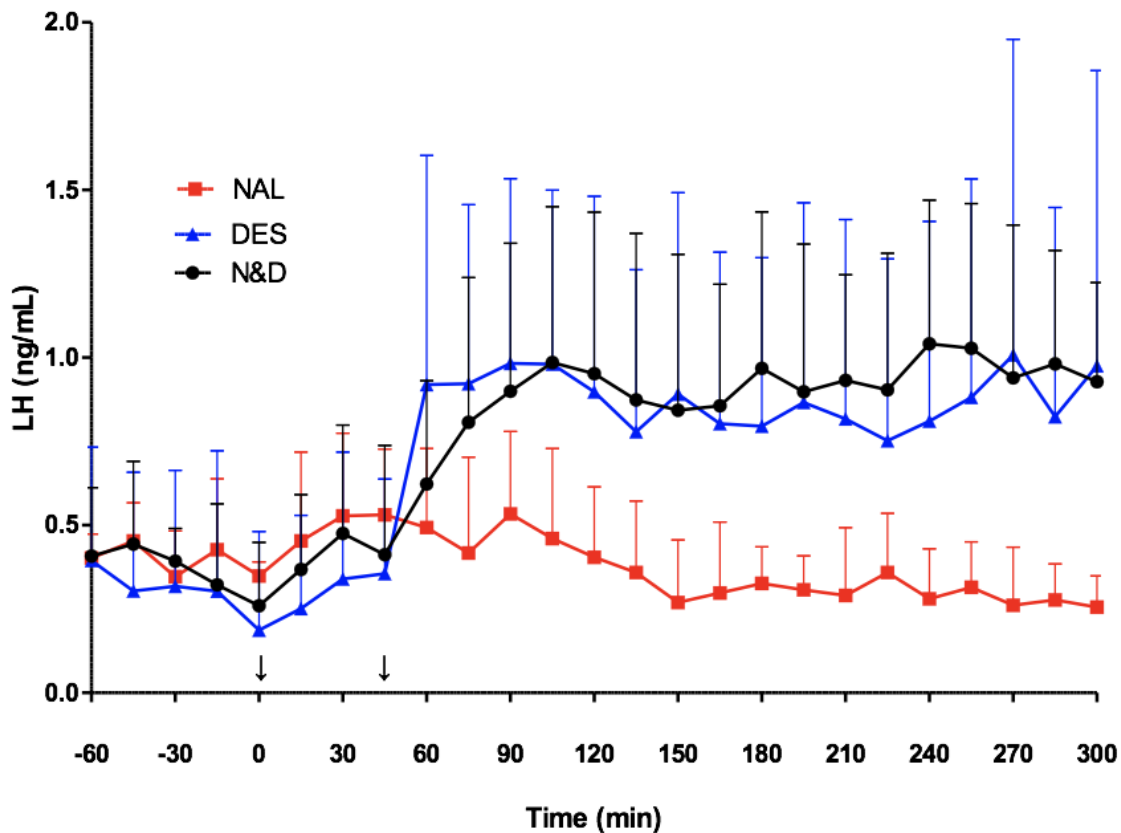


Figure 3.2 – Concentrations of LH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) on the last d of 15-d altrenogest supplementation period (P1). Naloxone was administered to NAL and N&D mares at 0 min. Deslorelin was administered to DES and N&D at 45 min. Treatment with N&D increased ($P < 0.05$) and DES tended ($P < 0.1$) to increase LH concentrations, whereas NAL did not ($P > 0.1$).

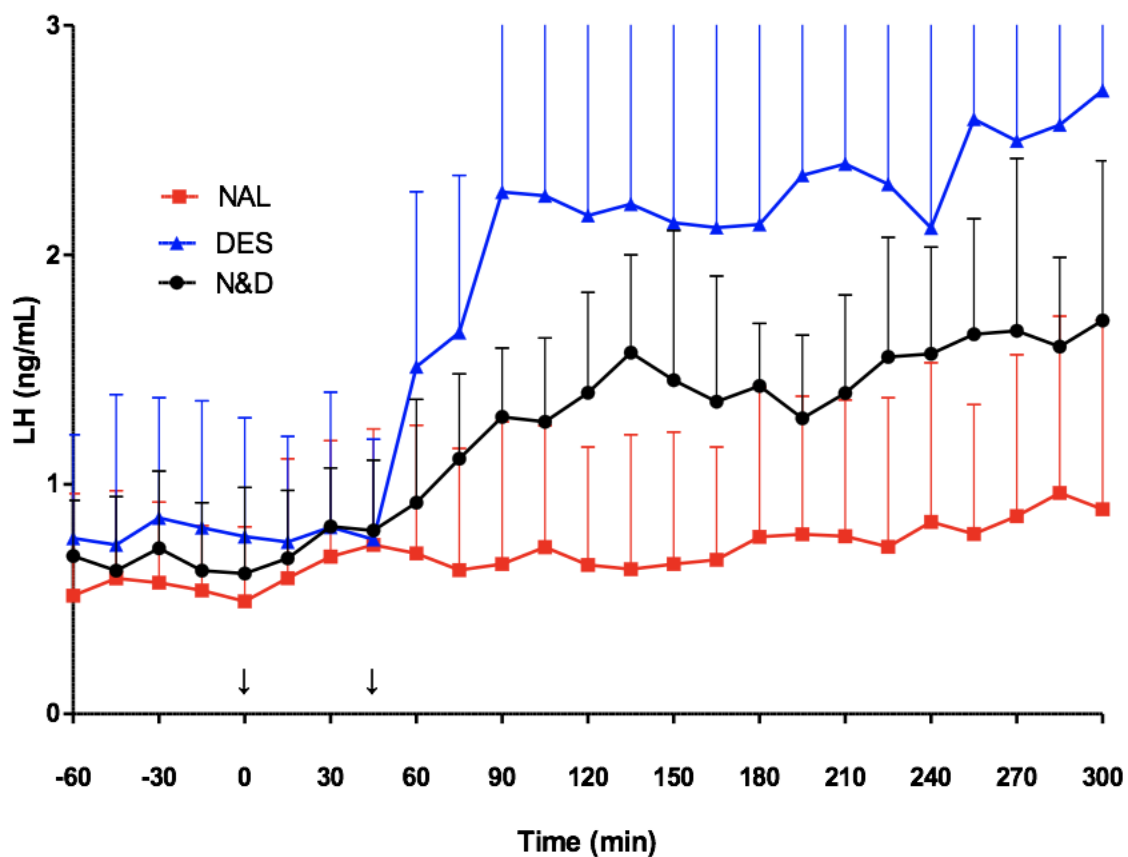


Figure 3.3 - Concentrations of LH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) upon detection of a follicle ≥ 35 mm in diameter (P2). Naloxone was administered to NAL and N&D at 0 min. Deslorelin was administered to DES and N&D mares at 45 min. Treatment with N&D and DES increased ($P < 0.05$) LH, but not NAL ($P > 0.1$).

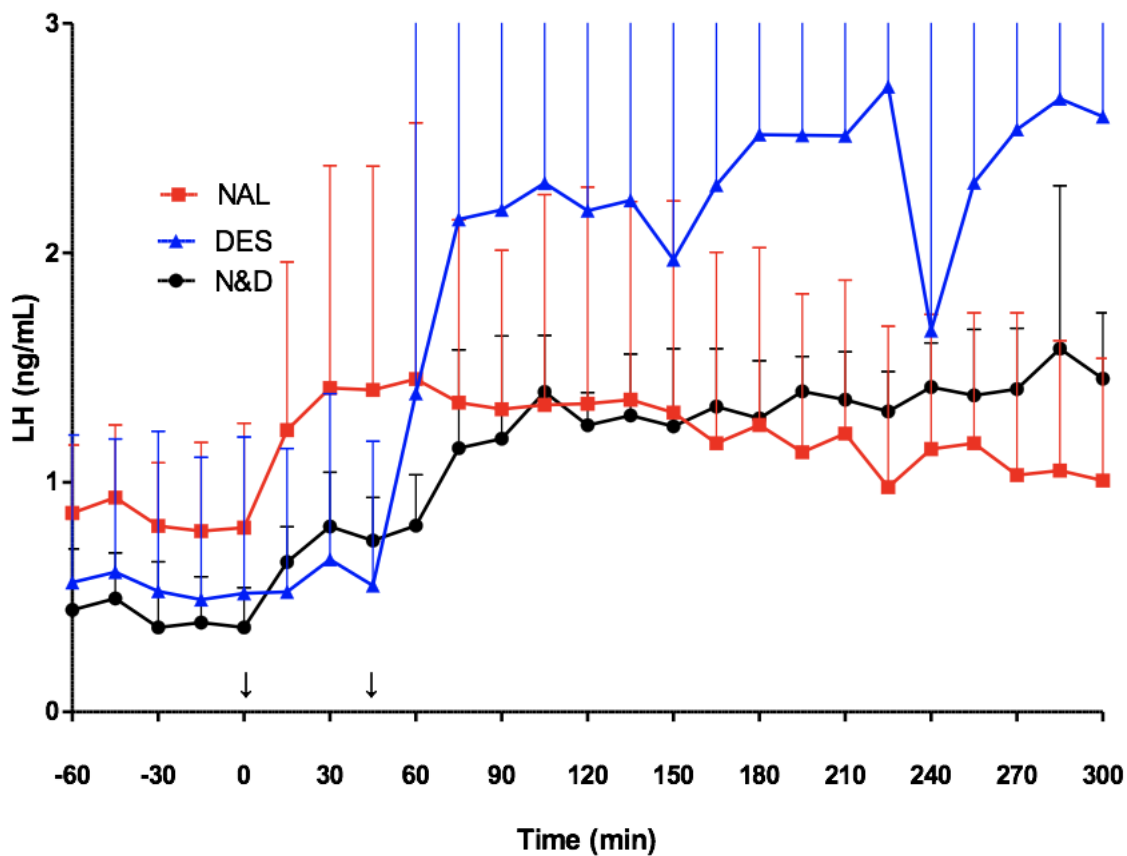


Figure 3.4 - Concentrations of LH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) 7 d post-ovulation (P3). Naloxone was administered to NAL and N&D at 0 min. Deslorelin was administered to DES and N&D mares at 45 min. All 3 treatments resulted in an increase ($P < 0.05$) in LH.

