

OVULATORY RESPONSE TO GNRH AGONIST DURING EARLY AND LATE FALL IN
MARES

BY

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THESIS

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ABSTRACT

This study aimed to assess the ovulatory response of deslorelin acetate during the fall and the response to PGF2 α 8 d post-ovulation. One hundred estrous cycles from 22 mares kept in 40° latitude were evaluated. Mares were checked by transrectal ultrasonography until a preovulatory follicle was detected and ovulation induced with deslorelin acetate. Ovulation was confirmed by ultrasonography performed at 24, 36 h post-induction and then repeated at 2-h intervals post-induction. Serum progesterone concentrations and luteal tissue area were determined daily to assess CL function. A dose of PGF2 α was administered 8 d post-ovulation and each cycle was repeated up to five times. The effects of local climate on endpoints were analyzed. Cycles were grouped as early (n=55) and late fall (n=45) based on the date of induction. Parametric data was analyzed with mixed models, ANOVA, Tukey's test, and Pearson's correlation. Non-parametric data was analyzed with Fisher's test, Kruskal-Wallis, and Dunn's test. The overall efficacy of deslorelin was 90%. Multiple ovulations were similar between early (n=5) and late (n=4) fall (P=0.87). There were no differences in spontaneous ovulations early (n=6) and late (n=2) fall (P=0.29). Two failures to respond to deslorelin by 48 h were recorded early and none in the late fall. The interval from induction to ovulation was similar in early (40.6 $\pm$ 0.4 h) and late (41.2 $\pm$ 0.5 h) fall (P=0.55). The percentage of mares ovulating between 36 and 48 h and 38 and 44 h post-deslorelin did not vary between early (91, 62%) and late (95, 60%) fall (P=0.21, P=0.69), respectively. Follicle size at induction tended to be smaller in early (36.4 $\pm$ 0.4mm) than late (37.4 $\pm$ 0.4mm) fall (P=0.07). Edema scores varied with time relative to ovulation (P<0.001) and were lower in late fall (P=0.01). Progesterone concentrations varied with time (P<0.001) but did not differ between early and late fall (P=0.73) and correlated weakly with the luteal area (r=0.13;

P=0.031). Follicles < 35mm at the PGF2 α (9.2 $\pm$ 0.5 d) had a shorter interval to the next ovulation than follicles $\geq$ 35mm (10.6 $\pm$ 1.2 d) (P=0.03). Lower temperature led to a smaller follicle size at induction (P=0.0021) and ovulation (P=0.009) and lower relative humidity yielded a larger follicle size at ovulation (P=0.032). In conclusion, cycling mares displayed a highly efficacious response to deslorelin acetate and apparently normal luteal function during the fall, despite lower edema scores late fall.

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CHAPTER 1: General introduction

The U.S. equine industry is a large and economically diverse industry which contributes significantly to the national economy. It is estimated that each year the horse industry has a direct economic impact of \$112.1 on the national level and sustains around 1.4 million jobs. Approximately 237,000 horse owners are directly involved in breeding, making reproduction one of the most economically impactful aspects of equine veterinary practice (American Horse Council Foundation 2005). Unlike many other livestock species, horses are not often genetically selected for reproductive efficiency. While most mares are fertile and able to be managed in a field situation, referral clinics see high caseloads consisting of the subfertile population. The demand for continued use of the mare as a performance athlete and possible income-source has increased the popularity of advanced reproductive technologies and enhanced reproductive efficiency.

There is economic pressure among multiple disciplines for having foals born early in the calendar year. Per many breed registry associations, the birth date of all foals is January 1 of each calendar year, regardless of actual foaling date. Therefore, earlier born foals have a size and maturity advantage when it comes to yearling sales and futurity competitions. Additionally, earlier born foals are at a decreased risk for infection with *Rhodococcus equi* and gastrointestinal parasites [1,2]. However, there are also disadvantages of having early born foals, especially in areas of cold climate. These include increased risk of osteochondrosis dissecans, pneumonia and delayed involution of the umbilical structures secondary to stall rest [3–5].

Significant efforts have been placed into maximizing pregnancy rates during late winter and early spring to generate early born foals. This can be challenging because mares are

transitioning during this time of the year. Specifically, mares are seasonally polyestrous long-day breeders, meaning that the physiological breeding season in the Northern Hemisphere typically takes place from March to October [6]. Before and after the physiological breeding season, mares will undergo transitional periods leading to cyclicity (spring transition) and anestrus (fall transition), respectively [6]. Conversely, in the Southern Hemisphere the physiological breeding season takes place from April to November [7].

Factors affecting duration of the ovulatory season and transition periods in mares include photoperiod, environmental temperature, nutrition, and diseases [8]. Daylight is the primary factor influencing seasonality in mares and an increased light to dark ratio is associated with cyclicity[9]. The second most relevant affecting factor is temperature. Under cold weather, there is an increased production of endogenous opioid, which leads to a reduction in GnRH and subsequent ovarian cyclic activity. Adequate feed intake is a requirement for normal reproductive activity. Well-fed mares will cycle earlier in the year than thin mares and overly conditioned mares tend to cycle year-round. Ocular problems impairing vision and the capture of changes in light, metabolic disturbances affecting reproductive hormones, emaciation resulting in a negative energy balance, and increased cortisol from pain or stress are all disease sequelae which could alter the normal reproductive cyclicity. As fall progresses in the Northern Hemisphere, daylight length and temperature decrease, and the nutritional value of pasture begins to depreciate.

Strategies such as extended artificial light exposure, pharmacological interventions, nutritional modulation, and a combination are successfully applied in the horse breeding industry to circumvent this physiological limitation [8,10,11]. Methods of artificial light therapy are implemented, with the most common being daylight extension, night breaks, T-cycle paradigms

and blue light therapy. Secondary to photomanipulation, use of pharmacological therapeutics, most notably steroid hormones, dopaminergic agents, GnRH agonists and gonadotropins, is the next most commonly employed method to alter cyclicity. Nutrition is often another main focus of breeding management. While it is widely understood that emaciation can lead to a termination of cyclicity, a more common problem in the U.S. is obesity in mares. Obesity and feeding of energy-dense diets can lead to an increase of mediators, such as growth hormone (GH), insulin-like growth factor (IGF-1), leptin, inhibin-A and vascular endothelial growth factor (VEGF) which have impact the reproductive cycle.

Induction of ovulation is an important tool for maximizing reproductive efficiency and fertility in the mare. The multitude of benefits to using an induction agent include hastening the interval from breeding to ovulation, facilitating the use of timed artificial insemination protocols, decreasing the number of breeding and reproductive examinations per estrous cycle, controlling the timing of breeding to facilitate semen and personnel availability, insuring at least one ovulation for each cycle, enhancing the progesterone production capabilities of subsequent corpora lutea and allowing for the estrous synchronization between embryo donors and recipients [12,13].

CHAPTER 2: Literature review

2.1 The Estrous Cycle

Physiology and follicular dynamics

The estrous cycle of mares is a complex physiological process which relies on various structural, endocrinological and behavioral changes to proceed. As a seasonally polyestrous long-day breeder, the mare goes through multiple estrous cycles throughout her physiological breeding season, which takes place during times of the year with increased daylength. The average length of the estrous cycle in the mare is 21 days, but can range from 18-24 days [14]. There are many factors that affect estrous length, also called interovulatory interval in the mare, including age, breed, and occurrence of diestrus ovulations. In light-breed mares in the Northern Hemisphere, aged mares have a longer interovulatory interval (23.0 ± 0.8 days) in comparison to middle-aged mares (21.6 ± 0.4 days) and both had a longer cycle than young mares (20.9 ± 0.5 days)[15].