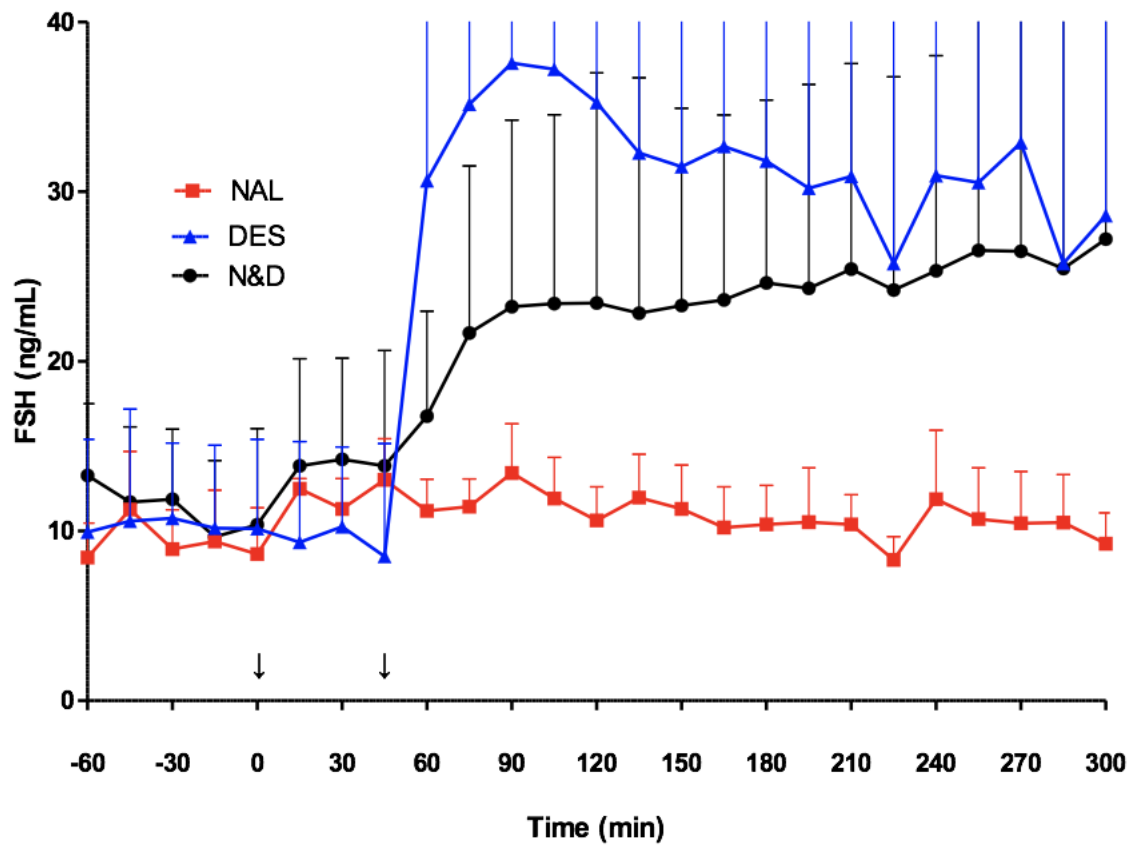


Figure 3.5 - Concentrations of FSH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) on the last d of altrenogest administration (P1). Naloxone was administered to NAL and N&D at 0 min. Deslorelin was administered to DES and N&D mares at 45 min. FSH increased to a greater ($P < 0.05$) extent in both N&D and DES compared with NAL mares.

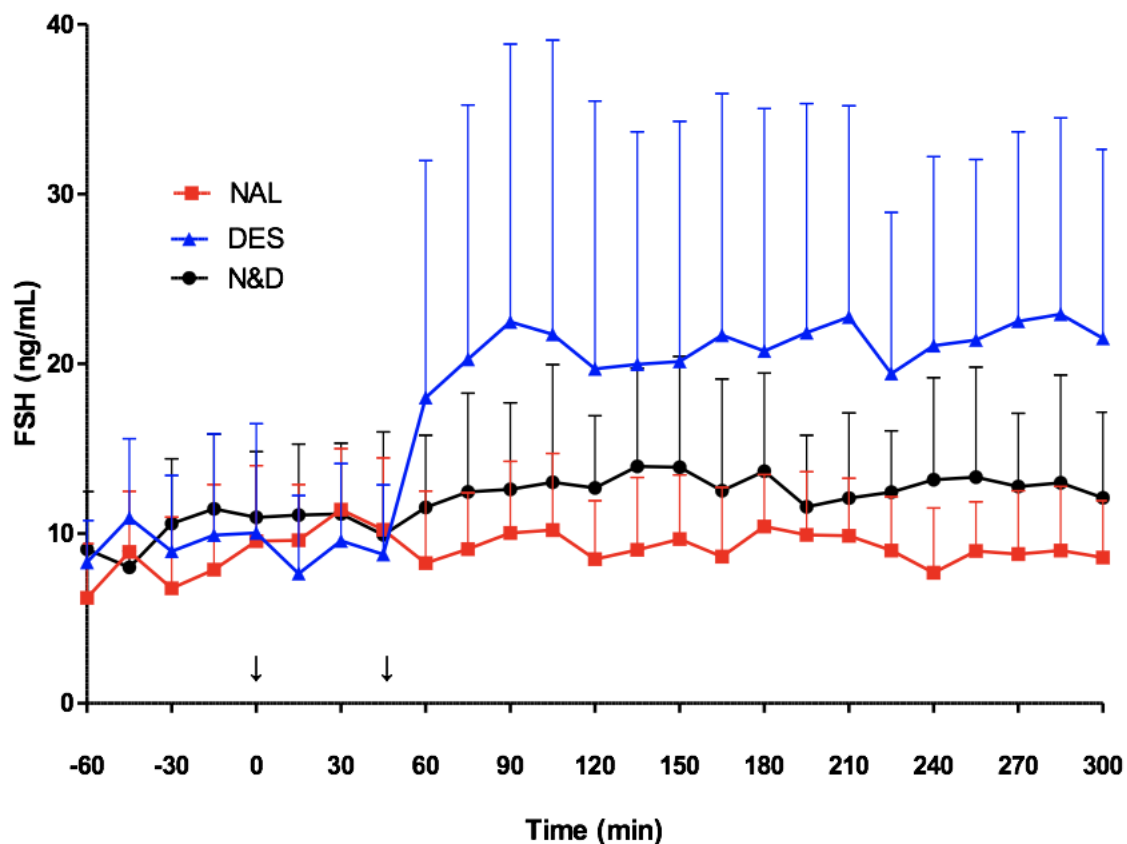


Figure 3.6 - Concentrations of FSH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) upon detection of a follicle ≥ 35 mm in diameter (P2). Naloxone was administered to NAL and N&D at 0 min. Deslorelin was administered to DES and N&D mares at 45 min. A greater ($P < 0.05$) FSH response was observed in DES compared with NAL, but not N&D.

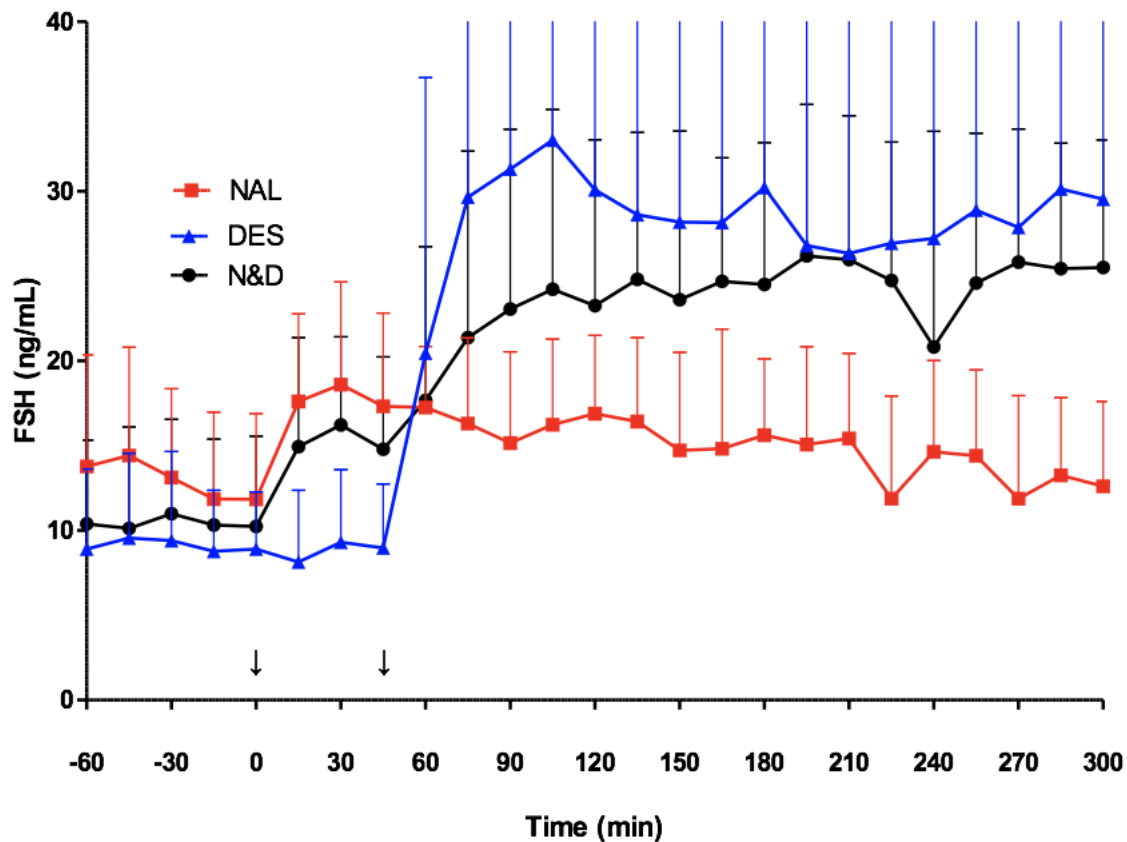


Figure 3.7 - Concentrations of FSH in transitional mares after treatment with deslorelin (DES), naloxone (NAL), or a combination of both (N&D) 7 d post-ovulation (P3). Naloxone was administered to NAL and N&D at 0 min. Deslorelin was administered to DES and N&D mares at 45 min. A greater ($P < 0.05$) FSH response was observed in DES compared with NAL, but not N&D.

Discussion

In an elevated exogenous progestin environment, naloxone alone failed to elicit an increase in LH and FSH. Previous studies administering naloxone to anestrus mares resulted in an increase in both of the gonadotropic hormones [183,184]. The dose of naloxone used in the current study was the same as or greater than the doses utilized in the previous studies. This indicates that opioidergic inhibition of gonadotropin hormones during the vernal transition may be largely reduced from that during anestrus. Because E2 concentrations before both P1 and P2

treatment administration did not differ, it does not seem that the E2 environment was responsible for the lack of gonadotropin response during P1. Therefore, altrenogest may not interact with the opioidergic system in the same manner as endogenous P4. The relative binding activity of altrenogest is only 45 and 20% in the hypothalamus and pituitary, respectively [198]. It is possible that altrenogest is not able to sufficiently mimic at the level of the hypophysis the elevated P4 environment typical of the luteal phase of the estrous cycle.

Although the interval from P1 to P2 was shorter in N&D mares compared with NAL alone, it was not different from DES, which was intermediate. Therefore, it does not seem that short-term suppression of EOP alone is sufficient to hasten estrus in transitional mares exposed to altrenogest. Naloxone alone did not result in increased LH or FSH concentrations during P2 treatment. These results are consistent with reports showing no gonadotropin response to naloxone during the follicular phase in cyclic mares [179,180]. The criteria for P2 treatment, development of a follicle ≥ 35 mm, would place mares in the category of late transitional. Thus, the results might indicate that the opioid inhibition of gonadotropin secretion is largely removed by this point. Consistent with reports in luteal phase mares, both LH and FSH were increased in response to naloxone in N&D and NAL [179,180]. This observation indicates that the EOP-mediated suppressive effect of P4 on gonadotropic hormones during the first luteal phase of the season is functional.

Deslorelin, either alone or in combination with naloxone, increased LH and FSH during all phases. Furthermore, with the exception of LH during P1, a more robust LH and FSH response was observed in DES compared with N&D. A study in cyclic mares using the GnRH analogue buserelin 2 h after administration of naloxone or saline demonstrated that the magnitude of response to buserelin was not different between mares that had previously been

given naloxone or those that had received saline in either the luteal or follicular phases [179]. Therefore, it does not seem likely that prior naloxone treatment elicits any suppressive effects of gonadotropin release in response to the GnRH agonist. Because pituitary LH content decreases through the fall transition into anestrus, and remains low throughout the vernal transition, it is possible that the intermediate LH response to deslorelin observed in the N&D group could be a result of less pituitary LH content being available for release after the initial increase in response to naloxone.

Regardless of treatment, mares ovulated on average 11 to 18 d after progestin withdrawal. These results are similar to reports in the literature utilizing various natural or synthetic progestin sources in transitional mares with a return to estrus approximately 7 to 10 d after withdrawal and ovulation occurring 12 to 24 d after P4 or progestin removal [187,188,191]. Therefore, the addition of a single treatment with deslorelin, naloxone, or a combination of both does not hasten the interval from withdrawal of progestin compared with progestin alone. One study [225] enrolled a large number of transitional mares with pregnancy risk as an outcome after intravaginal P4 treatment for 12 d, followed by either hCG or subcutaneous deslorelin implant upon detection of a 30-mm follicle and subsequent insemination. The authors reported an overall pregnancy risk of 56% in mares from the first breeding following administration of hCG or deslorelin compared with 44% of mares that ovulated unassisted following P4 removal [225]. Although the overall pregnancy risk was greater in that study at 73%, it likely resulted from mares being bred every 48 h until ovulation rather than just once. Pregnancy risk obtained in the present study indicates that treatment with naloxone or deslorelin did not negatively impact subsequent fertility of the first ovulation of the year.

Opioids have been shown to have suppressive effects on E2 secretion in women [226] and female rats [227]. Furthermore, administration of opioid antagonists reverses the suppressive effects of EOP on E2 [228,229]. Localization of μ -opioid receptors in the ovaries of mares, particularly in the granulosa cells, cumulus cells, and in the oocyte itself has been reported [186]. Furthermore, receptor content fluctuates with season, with greatest concentrations being observed during winter anestrus. Using oocytes harvested from mares during anestrus and the vernal transition period, one group noted that addition of EOP to the culture resulted in a slower rate of maturation, indicating that EOP are suppressive to the meiotic competence of oocytes [186]. The current study found that E2 concentrations were greater before P2 treatment in N&D and NAL mares compared with DES, and tended to be greater in the combination-treated mares before P3 treatment compared with both DES and NAL. It is possible that EOP may have a suppressive effect on steroidogenesis; however, this has not yet been studied in the mare. Based on previous work and the potential interaction between EOP and E2 observed in the current study, further investigation is warranted. Additional post-treatment samples from P2 and P3 are currently being analyzed for estradiol to determine if treatment with naloxone had any acute effects on E2 secretion.

Conclusions

Inhibition of gonadotropin hormones by EOP seems to wane as mares progress from anestrus through the vernal transition, as indicated by the lack of gonadotropin response to naloxone alone during both P1 and P2 treatment. Furthermore, an elevated exogenous progestin milieu in transitional mares does not produce the same response to naloxone as the high endogenous P4 environment during the luteal phase. Although the combination of naloxone and deslorelin failed to elicit a greater LH and FSH response compared with deslorelin alone, it did

result in a shorter interval from progestin removal to development of a 35-mm follicle. Compared with progestin treatment alone, it does not seem that deslorelin, naloxone, or a combination of the two hastens ovulation after progestin removal. It is unlikely that naloxone itself would be beneficial in an applied setting for transitional mares unless repeated injections proved to be more effective, which in any case would be cost-prohibitive. Nevertheless, further elucidation of how opioidergic systems interact with the hypothalamic-pituitary-axis would further our understanding of the physiological mechanisms involved in endocrine changes associated with seasonality in the mare.

References

- [1] Murphy, BA. Circadian and circannual regulation in the horse: Internal timing in an elite athlete. *J Equine Vet Sci* 2019; 76:14-21.
- [2] Sharp, DC. Vernal Transition into the Breeding Season. In: McKinnon, AO, Squires, EL, Vaala, WE, & Varner, DD, editors. *Equine reproduction*, United Kingdom: John Wiley & Sons; 2011, p. 1705-15.
- [3] Aurich, C. Reproductive cycles of horses. *Anim Reprod Sci* 2011; 124:220-28.
- [4] Hattar, S, Liao, HW, Takao, M, Berson, DM, & Yau, KW. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science* 2002; 295:1065-70.
- [5] Sharp, DC. Photoperiod. In: McKinnon, AO, Squires, EL, Vaala, WE, & Varner, DD, editors. *Equine reproduction*, United Kingdom: John Wiley & Sons; 2011, p. 1771-77.
- [6] Moore, RY, & Lenn, NJ. A retinohypothalamic projection in the rat. *J Comp Neurol* 1972; 146:1-14.
- [7] Rudeen, PK, Reiter, RJ, & Vaughan, MK. Pineal serotonin-N-acetyltransferase activity in four mammalian species. *Neurosci Lett* 1975; 1:225-29.
- [8] Nonno, R, Capsoni, S, Lucini, V, Møller, M, Fraschini, F, & Stankov, B. Distribution and characterization of the melatonin receptors in the hypothalamus and pituitary gland of three domestic ungulates. *J Pineal Res* 1995; 18:207-16.
- [9] Malpaux, B, Thiéry, JC, & Chemineau, P. Melatonin and the seasonal control of reproduction. *Reprod Nutr Dev* 1999; 39:355-66.

- [10] Sharp, DC. The effects of artificial lighting on reproduction. In: Robinson, NE, editor. Current Therapy in Equine Medicine, Philadelphia: Saunders; 1983, p. 399-401.
- [11] Ginther, OJ, Whitmore, HL, & Squires, EL. Characteristics of estrus, diestrus, and ovulation in mares and effects of season and nursing. *Am J Vet Res* 1972;33:1935-39.
- [12] Heidler, B, Aurich, JE, Pohl, W, & Aurich, C. Body weight of mares and foals, estrous cycles and plasma glucose concentration in lactating and non-lactating Lipizzaner mares. *Theriogenology* 2004;61:883-93.
- [13] van Niekerk, CH, Morgenthal, JC, & Gerneke, WH. Relationship between the morphology of and progesterone production by the corpus luteum of the mare. *J Rep Fer S* 1975;23:171.
- [14] Roberto da Costa, RP, Branco, V, Pessa, P, Silva, JR, & Ferreira-Dias, G. Progesterone receptors and proliferating cell nuclear antigen expression in equine luteal tissue. *Reprod Fert Develop* 2005;17:659-66.
- [15] Lofstedt, RM. Diestrus. In: McKinnon, AO, Squires, EL, Vaala, WE, & Varner, DD, editors. *Equine reproduction*, United Kingdom: John Wiley & Sons; 2011, p. 1728-31.
- [16] Karsch, FJ, Cummins, JT, Thomas, GB, & Clarke, IJ. Steroid feedback inhibition of pulsatile secretion of gonadotropin-releasing hormone in the ewe. *Biol Reprod* 1987;36:1207-18.
- [17] Ginther, OJ, Utt, MD, Bergfelt, DR, & Beg, MA. Controlling interrelationships of progesterone/LH and estradiol/LH in mares. *Anim Reprod Sci* 2006;95:144-50.
- [18] Alexander, SL & Irvine, CHG. FSH and LH. In: McKinnon, AO, Squires, EL, Vaala, WE, & Varner, DD, editors. *Equine reproduction*, United Kingdom: John Wiley & Sons; 2011, p. 1619-30.