The mare ovary is unique in structure from other mammalian species. Contrary to other species, the mare has an interior cortex, which is the reproductively active portion of the ovary that contains primordial germ cells and follicles. It also has an exterior medulla, containing connective tissues, blood vessels and lymphatics. This organization makes reliable rectal palpation of follicles and corpora lutea in the mare difficult compared with ruminant species. Another remarkable feature of the mare ovary is the ovulation fossa, a structure on the exterior surface of the ovary which is lined with superficial germinal epithelium through which all ovulations occur [7]. The ovulation fossa is not present in prepubertal animals and may become obstructed or disfigured in the presence of pathological conditions, including neoplasia, and is a

primary limiting factor for successful induction of superovulation and multiple pregnancies in mares. This limitation arises due the formation of a clot that physically occlude the fossa and prevents passage of follicular fluid from subsequent ovulations.

Follicular growth and recruitment are vital processes in the preparation of an ovulatory follicle. A synchronous growth period of a particular group, or cohort, of follicles is described as a follicular wave (primary or secondary). Emergence is the first step of a follicular wave, in which a rise in follicle stimulating hormone (FSH) stimulates development of multiple antral follicles from primary and secondary follicles. Mean follicular diameter at the beginning of emergence is 6.0-6.9 mm [16]. The cohort goes through a common growth phase for several days [7]. Next, deviation occurs, when a dominant follicle, measuring 21-24 mm in diameter, and a subordinate follicle, 19-21 mm, arise and continue to grow, while growth of the other follicles is reduced [16]. The dominant follicle either develops into a preovulatory follicle in a major wave or undergoes atresia during a minor wave [16,16–18]. On average, follicles grow at a rate of 2.5 to 5 mm per day [19] and anovulatory follicles regress at a rate of 1.0 to 1.5 mm per day [19].

Major waves can be classified as primary or secondary. A primary major wave is the main ovulation that occurs during estrus. A secondary major wave occurs during diestrus, either just after the major primary wave ovulation, or mid-late diestrus. Certain breeds, such as Thoroughbred and Warmbloods, are predisposed to development of major secondary waves [7]. Additionally, ovulation of a major secondary wave follicle can be induced by the administration of Prostaglandin F_{2α} (PGF_{2α}) or another ovulatory agent in the presence of a large follicle.

2.1.1 Estrus

Endocrinology

Gonadotrophin-releasing hormone (GnRH) is the primary reproductive stimulating factor in most species. GnRH is produced in the hypothalamus and acts on the pituitary to serve as the sole stimulator of gonadotropin (FSH and luteinizing hormone (LH)) release. Circulating GnRH concentrations are influenced by external factors, such as the stimulator kisspeptin [20] and inhibitory factors like gamma-aminobutyric acid (GABA) [21] and opioids [22]. The pattern of GnRH secretion changes throughout the estrous cycle. During diestrus, GnRH pulses are low in frequency and high in amplitude, and in estrus the pulses increase threefold in frequency, but decrease in amplitude [23,24].

FSH and LH are glycoproteins which are produced by the gonadotropic cells of the pituitary. The proteins are similar in structure, with identical α subunits and distinct β subunits. Receptors for these gonadotropins are found on the mare ovary, with FSH receptors residing in granulosa cells and LH receptors residing in thecal, granulosa and luteal cells. FSH is found to have a bimodal to trimodal secretion pattern which lends itself to the existence of 2-3 follicular waves throughout the estrous cycle [7]. In the bimodal pattern, two distinct FSH surges occur throughout the estrous cycle, at 10 -12 day intervals. Breeds with higher incidence of a trimodal pattern include Thoroughbred, Warmbloods and Standardbreds, while Arabians and Quarter Horses are more likely to display the bimodal pattern. As the name suggests, FSH acts on the granulosa cells to stimulate follicular growth in a cohort. It is downregulated by the excretion of inhibin from the dominant follicle. LH is secreted in a pulsatile fashion, in tandem with GnRH secretions. LH receptors are present in theca cells of all follicles and in the granulosa cells of mature follicles [25]. LH pulses increase in rate of frequency as ovulation approaches [7].

Peripheral concentrations of LH increase throughout estrus, with a significant rise starting two days prior to ovulation and peaking one or two days after ovulation.

As follicles mature, they produce estradiol (E_2) which acts on various parts of the reproductive and central nervous system to increase the mare's receptivity to mating and readiness of the uterus to receive an embryo. Estradiol concentrations in the blood have a similar trend to LH concentrations, increasing throughout estrus and peaking two days prior to ovulation [7].

Clinical signs of estrus

There are multiple diagnostic indicators, uterine tone and edema, which can be used as biomarkers to determine if a mare is in estrus. Uterine tone is greater when mares are in diestrus under the influence of progesterone than in estrus when they have high levels of estrogen [26]. Although not as specific as uterine edema, it can be used to subjectively assess which part of the estrous cycle a mare is in. Under the influence of estrogen the cervix also begins to soften and relax, allowing for influx and outflux of fluid. Endometrial edema, which will be discussed in detail in a forthcoming section, is another important indicator of estrus and follicular proximity to ovulation. Endometrial edema will increase upon onset of estrus, peak around day 3, then decrease prior to ovulation [27].

Vaginal speculum examination is a key diagnostic tool for evaluation of estrus. Visually, the cervix relaxes during estrus and sinks ventrally. Often the ventral frenulum is covered by the cervix and the dorsal frenulum hangs loosely. The mucosa of the cranial vagina and cervix are pink and plump and a thin layer of clear mucous coats the vaginal os of the cervix [7].

During estrus, mares exhibit several behaviors intended for signalment to stallions that they are ready to mate. Timing of copulation in feral populations is determined solely by mare behavior and is an important factor of reproductive efficiency for the stallion [28] and teasing is often used in breeding management situations for the same reason. As mares get further along in the estrous cycle and closer to the time of ovulation, they begin to approach and solicit stallions with increasing frequency, eventually allowing them to mount [28]. Behavioral signs of estrus include exhibiting a sawhorse stance, presenting the hind end to the stallion, urinating, clitoral winking and relaxing her head and facial muscles [29]. These behavioral cues are stimulated by estrogens and administration of exogenous estrogen has been shown to increase frequency of these behaviors and incidence of copulations in mares at liberty [30]. In contrast, exogenous progesterone administration decreases these behaviors [30,31]. Ovariectomized mares show behavioral signs of estrus with [30,31] and without [32] exogenous estrogen treatment.

2.1.2 Ovulation

Physiology

LH receptors are expressed on the theca cells of growing follicles and granulosa cells of the mature pre-ovulatory follicle account for the positive response to induction agents [25]. In the periovulatory period, LH pulse frequency increases from one pulse every two hours to one pulse every half hour [7]. While estradiol is initially an inhibitor of LH, as the levels of estradiol secreted from the large growing follicle increase, it becomes stimulatory and initiates the LH surge. LH is responsible for increasing the synthesis of prostaglandins within the follicle, upregulating the production of collagenase, and activating proteolytic enzymes to assist in the breakdown of the follicular wall at the time of rupture. While the complete pathophysiology of

ovulation has not been fully elucidated, many factors, such as growth factors, proteins and inflammatory cytokines are implicated to be involved in the process through their subsistence within the pre-ovulatory follicle. Recently, focus has been directed on the identification of specific inflammatory cytokines which play a role in ovulation, some of which include interleukin- 1β , interleukin-6, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor α , and nitric oxide [33–37].

Follicle Rupture

As the dominant follicle approaches ovulation, many changes occur. Blood flow increases to the follicle, which can be visualized on ultrasonography via increased Doppler color flow and the appearance of a hypoechoic ring surrounding the follicular wall. Additionally, a few hours prior to ovulation, the two layers of granulosa cells lining the follicle begin to separate, forming a serrated surface [18,38]. The follicle will also lose turgidity and spherical shape and may develop echogenic spots in the antrum. An apex or stigma is formed, which extends to the ovulation fossa for release of the oocyte. It is inferred that bands of connective tissue on the outer edge of the ovary may help to guide the growing follicle toward the ovulation fossa [7]. As the leading-edge progresses, the theca externa cells become hyperplastic and eventually coalesce with the theca interna cells forming a structure called the macula pellucida [39], also sometimes referred to as the thecal cone [7], which is the site of impending follicle rupture. The increased pressure of this area is exerted on the tunica albuginea and the tunica gradually disappears over this spot [39].

Follicle rupture is a fast process in the mare. In one study, there were two distinct evacuation patterns described- abrupt rupture which occurred over 60 seconds and gradual

rupture and subsequent loss of the majority of antral fluid occurring over 6 – 7.3 minutes [40]. In a majority of ruptures (7/10), a small amount of fluid persists in the follicle for 0.5-20 hours post-evacuation [7]. Follicular fluid is collected in infundibulum (fimbria), though some may leak into the peritoneal cavity.

2.1.3 Diestrus

CL formation

Following rupture of the ovulatory follicle and expulsion of the ovum and follicular fluid, the follicular wall immediately begins to collapse, folding in on itself, and capillaries begin to invade the developing structure [41]. This process is mediated by growth factors, such as fibroblast growth factor, platelet-derived growth factor, insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF) [41]. The granulosa cells of the follicle go on to form the small and large luteal cells of the CL. As its name suggests, luteinizing hormone (LH) is the principal mediator of CL formation. Compared to other species, mares have a prominent LH peak which is maintained until 2 days post-ovulation, which likely accounts for the rapid increase in circulating progesterone [7]. Alternatively, the rapid increase in circulating progesterone could be due to the high concentrations of progesterone from the pre-ovulatory follicle [42] being released into the peritoneal cavity and then reabsorbed.

CL area and progesterone profiles

Progesterone is secreted primarily by the large luteal cells of the CL. Multiple studies have found a correlation between the relative amount of luteal tissue and resultant progesterone production, although one study showed a stronger relationship between luteal blood flow and

progesterone concentrations [43]. In addition, evidence suggests that blood flow and diameter of the preovulatory follicle affect luteal diameter and progesterone production [44]. In general, following ovulation there is a rapid increase in luteal blood flow and area until a peak between day 5 and 10 [7,43], followed by a decrease until day 14-19 [43,45]. Maximum progesterone concentration occurred around day 8 and was an average of 12.8 ng/ml [45].

2.1.4 Luteolysis

Physiology

As with all mammalian species, Prostaglandin F2 α (PGF2 α) is the chief luteolysin. The main PGF metabolite in plasma is 13,14-dihydro-15-keto-PGF2 α (PGFM) [46] and is secreted by the uterine endometrium at day 14 of the estrous cycle [47]. Unique to the mare, the ovarian artery and vein are relatively straight and do not have a counter-current mechanism. This yields a bilateral ovarian effect as PGFM must travel systemically to reach the CL. Rather than having one discreet secretion of PGFM, it is released in pulses which increase in frequency and amplitude (peak 193 ± 36 pg/mL) during luteolysis [48]. A PGFM pulse in mares lasts approximately 6 hours [46]. One study found the total length of luteolysis to be 22.9 ± 0.9 hours [49]. Lysis of the CL is confirmed by progesterone concentrations < 4 ng/mL. During luteolysis, progesterone decrease is about six-fold greater than in the pre-luteolytic period [45]. About 2 days prior to luteolysis, estradiol begins to increase in the circulation and a new follicular wave begins to deviate in preparation for the upcoming estrus [49].

On occasion, luteolytic failure occurs resulting in spontaneously prolonged corpus luteum (SPCL). SPCL incidence increases during fall, with no causal mechanism yet identified. One study found that PGFM pulses were absent in mares who formed SPCLs, but that PGFM pulses

were able to be induced by intrauterine saline infusion [50]. Prolonged corpus lutea can also be induced pharmacologically. Multiple groups have suggested anti-luteolysis oxytocin protocols [51–55]. Exogenous administration of oxytocin prevents secretion of $\text{PGF2}\alpha$. However, administration of oxytocin on or after the time of luteolysis (day 11-15) evokes secretion of a PGFM pulse due to a positive feedback loop between oxytocin and $\text{PGF2}\alpha$ and results in luteolysis [51]. These protocols involve administration of 60 IU oxytocin once daily for a period of 7-30 days and have been shown to prolong CL function for 40-55 days [52–54]. Likewise, continuous oxytocin infusion via an osmotic minipump [56] and slow-release oxytocin formulations have been used effectively [55].

2.2 Seasonality

Reproductive seasonality has been adapted by many species to ensure the birth of offspring during times of favorable environmental conditions. The mare, with a relatively long gestation length, breeds during long days and gives birth during periods of warm ambient temperatures and availability of nutrient-dense food sources. Conversely, species with shorter gestations, such as the ewe and White-tailed doe, breed when daylight is shorter and experience parturition during the opposite seasons.

Defining seasons

Mares go through periods of reproductive cyclicity and acyclicity, with this circannual rhythm influenced by environmental cues. As long- day breeders, the natural reproductive season of the mare living in the Northern Hemisphere typically takes place from March to October [6]. Before and after the natural reproductive season, mares undergo transitional periods

leading to cyclicity (spring transition) and anestrus (fall transition), respectively [6]. In the Southern hemisphere, cyclicity takes place from April to November [7]. The lowest incidence of estrus occurred at the time of the winter solstice (June 22) in the Southern Hemisphere [57]. Variations in seasonal cyclicity among mares exist due to the influence of internal physiologic factors, such as nutrition, body condition, and age as well as external factors, such as photoperiod, and ambient temperature. Cyclicity can also be altered using pharmacological agents.

Physiology

Ginther splits the anovulatory season into three phases: 1) the receding phase (fall), 2) the inactive phase (winter) and 3) recrudescence (spring) [7]. During the receding phase and recrudescence, follicles continue to grow, but fail to ovulate, due to a progressive decrease in LH [58]. It has been speculated that this decrease in circulating LH is resultant from depletion of LH pituitary stores [59]. As the mare approaches the inactive phase, follicles fail to grow past 10-15 mm in diameter, reflecting the low levels of FSH. Due to a lack of large follicles, systemic estrogen and progesterone concentrations are also diminished. A subset of mares will continue to cycle year-round [7,57,60], although winter estruses have been reported to be longer than regular estruses [57] and an increased incidence of persistent corpus luteum, with an average of 77 days of luteal phase, have been reported [60]. During both transitional phases, many changes to the estrous cycle occur. The length of estrus has been shown to increase during the winter and fall in comparison to the summer, with no change in the length of diestrus [57].

Circannual rhythms in the mare are the result of a conglomerate of various physiologic pathways. Foremost, photoperiod is the primary zeitgeber of reproductive cyclicity. The photic

stimulus begins in the eye where signals from the retina are transferred to the suprachiasmatic nucleus (SCN) in the anterior hypothalamus. The SCN acts as the central pacemaker by regulating melatonin secretion from the pineal gland [61].