- [19] Aurich, C. Reproductive cycles of horses. *Anim Reprod Sci* 2011;124:220-28.
- [20] Daels, PF, Ammon, DC, Stabenfeldt, GH, Liu, IKM, Hughes, JP, & Lasley, BL. Urinary and plasma estrogen conjugates, estradiol and estrone concentrations in nonpregnant and early pregnant mares. *Theriogenology* 1991;35:1001-17.
- [21] Daels, PF, DeMoraes, JJ, Stabenfeldt, GH, Hughes, JP, & Lasley, BL. The corpus luteum: source of oestrogen during early pregnancy in the mare. *J Rep Fer S* 1991;44:501-08.
- [22] Ginther, OJ, Gastal, EL, Gastal, MO, Utt, MD, & Beg, MA. Luteal blood flow and progesterone production in mares. *Animal reproduction science* 2007;99:213-20.
- [23] Boerboom, D, Brown, KA, Vaillancourt, D, Poitras, P, Goff, AK, Watanabe, K, Doré, M & Sirois, J. Expression of key prostaglandin synthases in equine endometrium during late diestrus and early pregnancy. *Biol Reprod* 2004;70:391-99.
- [24] Bae, SE & Watson, ED. A light microscopic and ultrastructural study on the presence and location of oxytocin in the equine endometrium. *Theriogenology* 2003;60:909-21.
- [25] Makawiti, D. W., Allen, W. E., & Kilpatrick, M. J. Changes in oestrone sulphate concentrations in peripheral plasma of pony mares associated with follicular growth, ovulation and early pregnancy. *Reproduction* 1983;68:481-87.
- [26] Ndiaye, K, Fayad, T, Silversides, DW, Sirois, J, & Lussier, JG. Identification of downregulated messenger RNAs in bovine granulosa cells of dominant follicles following stimulation with human chorionic gonadotropin. *Biol Reprod* 2005;73:324-33.
- [27] Ginther, O, Beg, M, Donadeu, F, & Bergfelt, D. Mechanism of follicle deviation in monovular farm species. *Anim Reprod Sci* 2003;78:239-57.
- [28] Evans, A. Characteristics of ovarian follicle development in domestic animals. *Reprod Domest Anim* 2003;38:240-6.

- [29] Gastal, EL, Gastal, MO, Bergfelt, DR, & Ginther, OJ. Role of diameter differences among follicles in selection of a future dominant follicle in mares. *Biol Reprod* 1997;57:1320-27.
- [30] Ireland, J, Mihm, M, Austin, E, Diskin, M, & Roche, J. Historical perspective of turnover of dominant follicles during the bovine estrous cycle: Key concepts, studies, advancements, and terms. *J Dairy Sci* 2000;83:1648-58.
- [31] Ginther, OJ, Beg, MA, Bergfelt, DR, Donadeu, FX, and Kot, K. Follicle selection in monovular species. *Biol Reprod* 2001;65:638-47.
- [32] Donadeu, FX & Ginther, OJ. Follicular waves and circulating concentrations of gonadotrophins, inhibin and oestradiol during the anovulatory season in mares. *Reproduction* 2002;124:875-85.
- [33] Bergfelt, DR, Gastal, EL, & Ginther, OJ. Response of estradiol and inhibin to experimentally reduced luteinizing hormone during follicle deviation in mares. *Biol Reprod* 2001;65:426-32.
- [34] Belin, F, Goudet, G, Duchamp, G, & Gerard, N. Intrafollicular concentrations of steroids and steroidogenic enzymes in relation to follicular development in the mare. *Biol Reprod* 2000;62:1335-43.
- [35] Hodgen, GD. The dominant ovarian follicle. *Fertil Steril* 1982;38:281–300.
- [36] Mihm, M, Good, TEM, Ireland, JLH, Ireland, JJ, Knight, PG, & Roche, JF. Decline in serum follicle-stimulating hormone concentrations alters key intrafollicular growth factors involved in selection of the dominant follicle in heifers. *Biol Reprod* 1997;57:1328–37.

- [37] Jacob, JC, Gastal, EL, Gastal, MO, Carvalho, GR, Beg, MA, & Ginther, OJ. Temporal relationships and repeatability of follicle diameters and hormone concentrations within individuals in mares. *Reprod Domest Anim* 2009;44:92-99.
- [38] Senger, PL. The Follicular Phase. In: *Pathways to Pregnancy and Parturition*, Washington: Current Conceptions, Inc.; 2005, p. 164-187.
- [39] Yoon, MJ. The estrous cycle and induction of ovulation in mares. *J Anim Sci Technol* 2012;54:165-74.
- [40] Stout, TAE. Prostaglandins. In: McKinnon, AO, Squires, EL, Vaala, WE, & Varner, DD, editors. *Equine reproduction*, United Kingdom: John Wiley & Sons; 2011, p. 1642-47.
- [41] Townson, D & Ginther, O. Ultrasonic characterization of follicular evacuation during ovulation and fate of the discharged follicular fluid in mares. *Anim Reprod Sci* 1989;20:131-41.
- [42] Townson, D & Ginther, O. Duration and pattern of follicular evacuation during ovulation in the mare. *Anim Reprod Sci* 1987;15:131-38.
- [43] Carnevale, E, McKinnon, A, Squires, E, and Voxx, J. Ultrasonographic characteristics of the preovulatory follicle preceding and during ovulation in mares. *J Equine Vet Sci* 1988;8:428- 31.
- [44] King, SS. Autumnal Transition Out of the Breeding Season. In: McKinnon, AO, Squires, EL, Vaala, WE, & Varner, DD, editors. *Equine reproduction*, United Kingdom: John Wiley & Sons; 2011, p. 1732-53.
- [45] King, SS, Neumann, KR, Nequin, LG, & Weedman, BJ. Time of onset and ovarian state prior to entry into winter anestrus. *J Equine Vet Sci* 1993;13:512-15.

- [46] Nunes, MM, Gastal, EL, Gastal, MO, Rocha Filho, AN, Mellagi, AP, & Ginther, OJ. Follicle and gonadotropin relationships during the beginning of the anovulatory season in mares. *Anim Reprod* 2005;2:41-49.
- [47] Gentry, LR, Thompson Jr, DL, Gentry Jr, GT, Davis, KA, Godke, RA, & Cartmill, JA. The relationship between body condition, leptin, and reproductive and hormonal characteristics of mares during the seasonal anovulatory period. *J. Anim Sci* 2002;80: 2695-703.
- [48] King, SS, Nequin, LG, Drake, S, Hebner, TS, Roser, JF, & Evans, JW. Progesterone levels correlate with impending anestrus in the mare. *J Equine Vet Sci*, 1988;8:109-11.
- [49] Hart, PJ, Squires, EL, Imel, KJ, & Nett, TM. Seasonal variations in hypothalamic content of gonadotropin-releasing hormone (GnRH), pituitary receptors for GnRH, and pituitary content of luteinizing hormone and follicle-stimulating hormone in the mare. *Biol Reprod* 1984;30:1055-62.
- [50] Nequin, LG, King, SS, Roser, JF, Soderstrom, BL, Carnevale, EM, & Neumann, KR. Uncoupling of the equine reproductive axes during transition into anoestrus. *J Reprod Fertil S* 2000;56:153-161.
- [51] Murphy, BD & Rajkumar, K. Prolactin as a luteotropin. *Can J Physiol Pharm* 1985;63:257-64.
- [52] Fitzgerald, BP, Davison, LA, & McManus, CJ. Evidence for a seasonal variation in the ability of exogenous melatonin to suppress prolactin secretion in the mare. *Domest Anim Endocrinol* 2000;18:395-408.

- [53] King, SS, Jones, KL, Dille, EA, & Roser, JF. Prolactin receptors in the corpus luteum of mares. In: Proceedings of the 15th International Congress on Animal Reproduction 2004, p.39.
- [54] Weedman, BJ, King, SS, Neumann, KR & Nequin, LG. Comparison of circulating estradiol-17 β and folliculogenesis during the breeding season, autumn transition and anestrus in the mare. *J Equine Vet Sci* 1993;13:502-05.
- [55] Irvine, CH, Alexander, SL & McKinnon, AO. Reproductive hormone profiles in mares during the autumn transition as determined by collection of jugular blood at 6 h intervals throughout ovulatory and anovulatory cycles. *J Reprod Fertil* 2000;118:101-110.
- [56] King, SS, Douglas, BL, Roser, JF, Silvia, WJ & Jones, KL. Differential luteolytic function between the physiological breeding season, autumn transition and persistent winter cyclicity in the mare. *Anim Reprod Sci* 2010;117:232-40.
- [57] King, SS, Campbell, AG, Dille, EA, Roser, JF, Murphy, LL & Jones, KL. Dopamine receptors in equine ovarian tissues. *Domest Anim Endocrinol* 2005;28:405-15.
- [58] King, SS, Jones, KL, Mullenix, BA & Heath, DT. Seasonal relationships between dopamine D1 and D2 receptor and equine FSH receptor mRNA in equine ovarian epithelium. *Anim Reprod Science* 2008;108:259-66.
- [59] Ginther, OJ, Woods, BG, Meira, C, Beg, MA & Bergfelt, DR. Hormonal mechanism of follicle deviation as indicated by major versus minor follicular waves during the transition into the anovulatory season in mares. *Reproduction* 2003;126:653-60.
- [60] Nunes, MM, Gastal, EL, Gastal, MO & Rocha, AN. Influence of the autumn transitional phase on follicular development in mares. *Theriogenology* 2002;58:603-06.