Melatonin distributes to several sites for the entrainment of circadian rhythms, including the pars tubalis of the pituitary and the SCN in the mare [62] and, depending on the species, can be responsible for upregulation or downregulation of reproductive function. Intraperitoneal exogenous melatonin administration was found to reduce ovulation rates in rats [63]. In the mare, it is hypothesized that melatonin does not play as critical role in the regulation of seasonal reproductive activity. In pony mares with melatonin implants, there was a delay in time to the last ovulation in the season and, paradoxically, LH levels in melatonin treated mares were increased compared to control mares [64]. To explain this phenomenon, it has been suggested that mares experience a decreased or refractory response to melatonin during fall and winter. One group found that anovulatory mares under the treatment of a long-term melatonin osmotic mini pump, showed no difference in prolactin concentrations compared with control mares [65] and others showed that melatonin suppressed prolactin levels during the spring and summer months, but not in fall and winter [66], suggesting that mares become seasonally refractive to melatonin or that there are other physiologic mechanisms which supersede melatonin's control over prolactin. Also supporting this theory, other groups investigating melatonin concentrations found that there was no clear association between the degree of increase of nocturnal melatonin levels and daylight: nighttime ratio [67] and that the duration of elevated melatonin levels changed according to natural season, regardless of actual nighttime hours the mare is exposed to [68]. In addition, some individual mares show variation in melatonin patterns when exposed to the same photic stimuli as others in a group. In one study, some mares failed to exhibit a

nighttime rise in melatonin, a phenomenon which was suggested to be the reason some mares continue to cycle year-long [66].

Prolactin is another seasonally regulated hormone which has important functions in the female reproductive system. Prolactin concentrations vary in the mare throughout the year and coincide with periods of reproductive cyclicity [69]. Although the exact mechanism of prolactin control over the mare reproductive system has not yet been elucidated, exogenous prolactin administration to mares in anestrus leads to the development of a pre-ovulatory follicle [70] and hastening to the first ovulation of the year [71]. Systemic prolactin concentrations increase significantly in the peri-ovulatory period while concentrations of prolactin within the pre-ovulatory follicle simultaneously increase, lending to the thought that prolactin is a meaningful regulator of ovulation [72].

Dopaminergic agents, such as bromocriptine, provide inhibitory action on prolactin, while anti-dopaminergic agents, such as sulpiride and domperidone, increase prolactin levels. These relationships become clinically significant in cases of ergot toxicity, which commonly leads to agalactia in addition to a host of other late-term gestational problems, such as prolonged gestation length, placental edema, premature placental separation, dystocia, or weak foals. Conversely, anti-dopaminergic agents can be used to alter the mare's estrous cycle and seasonal cyclicity, as further described below.

2.3 Factors Affecting Cyclicity

Photoperiod

As the major zeitgeber of circannual rhythms in mares, photoperiod is frequently manipulated to accelerate the onset of the breeding season. The primary artificial photoperiod

manipulation used is the extension of daylight hours to mimic long days. This procedure was first described in the mare by Burkhart, *et al.*, in 1946 and has been studied by many groups since then [9,73]. In addition, photoperiod manipulation has been used to shorten the gestational length in mares [74] and cause hair coat shedding in show horses.

The most effective photoperiod manipulation protocols used at least 10 lux [75] of light ranging from 14.5:9.5 (14.5 hours light and 9.5 hours dark) to 16:8 [9,73,74,76]. An important concept is that extending daylight hours will only result in long-day responses if the daylight falls during a specific photoinductive phase, or time of the day that the animal is responsive to changes in light, of the circadian cycle [62]. As such, the recommended time to add artificial lighting is at the end of the daily natural light period (sunset) and conclude at 11 pm. Scaba and Ginther investigated the optimal time of the year to begin artificial light exposure and found that, in the Northern Hemisphere, starting on December 1st significantly hastened the onset of the first ovulation, with a mean of 65 days initiation of artificial light, compared to January 1st, but there was no benefit to starting exposure on November 1st [76]. The most common practice involves using artificial light daily starting on December 1st until resumption of cyclicity, although it has been shown that discontinuation of light therapy after 35 days produces similar results [77].

Another option for altering photoperiod in mares is using a night break protocol. Night break involves exposure to a very brief light pulse which interrupts a long night to induce a short-day response, despite a short total duration of light [62]. In one experiment, a short (1 minute) duration light pulse in the middle of the night suppressed melatonin secretion acutely following exposure and continued to suppress it 24 hours later in Djungarian hamsters [78]. In mares, interruption of a dark period with 1 hour of light is most effective 9.5 hours after dusk

(approximately 4am) when compared with other exposure times [73,76], suggesting that this is a photosensitive period.

Some photoperiod modifications not practically used in mares include resonance light cycles and T-cycle paradigms. Resonance light cycles involve repeated exposure to short (4-6 hours of light) light pulses in a background of darkness. As expected, when these exposures are repeated at 24-hour intervals, a short-day light response is observed. However, when the exposures are repeated at non-24-hour intervals, long-day effects are observed, further providing evidence that only photic stimuli provided at certain points within the circadian cycle will lead to long-day effects [62]. T-cycle paradigms are similar to resonance light cycles, instead utilizing very short light pulses (a few minutes duration) [62].

Recently, the effects of blue light therapy have been investigated. While traditional light therapy is applied in the form of overhead stall lights, blue light therapy is applied via a specialized mask. In one study, mares receiving blue light photic stimuli showed no difference in interval to cyclicity from mares housed under traditional stall lights [79]. Thoroughbred mares exposed to blue light at a minimum of 10 lux for 1 hour at night experienced an inhibition of melatonin production [80]. Moreover, photic stimulation of a single eye is as effective as blue light applied to both eyes [79,80].

Ambient temperature

While not the primary factor of entrainment of circannual rhythms in the mare, ambient temperature has been linked with seasonality in the mare. Due to the correlation between temperature and photoperiod in natural conditions, there is limited data describing effects of temperature solely. One retrospective study, which observed the reproductive cyclicity of

Thoroughbred mares over the course of several years found that the onset of the first ovulation of the year was closely related to both minimum and maximum temperature [81]. Another group reported a significant correlation between mean winter temperature and pregnancy rate of the first estrus post-partum (foal heat)[82]. While these findings are supportive of a downregulation of reproductive activity during times of very cold weather, the same seems to be true of very hot weather. In mares in Finland, increased ambient temperature in the summer was associated with decreased foaling rates [83]. Likewise, mares who were exercised for 30 minutes per day in hot (>30 °C) and humid (>50%) climates had a markedly decreased embryo recovery rate [84].

Nutrition

A third factor affecting reproductive cyclicity in mares is nutrition. While increased energy density of a diet does not directly influence whether a mare will cycle, it does have effects in relation to maintenance of healthy body condition score. In general, mares with thin body condition have poor reproductive efficiency, delayed onset of estrus and reduced pregnancy rates compared to moderately fleshed or obese mares. In general, mares with an increased body condition score (BCS > 5.0, on a scale of 1-9) have a shorter interval to the first ovulation than thin mares [85]. However, thin mares fed a nutrient-dense diet had a shorter interval to the first ovulation than thin mares who were fed nutrient-poor diets, but energy density did not affect intervals to ovulation in moderate and obese mares [86,87]. On the other end of the spectrum, obese mares who were fed an ad lib diet cycled longer during the fall than obese mares under feed restriction [88,89]. Additionally, obese mares have an increased interovulatory interval and luteal phase [88] and shorter duration of estrus [87]. One group found that well-fed mares have higher concentrations of growth hormone, IGF-1 and leptin levels, and that those levels were

highly correlated with the duration of ovulatory activity [89]. GH controls proliferation, apoptosis, growth and differentiation and the secretory activities of different reproductive organs and their response to GnRH [90]. IGF-1 stimulates the growth of ovarian follicles by granulosa cell proliferation and differentiation [91]. Leptin, a hormone produced by adipocytes, has influence over immune function, particularly T-cell immunity, and has been suggested to have a possible relationship with seasonal cyclicity [92]. Mares who were feed restricted had a lower follicular fluid concentration of leptin and increased concentrations of inhibin-A and VEGF as well as improved pre-ovulatory follicle vascularity than mares who were fed ad-lib, indicating that nutrition changes have effects at the level of the ovary [93]. Inhibin-A is primarily known for negative feedback of FSH. VEGF controls proliferation and migration, and inhibits apoptosis of endothelial cells, thereby mitigating bloodflow of many important reproductive processes such as ovulation and pregnancy maintenance [94].