- [61] Collins, SM, Zieba, DA & Williams, GL. Continuous administration of low-dose GnRH in mares: II. Pituitary and ovarian responses to uninterrupted treatment beginning near the autumnal equinox and continuing throughout the anovulatory season. *Theriogenology* 2007;68:673-81.
- [62] Silvia, PJ, Squires, EL & Nett, TM. Changes in the hypothalamic-hypophyseal axis of mares associated with seasonal reproductive recrudescence. *Biol Reprod* 1986;35:897-905.
- [63] Freedman, LJ, Garcia, MC & Ginther, OJ. Influence of photoperiod and ovaries on seasonal reproductive activity in mares. *Biol Reprod* 1979;20:567-74.
- [64] Cooper, DA. Reproductive neuroendocrine function in the mare as reflected in the intercavernous sinus during ovulatory, anovulatory, and transitional seasons. MS Thesis, Texas A&M University 2006.
- [65] Thompson Jr, DL, Johnson, L, George, RL & Garza Jr, F. Concentrations of prolactin, luteinizing hormone and follicle stimulating hormone in pituitary and serum of horses: effect of sex, season and reproductive state. *J Anim Sci* 1986;63:854-60.
- [66] Turner, DD, Garcia, MC & Ginther, OJ. Follicular and gonadotropic changes throughout the year in pony mares. *Am J Vet Res* 1979;40:1694-700.
- [67] Donadeu, FX & Ginther, OJ. Follicular waves and circulating concentrations of gonadotrophins, inhibin and oestradiol during the anovulatory season in mares. *Reproduction* 2002;124:875-85.
- [68] Donadeu, FX & Ginther, OJ. Interactions of follicular factors and season in the regulation of circulating concentrations of gonadotrophins in mares. *Reproduction* 2003;125:743-50.

- [69] Heidler, B, Aurich, JE, Pohl, W & Aurich, C. Body weight of mares and foals, estrous cycles and plasma glucose concentration in lactating and non-lactating Lipizzaner mares. *Theriogenology* 2004;61:883-93.
- [70] Nagy, P, Huszenicza, G, Juhasz, J, Kulcsar, M, Solti, L, Reiczigel, J & Abavary, K. Factors influencing ovarian activity and sexual behavior of postpartum mares under farm conditions. *Theriogenology* 1998;50:1109-19.
- [71] Bergfelt, DR & Ginther, OJ. Delayed follicular development and ovulation following inhibition of FSH with equine follicular fluid in the mare. *Theriogenology* 1983;24:99-108.
- [72] Irvine, CHG. Endocrinology of the estrous cycle of the mare: applications to embryo transfer. *Theriogenology* 1981;15:85-104.
- [73] Strauss, SS, Chen, CL, Kalra, SP & Sharp, DC. Localization of gonadotrophin-releasing hormone (GnRH) in the hypothalamus of ovariectomized pony mares by season. *J Reprod Fertil S* 1979;27:123-29.
- [74] Sharp, DC & Grubbaugh, WR. Use of push-pull perfusion techniques in studies of gonadotrophin-releasing hormone secretion in mares. *J Reprod Fertil S* 1987;35:289-96.
- [75] Silvia, PJ, Meyer, SL & Fitzgerald, BP. Pulsatile gonadotropin secretion determined by frequent sampling from the intercavernous sinus of the mare: possible modulatory role of progesterone during luteolysis. *Biol Reprod* 1995;53:438-46.
- [76] Sherman, GB, Wolfe, MW, Farmerie, TA, Clay, CM, Threadgill, DS, Sharp, DC & Nilson, JH. A single gene encodes the beta-subunits of equine luteinizing hormone and chorionic gonadotropin. *Mol Endocrinol* 1992;6:951-59.

- [77] Ginther, OJ. Occurrence of anestrus, estrus, diestrus, and ovulation over a 12-month period in mares. *Am J Vet Res* 1974;35:1173.
- [78] Asa, CS, Goldfoot, DA, Garcia, MC & Ginther, OJ. Sexual behavior in ovariectomized and seasonally anovulatory pony mares (*Equus caballus*). *Horm Behav* 1980a;14:46-54.
- [79] Asa, CS, Goldfoot, DA, Garcia, MC & Ginther, OJ. Dexamethasone suppression of sexual behavior in the ovariectomized mare. *Horm Behav* 1980b;14:55-64.
- [80] Pope, NS, Sargent, GF & Kesler, DJ. The dexamethasone induced suppression of androgen secretions suppressed estrous behavior in pony mares during the winter. *J Equine Vet Sci* 1995;15:119-23.
- [81] Bergfelt, DR & Ginther, OJ. Embryo loss following GnRH-induced ovulation in anovulatory mares. *Theriogenology* 1992;38:33-43.
- [82] Watson, ED, Heald, M, Tsigos, A, Leask, R, Steele, M, Groome, NP & Riley, SC. Plasma FSH, inhibin A and inhibin isoforms containing pro-and- α C during winter anoestrus, spring transition and the breeding season in mares. *Reproduction* 2002;123:535-42.
- [83] Ginther, OJ. Folliculogenesis during the transitional period and early ovulatory season in mares. *Reproduction*, 1990;90:311-20.
- [84] Davis, SD, Grubbaugh, WR & Weithenauer, J. Follicle integrity and serum estradiol-17 β patterns during sexual recrudescence in the mare. *Biol Reprod S* 1987;36:121.
- [85] Tucker, KE, Cleaver, BD & Sharp, DC. Does resumption of follicular estradiol synthesis during vernal transition in mares involve a shift in steroidogenic pathways. *Biol Reprod S* 1993;48:188.

- [86] Kenney, RM, Condon, W, Ganjam, VK & Channing, C. Morphological and biochemical correlates of equine ovarian follicles as a function of their state of viability or atresia. *J Reprod Fertil S* 1979;27:163-71.
- [87] Channing, CP. Studies on tissue culture of equine ovarian cell types: pathways of steroidogenesis. *J Endocrinol* 1969;43:403-14.
- [88] Davis, SD & Sharp, DC. Intra-follicular and peripheral steroid characteristics during vernal transition in the pony mare. *J Reprod Fertil S* 1991;44:333-40.
- [89] Watson, ED, Thomassen, R, Steele, M, Heald, M, Leask, R, Groome, NP & Riley, SC. Concentrations of inhibin, progesterone and oestradiol in fluid from dominant and subordinate follicles from mares during spring transition and the breeding season. *Anim Reprod Sci* 2002;74:55-67.
- [90] Watson, ED, Bae, SE, Steele, M, Thomassen, R, Pedersen, HG, Bramley, T, Hogg, CO & Armstrong, DG. Expression of messenger ribonucleic acid encoding for steroidogenic acute regulatory protein and enzymes, and luteinizing hormone receptor during the spring transitional season in equine follicles. *Domest Anim Endocrinol* 2004;26:215-30.
- [91] Silvia, PJ, Johnson, L & Fitzgerald, BP. Changes in the hypothalamic–hypophyseal axis of mares in relation to the winter solstice. *Reproduction* 1992;96:195-202.
- [92] Sharp, DC, Grubbaugh, WR, Weithenauer, J, Davis, SD & Wilcox, CJ. Effects of steroid administration on pituitary luteinizing hormone and follicle-stimulating hormone in ovariectomized pony mares in the early spring: pituitary responsiveness to gonadotropin-releasing hormone and pituitary gonadotropin content. *Biol Reprod* 1991;44:983-90.

- [93] Sharp, DC, Wolfe, MW, Cleaver, BD & Nilson, J. Effects of estradiol-17 β administration on steady-state messenger ribonucleic acid (mRNA) encoding equine α and LH/CG β subunits in pituitaries of ovariectomized pony mares. *Theriogenology* 2001;55:1083-93.
- [94] Sharp, DC, Garcia, MC & Ginther, OJ. Luteinizing hormone during sexual maturation in pony mares. *Am J Vet Res* 1979;40:584-86.
- [95] Peltier, MR, Robinson, G & Sharp, DC. Effects of melatonin implants in pony mares 2. Long-term effects. *Theriogenology* 1998;49:1125-42.
- [96] d'Anglemont de Tassigny, X & Colledge, WH. The role of kisspeptin signaling in reproduction. *Physiology* 2010;25:207-17.
- [97] Decourt, C, Tillet, Y, Caraty, A, Franceschini, I & Briant, C. Kisspeptin immunoreactive neurons in the equine hypothalamus: interactions with GnRH neuronal system. *J Chem Neuroanat* 2008;36:131-37.
- [98] Magee, C, Foradori, CD, Bruemmer, JE, Arreguin-Arevalo, JA, McCue, PM, Handa, RJ, Squires, EL & Clay, CM. Biological and anatomical evidence for kisspeptin regulation of the hypothalamic-pituitary-gonadal axis of estrous horse mares. *Endocrinology* 2009;150:2813-21.
- [99] Caraty, A, Smith, JT, Lomet, D, Ben Said, S, Morrissey, A, Cognie, J, Doughton, B, Baril, G, Briant, C, & Clarke, IJ. Kisspeptin synchronizes preovulatory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. *Endocrinology* 2007;148:5258-67.
- [100] Smith, JT, Coolen, LM, Kriegsfeld, LJ, Sari, IP, Jaafarzadehshirazi, MR, Maltby, M, Bateman, K, Goodman, RL, Tilbrook, AJ, Ubuka, T, Bentley, GE, Clarke, IJ, & Lehman, MN. Variation in kisspeptin and RFamide-related peptide (RFRP) expression and

- terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. *Endocrinology* 2008;149:5770-82.
- [101] Magee, C, Bruemmer, JE, Arreguin-Arevalo, JA, Nett, TM & Clay, CM. Effects of repeated injection of kisspeptide in diestrous and estrous mares. *Anim Reprod Sci S* 2010; 121:45-46.
- [102] Decourt, CV, Merzouki, Y, Duchamp, G, Bruneau, B, Caraty, A & Briant, C. Effects of constant intravenous kisspeptin administration on luteinizing hormone release in cyclic mares. *Anim Reprod Sci S* 2010;121:65-67.
- [103] Magee, C, Bruemmer, JE, Nett, TM, Squires, EL & Clay, CM. Kisspeptide in the estrous mare: Is it an appropriate ovulation-inducing agent?. *Theriogenology*, 2012;78:1987-96.
- [104] Smith, JT. The role of kisspeptin and gonadotropin inhibitory hormone in the seasonal regulation of reproduction in sheep. *Domest Anim Endocrinol* 2012;43:75-84.
- [105] Smith, JT, Cunningham, MJ, Rissman, EF, Clifton, DK & Steiner, RA. Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology*, 2005;146:3686-92.
- [106] Dungan, HM, Gottsch, ML, Zeng, H, Gragerov, A, Bergmann, JE, Vassilatis, DK & Steiner, RA. The role of kisspeptin–GPR54 signaling in the tonic regulation and surge release of gonadotropin-releasing hormone/luteinizing hormone. *J Neurosci* 2007;27:12088-95.
- [107] Roseweir, AK, Kauffman, AS, Smith, JT, Guerriero, KA, Morgan, K, Pielecka-Fortuna, J & Terasawa, E. Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation. *J Neurosci* 2009;29:3920-29.

- [108] Decourt, C, Caraty, A, Briant, C, Guillaume, D, Lomet, D, Chesneau, D, Lardic, L, Duchamp, G, Reigner, F, Monget, P, Dufourny, L, Beltramo, M. & Dardente, H. Acute injection and chronic perfusion of kisspeptin elicit gonadotropins release but fail to trigger ovulation in the mare. *Biol Reprod* 2014;90:1-12.
- [109] McGrath, BM, Scott, CJ, Wynn, PC, Loy, J & Norman, ST. Kisspeptin stimulates LH secretion but not ovulation in mares during vernal transition. *Theriogenology* 2016;86:1566-72.
- [110] d'Anglemont de Tassigny, X, Fagg, LA, Carlton, MB & Colledge, WH. Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. *Endocrinology* 2008;149:3926-32.
- [111] Seminara, SB, DiPietro, MJ, Ramaswamy, S, Crowley, WF & Plant, TM. Continuous human metastatin 45–54 infusion desensitizes G protein-coupled receptor 54-induced gonadotropin-releasing hormone release monitored indirectly in the juvenile male Rhesus monkey (*Macaca mulatta*): a finding with therapeutic implications. *Endocrinology* 2006;147:2122-26.
- [112] Shahed, A & Young, KA. Differential ovarian expression of KiSS-1 and GPR-54 during the estrous cycle and photoperiod induced recrudescence in Siberian hamsters (*Phodopus sungorus*). *Mol Reprod Dev* 2009;76:444-52.
- [113] Smith, JT. The role of kisspeptin and gonadotropin inhibitory hormone in the seasonal regulation of reproduction in sheep. *Domest Anim Endocrinol* 2012;43:75-84.
- [114] Williams, GL, Thorson, JF, Prezotto, LD, Velez, IC, Cardoso, RC & Amstalden, M. Reproductive seasonality in the mare: neuroendocrine basis and pharmacologic control. *Domest Anim Endocrinol* 2012;43:103-115.

- [115] Thorson, J, Prezotto, L, Cardoso, R, Alves, B, Liu, S, Edwards, J, Welsh, T, Amstalden, M & Williams, G. Effects of RF-Amide Related Peptide 3 (RFRP-3), an Equine Homolog of Avian Gonadotropin-Inhibiting Hormone, on Adenohypophyseal Responsiveness to GnRH in Mares. *Biol Reprod S* 2011;85:607.
- [116] Prezotto, LD, Thorson, JF, Cardoso, RC, Amstalden, M & Williams, GL. Failure of an Equine Homologue of Avian Gonadotropin Inhibiting Hormone to Alter Secretion of Luteinizing Hormone in the Mare. *Biol Reprod S* 2011;85:608.
- [117] Thorson, JF, Prezotto, LD, Cardoso, RC, Sharpton, SM, Edwards, JF, Welsh Jr, TH, Riggs, PK, Caraty, A, Amstalden, M & Williams, GL. Hypothalamic distribution, adenohypophyseal receptor expression, and ligand functionality of RFamide-related peptide 3 in the mare during the breeding and nonbreeding seasons. *Biol Reprod* 2014;90:1-9.
- [118] Korthanke, CM, Thorson, JF, Prezotto, LD, Welsh Jr, TH, Cardoso, RC & Williams, GL. Secretion of Gonadotropins in Response to a Novel Kiss-1 Receptor Agonist, RF9 in the Mare: Modulation by Estradiol-17 β and Half-Life of RF9 in the Peripheral Circulation. *J Equine Vet Sci* 2017;57:100-06.
- [119] Liu, X & Herbison, AE. RF9 excitation of GnRH neurons is dependent upon Kiss1r in the adult male and female mouse. *Endocrinology* 2014;155:4915-24.
- [120] Decourt, C, Anger, K, Robert, V, Lomet, D, Bartzen-Sprauer, J, Caraty, A, Dufourny, L, Anderson, G & Beltramo, M. No evidence that RFamide-related peptide 3 directly modulates LH secretion in the ewe. *Endocrinology*, 2016;157:1566-75.

- [121] Guillaume, D & Palmer, E. Effect of oral melatonin on the date of the first ovulation after ovarian inactivity in mares under artificial photoperiod. *J Reprod Fertil S* 1991;44: 249-57.
- [122] Guillaume, D, Arnaud, G, Camillo, F, Duchamp, G & Palmer, E. Effect of melatonin implants on reproductive status of mares. *Biol Reprod* 1995;52:435-42.
- [123] Fitzgerald, BP & McManus, CJ. Photoperiodic versus metabolic signals as determinants of seasonal anestrus in the mare. *Biol Reprod* 2000;63:335-40.
- [124] Burkhardt, J. Transition from anoestrus in the mare and the effects of artificial lighting. *J Agr Sci* 1947;37:64-68.
- [125] Nagy, P, Guillaume, D & Daels, P. Seasonality in mares. *Anim Reprod Sci* 2000;60:245-62.
- [126] Kooistra, LH & Ginther, OJ. Effect of photoperiod on reproductive activity and hair in mares. *Am J Vet Res* 1975;36:1413-19.
- [127] Palmer, E, Driancourt, MA & Ortavant, R. Photoperiodic stimulation of the mare during winter anoestrus. *J Reprod Fertil S* 1982;32:275-82.
- [128] Scraba, ST & Ginther, OJ. Effects of lighting programs on onset of the ovulatory season in mares. *Theriogenology* 1985;24:667-79.
- [129] Malpaux, B, Robinson, JE, Brown, MB & Karsch, FJ. Importance of changing photoperiod and melatonin secretory pattern in determining the length of the breeding season in the Suffolk ewe. *Reproduction* 1988;83:461-70.
- [130] Pelletier, J & Almeida, G. Short light cycles induce persistent reproductive activity in Île-de-France rams. *J Reprod Fertil S* 1987;34:215-26.

- [131] Palmer, E & Guillaume, D. Photoperiodism in the equine species—what is a long night?. *Anim Reprod Sci* 1992;28:21-30.
- [132] Robinson, JE & Karsch, FJ. Photoperiodic history and a changing melatonin pattern can determine the neuroendocrine response of the ewe to daylength. *J Reprod Fertil* 1987;80:159-65.
- [133] Palmer, E & Driancourt, MA. Some interactions of season of foaling, photoperiod and ovarian activity in the equine. *Livest Prod Sci* 1981;10:197-210.
- [134] Malinowski, K, Johnson, AL & Scanes, CG. Effects of interrupted photoperiods on the induction of ovulation in anestrus mares. *J Anim Sci* 1985;61:951.
- [135] Evans, MJ & Irvine, CHG. Induction of follicular development, maturation, and ovulation by gonadotrophin releasing hormone administration to acyclic mares. *Biol Reprod* 1977;16:452-62.
- [136] Evans, MJ & Irvine, CHG. Induction of follicular development and ovulation in seasonally acyclic mares using gonadotrophin releasing hormone and progesterone. *J Reprod Fertil S* 1979;27:113-21.
- [137] Johnson, AL. Gonadotropin-releasing hormone treatment induces follicular growth and ovulation in seasonally anestrus mares. *Biol Reprod* 1987;36:1199-1206.
- [138] Becker, SE & Johnson, AL. Effects of gonadotropin-releasing hormone infused in a pulsatile or continuous fashion on serum gonadotropin concentrations and ovulation in the mare. *J Anim Sci* 1992;70:1208-15.
- [139] Hyland, JH, Wright, PJ, Clark, IJ, Carson, RS, Langsford, DA & Jeffcott, LB. Infusion of gonadotropin-releasing hormone (GnRH) induces ovulation and fertile oestrus in mares during seasonal anestrus. *J Reprod Fertil S* 1987;35:211-20.