In post-foaling mares, a high body condition score (>5.0) is associated with enhanced breeding efficiency [95]. Similarly, a moderate body condition score (>3.0) was found to have a predictive value of fertility of 91% in Lusitano mares [96]. In one study looking at 3- and 4-year old mares, circulating leptin levels, which are often associated with obesity and high energy diets, increased significantly in summer, peaked in August then declined in fall. In addition, leptin concentrations were lower in anestrus fillies than those who continued cycling throughout winter, lending to the idea that leptin is associated with seasonality [60]. Although high body condition scores are associated with increased fertility, one experiment showed that obese Shetland pony mares fed a high energy diet produced embryos with a higher incidence of early embryonic death [97].

Van Niekirk et al. investigated the effects of protein quality on reproductive performance and found that mares receiving a high protein diet ovulated 2-3 weeks earlier in the breeding season than those eating a low protein diet [98].

Disease

Disease status of a mare may affect the reproductive cycle in various ways. Foremost, certain ailments may directly disrupt a physiological mechanism involved in cyclicity, as, for example, in the case of blindness blocking photic stimuli to the mare and resulting in loss of normal seasonal patterns of cyclicity, as exhibited by other species [99,100]. Other conditions, such as equine metabolic syndrome and pituitary pars intermedia dysfunction result in endocrine changes and hormone derangements which affect the reproductive cycle. Equine Metabolic Syndrome (EMS) is a highly prevalent condition of horses in first world countries, stemming from widespread obesity. EMS is characterized by insulin resistance, increased levels of leptin and pro-inflammatory cytokines and decreased adiponectin [101]. While the chief concerning sequela is laminitis, disruption of normal cyclicity and fertility can also occur in mares with EMS. One group found a change in expression of genes related to metabolism, follicle maturation, and oocyte maturation in mares with EMS as well as increased concentrations of insulin, leptin, adiponectin, and cytokines in both systemic circulation and follicular fluid [102]. Pituitary pars intermedia dysfunction (PPID) is a degenerative disease of the hypothalamic neurons of the paraventricular and supraoptic nuclei which results in decreased dopaminergic signaling [103]. Due to this decrease, melatonin profiles in horses with PPID showed less seasonal variation, diminishing the natural ability to distinguish between time of year [104]. While mares with PPID typically continue to cycle, administration of the PPID treatment

pergolide, a dopaminergic agonist, may lead to anestrus by blockade of the GnRH signaling pathway. Other disease conditions may act even more indirectly on the reproductive system via a rise in systemic cortisol. Glucocorticoids are known to suppress estrous, follicular growth, LH secretion and ovulation [31]. A comparable scenario is suspected to occur in mares who undergo periods of stress, but this has not been proven. A group of mares who were transported by trailer for 17 hours during the pre-ovulatory period of estrous were found to have increased cortisol and LH concentrations than mares who were not transported, but there was no difference in number of mares ovulating, estrus behavior, duration of the estrous cycle or pregnancy rate between groups [105].

Age

Age of a mare plays a factor in seasonal cyclicity. Aside from reaching a reproductive senescence, older mares show less follicular activity throughout the year [86]. In addition, older mares are more likely to undergo seasonal anestrus and enter into it earlier in the year than younger mares [69]. Mares also ovulate smaller follicles as they age above 20 years [86].

2.4 Pharmacological manipulation of the estrous cycle

Steroid hormones

Two steroid hormones, progesterone and estrogen, which are produced by the corpus luteum and growing follicles of the ovary, respectively, and their analogs are habitually used in reproductive practice for the purposes of hastening the first ovulation of the season, synchronizing embryo donors and recipients, delaying ovulation and maintaining pregnancy, among other things.

Many formulations and vehicles for each hormone are available. Progesterone in the United States can be found as a daily intramuscular injection in oil or a sustained-release intramuscular injection (BioRelease P4 LA, BET Pharmacy, Lexington, KY). This sustained release formulation was shown to elevate progesterone levels ($> 1 \text{ ng/mL}$) for an average of 7.6 ± 2.2 days [106]. Several synthetic progestins are also commercially available. The most popular, Altrenogest can be found in both daily oral (Regu-Mate®, Merck Animal Health, Millsboro, DE) and long-acting injectable formulations (Altrenogest, BET Pharmacy, Lexington, KY). Other progestins, such as medroxyprogesterone acetate (Depo-Provera®, Pfizer, New York, NY) and melengestrol acetate (MGA, Zoetis, Parsipanny, NJ) do not produce circulating progesterone levels high enough to maintain pregnancy or alter the estrous cycle [107]. In addition, intravaginal progesterone-releasing devices (IPRDS) are available, including the CIDR-B (Eazi-Breed CIDR®, Zoetis, Parsipanny, NJ) and Cue-Mare (Vetoquinol Australia, Brisbane, Australia) and progesterone-impregnated vaginal sponges. It is important to note that vaginitis can result from the use of IPRDs, but is usually transient and has does not affect fertility [108]. Estrogens are available in two injectable formulations- Estradiol 17β , (BET Pharmacy, Lexington, KY) a daily injection, and Estradiol cypionate (ECP, BET Pharmacy, Lexington, KY).

Secondary to alterations of photoperiod, the administration of steroid hormones is the most common method of decreasing the interval to the first estrus and ovulation of the season. The first investigation into the effects of progesterone on resumption of cyclicity was performed in 1973. A group treated fourteen anestrus mares with 100 mg progesterone in oil injections once daily for 7 days. Mares in late transition ovulated within 6 -7 days following cessation of therapy and obtained a conception rate of 75-80%. Mares that had only a small amount of

follicular activity responded to treatment and ovulated an average of 8 days following cessation of therapy, while mares who were in deep anestrus did not respond [109]. Similar effects were found in another study performed in the Southern Hemisphere where mares were administered one dose of 300 mg sustained-release progesterone and ovulated an average of 18.6 ± 8.7 days, with a range of 10 – 24 days, following treatment. This study also showed that there was a better response to progesterone administration later in transition (83%) than in early transition/anestrus (0%) [106]. In combination with 10 mg estradiol 17β once daily for 10 days, 150 mg progesterone once daily resulted in a mean interval to ovulation of 9 - 13 days following treatment [110]. Another study compared the effects of different steroid hormones and found that treatment with 26 mg altrenogest once daily for 15 days was superior to another progestin, norgestomet, and that combined treatment of altrenogest plus 10 mg estradiol 17β once daily yielded the best results for induction of ovulation [111]. Other groups studied the use of IPRDs with [108,112] and without [113] estradiol 17β and found that interval to ovulation was reduced to a mean of about 14 days [113] overall or 6 days after removal of IPRD [108] and pregnancy rates were enhanced following the use of the intravaginal device [112].

Estrus synchronization, while not performed on a large-scale basis for the purposes of timed insemination like in ruminant species, is a crucial component of embryo transfer programs. While some programs utilize natural cyclicity and detection of ovulation for synchrony between the embryo donor and recipient, many programs, employ the use of steroid hormones. Beginning in the 1970's mare synchronization was achieved using daily progesterone injections [114] and then later feed-through altrenogest supplementation [115]. While oral and injectable forms of progesterone are still used, IPRDs are becoming increasingly popular due to ease of use and have been studied by multiple groups with promising results [116–119]. In addition,

estrogens and prostaglandins have been used in synchronization protocols to further mimic a natural estrous cycle [119–121]. Recently, groups have explored the effects of endometrial edema on pregnancy [122,123]. Silva has shown that estrogen exposure of the uterus affects the production and gene expression of uterocalin, a protein known to play an important role in providing lipids to the embryo. In short, mares exhibiting high endometrial edema scores, a measure of estrogen exposure, for 6 or more consecutive days produce more uterocalin than other mares [122,123]. Also of importance, progesterone and estrogen combined protocols allow for the use of recipient mares in any stage of the estrous cycle, including anestrus [119,124].

In some cases, breeding on the “foal heat”, the first estrus following parturition, is desirable to hasten the foaling date in the following season and decrease the risk of the mare entering anestrus. However, the first estrus in the post-partum period has been shown to have reduced fertility, with pregnancy rates as low as 39% [125] and about 13% lower than those of the second ovulation of the year [126]. The immediate post-partum period is important for rapid uterine involution, clearance of fluid, debris and pathogens and healing of injuries that may have been sustained during parturition. One study found that in post-partum mares, there was an advantage in pregnancy rates for those mares whose first ovulation occurred after a certain time period (no sooner than 12.3 days). However, they also found that 43% of mares ovulated by 9 days after foaling which is well before this advantageous period [110]. To allow the uterus sufficient time for involution, some groups have attempted to use progesterone to delay the foal heat [127,128]. The combined use of exogenous progesterone (150-300 mg) and estradiol 17 β (10-20 mg) post-foaling successfully delayed ovulation for 10 days in 100% of mares in one study but had no effect on uterine involution [128]. In another study, mares who were treated with a IPRD post-partum and bred on the first ovulation had similar pregnancy rates to those

who were not treated and bred on the first and second ovulations. Interestingly, the group of mares who were treated with the IPRD had better responsiveness to human chorionic gonadotropin (hCG) induction of ovulation and, therefore, less breedings per cycle [127].

Besides delay of foal heat ovulation, progesterone has been investigated to delay ovulation as it pertains to regular cycles. There is economic merit to this idea due to the frequency in which semen shipments are lost or delayed after a mare has a large, pre-ovulatory follicle. In 1966, Loy used 100 mg progesterone in oil to successfully block estrus and ovulation in mares. Similarly, mares given altrenogest between days 10 - 20 of the estrous cycle had an increased luteal phase [129]. A more recent study treated mares that had a large, pre-ovulatory follicle (>35mm) with either a CIDR or 0.044 mg/kg altrenogest for 2 days found that neither treatment delayed ovulation, but both stopped estrus behavior [130].

Luteal insufficiency is a major cause of early pregnancy loss in the mare. Fetal death rates between 10 and 15% are reported with the majority occurring during the first 40 days of gestation when progesterone is supplied exclusively by the primary CL [131]. Serum progesterone concentrations of 4.0 ng/mL are required for pregnancy maintenance [131] and decreases in progesterone concentrations below 2.56 ng/mL are associated with subsequent embryo loss [107]. While the pathophysiology of luteal insufficiency leading to abortion has been disputed [131], cases have been reported [132,133]. Nevertheless, it is common practice to supplement progesterone in early gestation mares whose serum progesterone concentrations are less than 4.0 ng/mL or who have a history of pregnancy loss with no discernable cause. McKinnon explored the exogenous progesterone requirements for ovariectomized mares who produced no endogenous progesterone and found that mares given 200 mg of progesterone in oil daily maintained pregnancies, but began to lose pregnancies at a dose of 100 mg. Mares treated

with altrenogest maintained pregnancy at the label dose (0.044 mg/kg) and half the label dose (0.022 mg/kg) but started losing pregnancies at doses of 0.011 mg/kg and below [107].

Likewise, embryo recipient mares, who are often synchronized using pharmacological protocols and do not have an active CL at the time of the transfer, are often successfully maintained with progesterone or altrenogest [134,135] until the fetus takes over progesterone production.

Prostaglandin F2 α

Predominantly secreted by the endometrial cells of the uterus, Prostaglandin F2 α (PGF2 α) functions primarily in lysis of the corpus luteum. When administered exogenously, PGF2 α and its analogs are used for induction of luteolysis, prevention of luteogenesis. Less commonly, prostaglandins are also used for induction of ovulation, which will be discussed in further detail in the next section. Two formulations are widely available in the U.S. The natural PGF2 α , dinoprost tromethamine (Lutalyse, Zoetis, Parsipanny, NJ) and the synthetic cloprostenol (Estrumate®, Merck Animal Health, Millsboro, DE) are both labelled for intramuscular injection.

The use of PGF2 α is customary for luteolysis and reduction of the luteal phase of the cycle, a process called “short-cycling”. Minimal effective dose of dinoprost for the mare is 8.8 mg/kg and 7.3 mg/kg for ponies [7]. While systemic administration of PGF2 α is typically performed, intrauterine administration is equally effective. This contrasts with ruminants who are much more sensitive to intrauterine PGF2 α administration and require reduced doses [41]. The ruminant dose is 1/18th that of horses. Doses of PGF2 α are almost exclusively given in bolus. However, it has been shown that boluses of PGF2 α lead to an increase of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and cortisol concentrations within 10

minutes of administration, which is not repeatable when the same dose is given intravenously slowly over the course of an hour to mimic physiologic conditions [46]. This could explain the mechanism by which $\text{PGF2}\alpha$ stimulates ovulation in mares and why there is an increased incidence of development of hemorrhagic anovulatory follicles when $\text{PGF2}\alpha$ is administered in the presence of a large follicle, even during diestrus. It has also been shown that administration of two small doses (5 mg dinoprost) 24 hours apart is just as effective as one large dose (10 mg dinoprost tromethamine) and reduces side effects [23]. Similarly, microdoses of $\text{PGF2}\alpha$ (0.5 mg dinoprost tromethamine) have been administered at the BAI HUI acupuncture point with acceptable effectiveness for induction of luteolysis [136].

Shortening of the interovulatory interval is a chief goal of short cycling in mares. This interval is especially important when it comes to maximizing the reproductive efficiency of embryo donor mares. Furthermore, it has been shown that interovulatory interval has an effect on pregnancy rate of the subsequent cycle. One group found that mares who were induced for luteolysis at an interval of less than 6 days had a pregnancy rate of 26.6%, while mares who had intervals of 6 to 8 days and greater than 8 days had pregnancy rates of 39.4% and 55.9%, respectively [127]. In the natural estrous cycle, interovulatory interval varies from 16 to 25 days, with a mean of 21 days [137]. It has been shown that administration of $\text{PGF2}\alpha$ two days prior to embryo flushing effectively decreases the interovulatory interval in embryo donor mares, without impacting embryo recovery rate. Mares who received cloprostenol between days 5 and 12 after ovulation had an average interovulatory interval of 8.4 days [138]. Similarly, mares given $\text{PGF2}\alpha$ at day 3 and day 10 post-ovulation had a shorter interovulatory interval following day 3 administration vs. day 10 (average 13.2 vs. 19.2 days) [139].

In general, PGF2 α is administered on or after day 5 post-ovulation, once the CL is fully mature. In a group of mares given PGF2 α on day 3, a functional resurgence of the CL was detected in 75% of the mares beginning 3 days after administration [139]. Likewise, mares given dinoprost tromethamine on day 2 of estrous had an increased number of partially regressed CL's as compared with administration on day 8 [140]. To circumvent this waiting period, an anti-luteogenesis protocol has been established, consisting of administration of 2.5 mg dinoprost tromethamine on days 2, 3 and 4 following ovulation [141] or days 1, 2 and 3 [142].