- [140] Hyland, JH & Jeffcott, LB. Control of transitional anestrus in mares by infusion of gonadotropin releasing hormone. *Theriogenology* 1988;29:1383-1391.
- [141] McCue, PM, Warren, RC, Appel, RD, Stabenfeldt, GH, Hughes, JP & Lasley, BL. Pregnancy rates following administration of GnRH to anestrus mares. *J Equine Vet Sci* 1992;12:21-23.
- [142] Harrison, LA, Squires, EL, Nett, TM & McKinnon, AO. Use of gonadotropin-releasing hormone for hastening ovulation in transitional mares. *J Anim Sci* 1990;68:690-99.
- [143] Ginther, OJ & Bergfelt, DR. Effect of GnRH treatment during the anovulatory season on multiple ovulation rate and on follicular development during the ensuing pregnancy in mares. *Reproduction* 1990;88:119-26.
- [144] McCue, PM, Troedsson, MH, Liu, IK, Stabenfeldt, GH, Hughes, JP & Lasley, BL. Follicular and endocrine responses of anoestrous mares to administration of native GnRH or a GnRH agonist. *J Reprod Fertil S* 1991;44:227-33.
- [145] Fitzgerald, BP, Meyer, SL, Affleck, KJ & Silvia, PJ. Effect of constant administration of a gonadotropin-releasing hormone agonist on reproductive activity in mares: induction of ovulation during seasonal anestrus. *Am J Vet Res* 1993;54:1735-1745.
- [146] Mumford, EL, Squires, EL, Peterson, KD, Nett, TM & Jasko, DJ. Effect of various doses of a gonadotropin-releasing hormone analogue on induction of ovulation in anestrus mares. *J Anim Sci* 1994;72:178-83.
- [147] Rahmanian, MS, Thompson Jr, DL & Melrose, PA. Immunocytochemical localization of prolactin and growth hormone in the equine pituitary. *J Anim Sci* 1997;75:3010-18.

- [148] Worthy, K, Escreet, R, Renton, JP, Eckersall, PD, Douglas, TA & Flint, DJ. Plasma prolactin concentrations and cyclic activity in pony mares during parturition and early lactation. *Reproduction* 1986;77:569-74.
- [149] Worthy, K, Colquhoun, K, Escreet, R, Dunlop, M, Renton, JP & Douglas, TA. Plasma prolactin concentrations in non-pregnant mares at different times of the year and in relation to events in the cycle. *J Reprod Fertil S* 1987;35:269-76.
- [150] Morresey, P. Oxytocin, Inhibin, Activin, Relaxin and Prolactin. In: McKinnon, AO, Squires, EL, Vaala, WE, & Varner, DD, editors. *Equine reproduction*, United Kingdom: John Wiley & Sons; 2011, p. 1679-86.
- [151] Brendemuehl, JP & Cross, DL. Influence of the dopamine antagonist domperidone on the vernal transition in seasonally anoestrous mares. *J Reprod Fertil S* 2000;56:185-93.
- [152] King, SS, Campbell, AG, Dille, EA, Roser, JF, Murphy, LL & Jones, KL. Dopamine receptors in equine ovarian tissues. *Domest Anim Endocrinol* 2005;28:405-15.
- [153] King, SS, Jones, KL, Mullenix, BA & Heath, DT. Seasonal relationships between dopamine D1 and D2 receptor and equine FSH receptor mRNA in equine ovarian epithelium. *Anim Reprod Sci* 2008;108:259-66.
- [154] Oberhaus, EL, Jones, KL & King, SS. Immunohistochemical localization of prolactin receptors within the equine ovary. *J Equine Vet Sci* 2015;35:7-12.
- [155] McCue, PM, Buchanan, BR, Farquhar, VJ, Squires, EL & Cross, DL. Efficacy of domperidone on induction of ovulation in anestrous and transitional mares. *Proc Am Assoc Equine Practnrs* 1999;45:217-18.
- [156] McCue, PM, Logan, NL & Magee, C. Management of the transition period: hormone therapy. *Equine Vet Educ* 2007;19:215-21.

- [157] Besognet, B, Hansen, BS & Daels, PF. Induction of reproductive function in anestrus mares using a dopamine antagonist. *Theriogenology* 1997;47:467-80.
- [158] Duchamp, G & Daels, PF. Combined effect of sulpiride and light treatment on the onset of cyclicity in anestrus mares. *Theriogenology* 2002;58:599-602.
- [159] Thompson Jr, DL & DePew, CL. Prolactin, gonadotropin, and hair shedding responses to daily sulpiride administration in geldings in winter. *J Anim Sci* 1997;75:1087-91.
- [160] Donadeu, FX & Thompson Jr, DL. Administration of sulpiride to anovulatory mares in winter: effects on prolactin and gonadotropin concentrations, ovarian activity, ovulation and hair shedding. *Theriogenology* 2002;57:963-76.
- [161] Nagy, P, Bruneau, B, Duchamp, G, Daels, PF & Guillaume, D. Dopaminergic control of seasonal reproduction in mares. *Reprod Dom Anim Suppl* 1999;6:33.
- [162] Mari, G, Morganti, M, Merlo, B, Castagnetti, C, Parmeggiani, F, Govoni, N, Galeati, C & Tamanini, C. Administration of sulpiride or domperidone for advancing the first ovulation in deep anestrus mares. *Theriogenology* 2009;71:959-65.
- [163] Thompson Jr, DL, Garza Jr, F, George, RS, Rabb, MH, Barry, BE & French, DD. Relationships among LH, FSH and prolactin secretion, storage and response to secretagogue and hypothalamic GnRH content in ovariectomized pony mares administered testosterone, dihydrotestosterone, estradiol, progesterone, dexamethasone or follicular fluid. *Domest Anim Endocrinol* 1991;8:189-99.
- [164] Kelley, KK, Thompson Jr, DL, Storer, WA, Mitcham, PB, Gilley, RM & Burns, PJ. Estradiol interactions with dopamine antagonists in mares: prolactin secretion and reproductive traits. *J Equine Vet Sci* 2006;26:517-28.

- [165] Mitcham, PB, Thompson Jr, DL, Thompson, TT, Bennett, SD, Burns, PJ & Caltabilota, TJ. Stimulation of ovulation in seasonally anovulatory and vernal transitional mares with estradiol and domperidone: dose and combination studies. *J Equine Vet Sci* 2010;30:244-48.
- [166] Oberhaus, EL, Thompson Jr, DL & Pham, CK. Factors affecting the ovarian response to a combined estradiol-sulpiride treatment in seasonally anovulatory mares. *J Equine Vet Sci* 2017;50:129-38.
- [167] Thompson Jr, DL, Hoffman, R & DePew, CL. Prolactin administration to seasonally anestrous mares: reproductive, metabolic, and hair-shedding responses. *J Anim Sci* 1997;75:1092-99.
- [168] Chowdhury, M, Kabir, SN, Pal, AK & Pakrashi, A. Modulation of luteinizing hormone receptors: effect of an inhibitor of prolactin secretion, p-coumaric acid. *J Endocrinol* 1983;98:307-11.
- [169] Grosdemouge, I, Bachelot, A, Lucas, A, Baran, N, Kelly, PA & Binart, N. Effects of deletion of the prolactin receptor on ovarian gene expression. *Reprod Biol Endocrin* 2003;1:12.
- [170] Quigley, ME & Yen, SSC. The role of endogenous opiates on LH secretion during the menstrual cycle. *J Clin Endocr Metab* 1980;51:179-81.
- [171] Orstead, KM, Hess, DL & Spies, HG. Opiatergic inhibition of pulsatile luteinizing hormone release during the menstrual cycle of rhesus macaques. *Proc Soc Exp Biol Med* 1987;184:312-19.
- [172] Short, RE, Brooks, AN, Peters, AR & Lamming, GE. Opioid modulation of LH secretion during the oestrous cycle of heifers. *Reproduction* 1987;80:213-19.

- [173] Currie, WD & Rawlings, NC. Naloxone enhances LH but not FSH release during various phases of the estrous cycle in the ewe. *Life Sci* 1987;41:1207-14.
- [174] Barb, CR, Kraeling, RR & Rampacek, GB. Opioid modulation of FSH, growth hormone and prolactin secretion in the prepuberal gilt. *Journal of endocrinology*, 1992;133:13-19.
- [175] Petraglia, F, Locatelli, V, Facchinetti, F, Bergamaschi, M, Genazzani, AR & Cocchi, D. Oestrous cycle-related LH responsiveness to naloxone: effect of high oestrogen levels on the activity of opioid receptors. *J Endocrinol* 1986;108:89-94.
- [176] Caraty, A, Locatelli, A & Schanbacher, B. Naloxone increases the frequency and amplitude of LH-RH pulses in the hypothalamic-pituitary portal blood in castrated ram. *Proc Acad Sci* 1987;305:369-74.
- [177] Horton, RJE, Cummins, JT & Clarke, IJ. Naloxone evokes large-amplitude GnRH pulses in luteal-phase ewes. *Reproduction* 1987;81:277-86.
- [178] Aurich, JE, Hoppen, HO, Hoppe, H & Aurich, C. Endogenous opioids and reproductive functions in the horse. *Anim Reprod Sci* 1996;42:119-29.
- [179] Behrens, C, Aurich, JE, Klug, E, Naumann, H & Hoppen, HO. Inhibition of gonadotrophin release in mares during the luteal phase of the oestrous cycle by endogenous opioids. *Reproduction* 1993;98:509-14.
- [180] Turner, JE, Irvine, CHG & Alexander, SL. Regulation of seasonal breeding by endogenous opioids in mares. *Biol Reprod* 1995;55:443-48.
- [181] Aurich, C, Daels, PF, Ball, BA & Aurich, JE. Effects of gonadal steroids on the opioid regulation of LH and prolactin release in ovariectomized pony mares. *J Endocrinol* 1995;147:195-202.

- [182] Hammer Jr, RP, Zhou, L & Cheung, S. Gonadal steroid hormones and hypothalamic opioid circuitry. *Horm Behav* 1994;28:431-37.
- [183] Aurich, C, Schlote, S, Hoppen, HO, Klug, E, Hoppe, H & Aurich, JE. Effects of the opioid antagonist naloxone on release of luteinizing hormone in mares during the anovulatory season. *J Endocrinol* 1994;142:139-44.
- [184] Irvine, CHG, Alexander, SL & Turner, JE. Differential effects of graded doses of naloxone on the reproductive and adrenal axes in seasonally anestrous mares. *Endocrine* 1994;2:913-19.
- [185] Davison, LA, McManus, CJ & Fitzgerald, BP. Gonadotropin response to naloxone in the mare: effect of time of year and reproductive status. *Biol Reprod* 1998;59:1195-99.
- [186] Dell'Aquila, ME, Albrizio, M, Guaricci, AC, De Santis, T, Maritato, F, Tremoleda, JL, Colenbrander, B, Guerra, L, Casavola, V & Minoia, P. Expression and localization of the μ -opioid receptor (MOR) in the equine cumulus–oocyte complex and its involvement in the seasonal regulation of oocyte meiotic competence. *Mol Reprod Dev* 2008;75:1229-46.
- [187] Squires, EL, Stevens, WB, McGlothlin, DE & Pickett, BW. Effect of an oral progestin on the estrous cycle and fertility of mares. *J Anim Sci* 1979;49:729-35.
- [188] Allen, WR, Urwin, V, Simpson, DJ, Greenwood, RES, Crowhurst, RC, Ellis, DR, Ricketts, SW, Hunt, MDN & Digby, N. W. Preliminary studies on the use of an oral progestogen to induce oestrus and ovulation in seasonally anoestrous Thoroughbred mares. *Equine Vet J* 1980;12:141-45.

- [189] Squires, EL, Heesemann, CP, Webel, SK, Shideler, RK & Voss, JL. Relationship of altrenogest to ovarian activity, hormone concentrations and fertility of mares. *J Anim Sci* 1983;56:901-10.
- [190] McCue, PM, Nickerson, KC, Squires, EL, Farquhar, VJ & Nett, TM. Effect of altrenogest on luteinizing hormone concentrations in mares during the transition period. *Proc of Am Assoc Equine Pract* 2001;47:249-51.
- [191] Staempfli, SA, Clavier, S, Thompson, DL, Burns, PJ, Lyle, SK & McKinnon, AO. Effect of a single injection of long-acting progesterone on the first ovulation in early and late spring transitional mares. *J Equine Vet Sci* 2011;31:744-48.
- [192] Newcombe, JR, Handler, J, Klug, E, Meyers, PJ & Jöchle, W. Treatment of transition phase mares with progesterone intravaginally and with deslorelin or hCG to assist ovulations. *J Equine Vet Sci* 2002;22:57-64.
- [193] Arbeiter, K, Barth, U & Jöchle, W. Observations on the use of progesterone intravaginally and of deslorelin STI in acyclic mares for induction of ovulation. *J Equine Vet Sci* 1994;14:21-25.
- [194] Hanlon, DW, Evans, MJ & Firth, EC. Effects of intravaginal progesterone on follicular dynamics and FSH, LH and progesterone concentrations in transitional mares. *Anim Reprod Sci* 2010;121:32-34.
- [195] Grimmett, JB, Hanlon, DW, Duirs, GF & Jochle, W. A new intra-vaginal progesterone-releasing device (Cue-Mare) for controlling the estrous cycle in mares. *Theriogenology* 2002;58:585-87.

- [196] Hanlon, DW & Firth, EC. The reproductive performance of Thoroughbred mares treated with intravaginal progesterone at the start of the breeding season. *Theriogenology* 2012;77:952-58.
- [197] Burns, PJ, Morrow, C & Abraham, J. Evaluation of Biorelease P4LA 300 in the mare. *Proc Equine Embryo Transfer Symposium* 2008;71.
- [198] Gelez, H, Fabre-Nys, C. The “male effect” in sheep and goats: a review of the respective roles of the two olfactory systems. *Horm Behav* 2004;46:257-71.
- [199] Delgadillo, JA, Gelez, H, Ungerfeld, R, Hawken, PA, Martin, GB. The ‘male effect’ in sheep and goats—revisiting the dogmas. *Behav Brain Res* 2009;200:304-14.
- [200] Martin, GB, Oldham, CM, Lindsay, DR. Increased plasma LH levels in seasonally anovular Merino ewes following the introduction of rams. *Anim Reprod Sci* 1980;3:125-32.
- [201] Martin, GB, Scaramuzzi, RJ. The induction of oestrus and ovulation in seasonally anovular ewes by exposure to rams. *J Steroid Biochem* 1983;19:869-75.
- [202] Oldham, CM, Martin, GB, Knight, TW. Stimulation of the seasonally anovular merinos ewes by rams: I. Time from introduction of the rams to the preovulatory surge and ovulation. *Anim Reprod Sci* 1979;1:283-90.
- [203] Chemineau, P. Effect on oestrus and ovulation of exposing creole goats to the male at three times of the year. *J Reprod Fertil* 1983;67:65-72.
- [204] Wespi, B, Sieme, H, Wedekind, C, Burger, D. Exposure to stallion accelerates the onset of mares' cyclicity. *Theriogenology* 2014;82:189-94.

- [205] Thompson Jr, DL, Godke, RA, Squires, EL. Testosterone effects on mares during synchronization with altrenogest: FSH, LH, estrous duration and pregnancy rate. *J Anim Sci* 1983; 56:678-86.
- [206] Colborn, DR, Thompson Jr, DL, Roth, TL, Capehart, JS, White, KL. Responses of cortisol and prolactin to sexual excitement and stress in stallions and geldings. *J Anim Sci* 1991; 69:2556-62.
- [207] Underwood, EJ, Shier, FL, Davenport, N. Studies in sheep husbandry in Western Australia, V. The breeding season of Merino Crossbred and British Breed Ewes in the Agricultural Districts, Department of Agriculture, Western Australia 1944;11:135-43.
- [208] Oldham, CM, Cognie, Y. Do ewes continue to cycle after teasing. *Proc Aust Soc Anim Prod* 1980;13:82-85.
- [209] Martin, GB, Oldham, CM, Cognié, Y, Pearce, DT. The physiological responses of anovulatory ewes to the introduction of rams—a review. *Livest Prod Sci* 1986;15:219-47.
- [210] Knight, TW. Ram induced stimulation of ovarian and oestrus activity in anoestrus ewes – a review. *Proc NZ Soc Anim Prod* 1983;43:7-11.
- [211] Rosa, HJD., Bryant, MJ. The ‘ram effect’ as a way of modifying the reproductive activity in the ewe. *Small Ruminant Res* 2002;45:1-16.
- [212] Sharp, DC, Kooistra, L, Ginther, OJ. Effects of artificial light on the oestrous cycle of the mare. *J Reprod Fertil* 1975;23:241-246.
- [213] Guerin, MV, Wang, XJ. Environmental temperature has an influence on timing of the first ovulation of seasonal estrus in the mare. *Therogenology* 1994;42:1053-60.
- [214] Tucker HA, Oxender, WD. Seasonal aspects of reproduction, growth and hormones in cattle and horses. *Prog Reprod Biol* 1980;5:155-80.

- [215] Alvarez, L, Martin, GB, Galindo, F, Zarco, LA. Social dominance of female goats affects their response to the male effect. *Appl Anim Behav Sci* 2003;84:119-26.
- [216] Martin, GB, Oldham, CM, Lindsay, DR. Increased plasma LH levels in seasonally anovular Merino ewes following the introduction of rams. *Anim Reprod Sci* 1980;3:125-32.
- [217] Poindron, P, Cognie, Y, Gayerie, F, Orgeur, P, Oldham, CM, and Ravault, JP. Changes in gonadotrophins and prolactin levels in isolated (seasonally or lactationally) anovular ewes associated with ovulation caused by the introduction of rams. *Physiol Behav* 1980;25:227-36.
- [218] Chemineau, P, Normant, E, Ravault, JP, Thimonier, J. Induction and persistence of pituitary and ovarian activity in the out-of-season lactating dairy goat after a treatment combining a skeleton photoperiod, melatonin and the male effect. *Reproduction* 1986;78:497-504.
- [219] Chemineau, P, Levy, F, Thimonier, J. Effects of anosmia on LH secretion, ovulation and oestrous behaviour induced by males in the anovulatory Creole goat. *Anim Reprod Sci* 1986;10:125-32.
- [220] Watson, RH, Radford, HM. The influence of rams on onset of oestrus in Merino ewes in the spring. *Aust J Agri Res* 1960;11:65-71.
- [221] Morgan, PD, Arnold, GW, Lindsay, DR. A note on the mating behaviour of ewes with various senses impaired. *J Reprod Fertil* 1972;30:151-52.
- [222] Knight, TW, Lynch, PR. Source of ram pheromones that stimulate ovulation in the ewe. *Anim Reprod Sci* 1980;3:133-36.

- [223] Tucker, HA, Wettemann, RP. Effects of ambient temperature and relative humidity on serum prolactin and growth hormone in heifers. *Proc Soc Exp Biol Med* 1976;151:623-26.
- [224] Wettemann, RP, Tucker, HA. Relationship of ambient temperature to serum prolactin in heifers. *Proc Soc Exp BiolMed* 1974;146:908-11.
- [225] Wiepz, GJ, Squires, EL & Chapman, PL. Effects of norgestomet, altrenogest, and/or estradiol on follicular and hormonal characteristics of late transitional mares. *Theriogenology* 1988;30:181-93.
- [226] Wiesenfeld-Hallin, Z & Sodersten, P. Spinal opiates affect sexual behavior in rats. *Nature* 1984;309:257-58.
- [227] Abs, R, Verhelst J, Maeyaert, J, Van Buyten, JP, Opsomer, F, Adriaensen, H, Verlooy, J, Van Havenbergh, T, Smet, M & Van Acker, K. Endocrine consequences of long-term intrathecal administration of opioids. *J Clin Endocrinol Metab* 2000;85:2215-22.
- [228] Forsberg, G, Eneroth, P & Sodersten, P. Naloxone stimulates sexual behavior in lactating rats. 1987;113:423-27.
- [229] Metera, C, Freda, PU, Ferin, M & Wardlaw, SL. Effect of chronic opioid antagonism on the hypothalamic-pituitary-ovarian axis in hyperprolactinemic women. *J Clin Endocrinol Metab* 1995;80:540-45.