Prostaglandins have further use as ecbolic agents due to their prolonged contractile activity. As compared with oxytocin, which increases intrauterine pressure within 60 seconds of administration and lasts for 40 - 60 minutes [143], dinoprost tromethamine produces a response within 10 - 20 minutes after administration which lasts for 60 - 240 minutes [143]. One study, which looked at uterine clearance of a radiocolloid, found that the prostaglandins dinoprost, cloprostenol and fenprostalene all successfully cleared the colloid by 60 minutes, with cloprostenol having the most consistent results [144].

Dopaminergic agents

Dopamine plays an important, albeit poorly understood, function in seasonal reproduction. Dopamine-1 and Dopamine-2 receptors have been found in ovarian tissues in mares in higher concentrations during times of cyclicity vs. acyclicity [145] and high concentrations of dopamine have been found in growing follicles [145]. Further evidence of dopamine's importance exists in the case when an anti-dopaminergic agent, such as ergot alkaloid or pergolide, inhibit cyclicity in the mare. There are two main Dopamine-2 agonists available in the U.S.- the injectable sulpiride

(human trade name: Dogmatil) and oral paste domperidone (Equidone® gel, Dechra, Overland Park, KS).

While sulpiride has been used successfully to hasten ovulation in transitional mares, domperidone use has not been successful. In one study, the first ovulation was advanced for mares treated with daily sulpiride by a mean of 33 days and LH and FSH levels were higher at the time of the LH surge in sulpiride-treated mares [146]. Another study showed similar effects on LH and FSH concentrations, with a mean time to ovulation 21 days shorter than non-treated mares [147]. In a comparative study, domperidone was not found to advance the first ovulation to the extent of sulpiride and mean intervals to ovulation were 46 and 16 days, respectively. Pre-treatment with estrogen greatly enhances the response to sulpiride [148].

GnRH and GnRH agonists

As stimulants of both FSH and LH release, GnRH and its agonists are useful in the advancement of the first estrus and ovulation in transition mares. GnRH itself is a decapeptide released from the hypothalamus which functions in the stimulation of gonadotropin (LH and FSH) release from the anterior pituitary gland. One of the early studies on this topic challenged pre-slaughter mares at different times of the season with a single injection of GnRH. Mares at all points in cyclicity responded to GnRH administration, however, as breeding season approached, the amount of FSH was reduced, suggesting a regulatory mechanism of FSH secretion during this time [149]. Another study, which analyzed the effects of different dosages of native GnRH (50, 100 and 200 ng/kg/h) infused via osmotic minipumps on anestrus mares, found that time to ovulation was inversely related to dose [150]. Time to the first ovulation was also advanced in

anestrus mares given the GnRH agonists deslorelin [108,151] and busreltin [152] but not goserelin [153].

Gonadotropins

Gonadotropins were first used to induce ovulation in mares in 1939 via administration of pregnant mare urine extract and pregnant mare serum gonadotropin [154]. Today, hCG (Chorulon®, Intervet/Merck Animal Health, Madison, New Jersey, U.S.) is the only product labelled for horses in the U.S. Aside from their use as potent ovulation induction agents, which will be discussed in the next section, gonadotropins are stimulatory to CL development and progesterone production. In one study, 1000 i.u. of hCG was administered to mares on days 3, 4, 5 post-ovulation and led to increased progesterone concentrations between days 7 and 14 [155]. In another, 1500 i.u. hCG was administered to pony mares on day 8 of the estrous cycle, resulting in a precipitous rise in mean progesterone concentrations from 9.65 to 12.63 ng/mL via an increase of progesterone pulse amplitude, but not frequency [156]. Similarly, in the rabbit, administration of 10 i.u. hCG on day 3 post-ovulation resulted in a threefold increase in progesterone concentrations, but no changes in luteal blood flow [157].

2.5 Induction of Ovulation

Pharmaceutical induction of ovulation is one of the most important tools on the reproductive veterinarian's belt. It can lead to both a reduction of interval to ovulation, typically by 1-3 days, thereby improving reproductive efficiency in embryo donors and a narrowing time period of ovulation. This programmed window of ovulation allows for the use of timed insemination protocols, scheduling stallion appointments for live cover mating, ordering cooled semen,

synchronizing embryo recipients with donors and decreasing number of inseminations for persistent-breeding endometritis susceptible mares. As with most mammalian species, ovulation in mares occurs via a series of complex physiologic events. In short, an increase in GnRH produced by the hypothalamus causes an increase of follicle stimulating hormone and luteinizing hormone production by the anterior pituitary. Follicle stimulating hormone stimulates growth of the follicles, while the luteinizing hormone surge initiates ovulation. The mare is unique in that the LH surge is prolonged and peak occurs 2 days post-ovulation, which provides a set of challenges in inducing ovulation that are not apparent in other domestic livestock species.

Ovulatory agents

One of the earliest pharmaceutical agents used to induce ovulation in the mare, with first reports of administration dating back to the 1930's, human chorionic gonadotropin (hCG) is still widely popular today. A glycoprotein secreted by the chorionic villi in the woman's placenta, hCG mimics the actions of its pituitary-derived analog, LH, by binding to LH receptors on the granulosa cells of the preovulatory follicle and initiating ovulation. Interestingly, hCG is shown to have a dual mechanism of action by also stimulating an increase in systemic LH concentrations and a decrease in estradiol 17β concentrations. Following administration of hCG, mares with a pre-ovulatory follicle of at least 30 mm in diameter are expected to ovulate within a 48-hour time window, with the average being 36 hours. hCG is available in the U.S. under the trade name Chorulon® (Intervet/Merck Animal Health, Madison, New Jersey, USA) as a freeze-dried preparation with sterile water for reconstitution. Doses commonly used range from 1500-3000 iu/ horse [158], though a dose of 750 iu has been shown in one study to be effective in inducing ovulation in mares [159] while 500 iu was found to be ineffective [158]

Despite its popularity, there are drawbacks associated with the use of hCG, foremost being the development of anti-hCG antibodies in mares following repeated use. Wilson et al investigated this phenomenon in mares administered hCG multiple times over the course of two breeding seasons. They found that following 2 - 5 injections in the first year, 50% of mare's individual ovulation times did not differ from control cycles and after 1 - 4 injections during the second season, 57.1% of mares showed reduced efficacy. Additionally, they found that all treated mares were positive for anti-hCG antibodies after 1 - 4 injections, but there did not seem to be a correlation between the magnitude of immune response and increased time to ovulation or pregnancy rate [160]. This immune response to hCG can also manifest as a Type III hypersensitivity reaction, leading to life-threatening complications. Moreover, efficacy of hCG decreases in relation with mare's age. Being of human-derived origin, hCG has shown variations of purity and consistency of product as well as periods of unavailability.

Another immensely popular group of reproductive pharmaceuticals, GnRH agonists work to mimic the action of GnRH at the level of the pituitary, causing a release of LH and FSH, which ultimately results in ovulation in appropriate doses and in the presence of a pre-ovulatory follicle. GnRH is commonly used for induction of ovulation in other livestock species. Unique to the mare, the LH surge lasts several days and often peaks 2 days after ovulation. Therefore, the need arose for a pharmaceutical agent or administration protocol that stimulated this prolonged increase. One group looked at the use of GnRH osmotic mini pumps for the transition to estrus and ovulation in anestrus mares using dose rates of 50 ng/kg/h, 100 ng/kg/h and 200 ng/kg/h [150] with the higher doses being more efficacious. In 1992, Becker and Johnson compared the effects of continuous versus pulsatile infusion of GnRH by administering either 20 ug/hr of GnRH continuously or giving 20 ug of GnRH each hour. They found that mares

receiving pulsatile administration ovulated 2.8 days sooner than those receiving continuous infusion [161]. Apart from ovulation induction, GnRH and its agonists have been used in the mare to hasten the spring transition period [158], increase follicular growth, suppress estrus and promote multiple ovulations [162,163].

Deslorelin acetate is a synthetic nonapeptide of the natural gonadotropin releasing hormone. The first commercially available deslorelin acetate product available for use in the mare was the Ovuplant (Peptide Technologies, Sydney, Australia), a subcutaneous implant that met the need for a long-acting GnRH pharmaceutical that would induce a prolonged LH surge. Originally implanted under the neck skin of mares, the Ovuplant contains 2.1 mg deslorelin acetate and is typically imbedded within the vulvar mucosa. Multiple studies investigated different doses of deslorelin acetate implants. McKinnon et al. placed implants containing 1.3 mg, 1.6 mg or 2.2 mg subcutaneously in the necks of mares and found the 2.2 mg dose to be most effective [164]. This finding was also mimicked by Squires et al [165]. Meanwhile, Meinert et al. found that implants containing 1.5 mg and 2.25 mg deslorelin acetate had the similar efficacy when being used to induce ovulation in Hanoverian mares with follicles > 40 mm [166]. Later, Mumford et al. placed multiple (0, 1, 3, or 5) implants containing 2.2 mg of deslorelin acetate in mares with a pre-ovulatory follicle > 30 mm and found that all groups treated with an implant had decreased intervals to ovulation, increased proportions of mares ovulating within 48 hours and a decreased follicular diameter at the time of ovulation compared with the control group [167]. However, prolonged use of a GnRH analogue is known to cause internalization and downregulation of receptors, thereby suppressing FSH and lengthening the interestrus interval in mares. This concern was remedied with the work by McCue et al. which showed that mares from which the implant was removed after 48 hours had a normal ovulation rate and interval to ovulation [168].

Although the implant is no longer commercially available in the U.S., an FDA-approved injectable formulation of deslorelin acetate has been on the market since 2010. SucroMate (Thorn Biosciences, Louisville, KY) is a sustained release product consisting of 1.8 mg deslorelin acetate/mL in a sucrose base and is indicated for inducing ovulation within 48 hours of treatment in mares with a follicle between 30 - 40 mm in diameter. Ferris found that Quarter Horse mares with follicles ≥ 35 mm, induced with SucroMate had a higher ovulation rate (89.9%) than those induced with hCG (82.8%) [169]. Additionally, a similar product, BioRelease Deslorelin (Dechra, Australia) was found to be equally effective at inducing ovulation as Ovuplant [170]. In addition to deslorelin acetate, a host of other injectable GnRH agonists have been used in the mare. BioRelease Histrelin acetate (BioRelease Technologies, Lexington, KY), an analogue more potent than deslorelin, has been found to be equally effective in inducing ovulation at a dose of 0.25 mg [171], 0.5 mg [171,172] and 1.0 mg [172], as has the analogue busrelin which has been given in multiple, frequent doses [173–176].

A third and less commonly used group of ovulation induction agents include prostaglandin $F_{2\alpha}$ and its analogues. In the U.S., this includes the natural $PGF_{2\alpha}$, dinoprost (Lutalyse, Zoetis, Inc., Kalamazoo, MI, US) and cloprostenol (Estrumate, Merck Animal Health, Madison, NJ, USA). Loy et al found that 81% of estrus mares given 250 mcg of the $PGF_{2\alpha}$ Fenprostalene intravenously ovulated within 48 hours [110]. Similarly, Jochle et al. found that, following intramuscular administration of 7.5 mg luprostiol, pituitary venous concentrations of FSH and LH peaked instantly and remained elevated for 60-120 minutes [177]. However, others have demonstrated the administration of prostaglandins is associated with the formation of hemorrhagic anovulatory follicles (HAFs) [137,159]. Arango and Newcombe found that the incidence of HAF formation correlated with an increasing dose of cloprostenol, with the average

dose given in HAF-resultant cycles being 375 mcg, while the average dose in non-HAF cycles was 254 mcg. They speculated that this association is likely due to an interference with intra-follicular metabolism of prostanoids and proteolytic enzymes [178]. Other ovulatory agents that have been studied, but are not widely available for use in clinical practice include recombinant LH, equine pituitary extract and kisspeptin, which has not been shown to be effective in the mare [179].

Ovulation induction protocols and reproductive efficiency

When it comes to induction of ovulation and insemination, timing is everything. In the Thoroughbred industry, all matings are produced by live cover and, therefore, ovulation programming is important to avoid multiple breedings and increasing the demand on the stallion. Common practice for natural cover situations is to administer an induction agent, breed 24 hours later and then repeat the breeding every 48 hours until an ovulation is detected. Woods et al. found that mares had a higher pregnancy rate when bred 3 or less days prior to ovulation and within the first 12 h after ovulation, with the highest pregnancy rates within 6 hours after ovulation [180]. Similarly, with the use of cooled-shipped semen, chute fees and shipping costs can become economically burdensome on mare owners and lessening the number of shipments is advantageous.

The use of frozen semen, while advantageous in allowing the propagation of genetics from stallions who are not actively collecting or from a geographically isolated or distant location, can present with many challenges. Pregnancy rates of mares bred with frozen semen range from 30 - 40% [181]. Frozen semen has a shorter lifespan than fresh or chilled, likely due to the capacitation-like changes that the sperm undergo during the cryopreservation process.

Due to this shortened lifespan, mares should be bred within 12 hours of ovulation. One study showed that there is no difference in pregnancy rates between mares bred prior to or just after ovulation [182]. Multiple considerations need to be made when breeding with frozen semen, such as number of inseminations and which insemination timing protocol to use. It has been shown that breeding with two doses of frozen semen shows an increase in fertility when compared to one [183]. If only one dose is available, similar rates have been achieved by splitting the dose, providing there is adequate volume to perform the insemination technique. In the case of two inseminations, a variety of timed protocols can be used. Firstly, a dose of a GnRH agonist or hCG can be administered. The mare is bred at 24 hours post-induction and checked via transrectal ultrasonography 40 hours post-induction and bred again if a fresh ovulation is present. If the mare has not ovulated, she will be rechecked at 4 - 6 hour intervals until ovulation occurs and the mare is bred. Another approach would be to breed at 24 hours post-induction, then ultrasound 12 hours later, repeating the ultrasound examination until an ovulation is identified and the mare is bred again. This method ensures that the second dose of semen is inseminated within 6 hours of ovulation, to allow ample time for the semen to migrate to the oviduct while the oocyte is still viable. Alternatively, the mare can be induced, checked via transrectal ultrasonography at 24 hours post-induction and then rechecked every 2 - 6 hours to breed as close to ovulation as possible. The first dose of semen is administered when the follicle shows signs of impending ovulation, as previously described, and the second dose is given when fresh ovulation is detected. When a single dose is used, it is most common for practitioners to induce ovulation, perform transrectal ultrasonography 24 hours later and recheck every 4 - 6 hours to breed when a fresh ovulation is seen.

Prerequisites for induction of ovulation

Before administration of an ovulatory agent, a follicle must be identified that will be receptive to the agent. In general, follicles that are responsive to deslorelin acetate measure ≥ 35 mm in diameter, while follicles that will respond to hCG measure ≥ 30 mm [184]. There is great variation in size of preovulatory follicles due to the physiological individuality of mares and variations among breeds. Additionally, size of pre-ovulatory follicle is affected by mare age, season and the presence of multiple pre-ovulatory follicles. A study by Mores et al. that examined 1,492 Thoroughbred mares found that younger mares, aged 2 - 4 years, had a larger mean pre-ovulatory follicular diameter (38.95 ± 5.61 mm), while older mares, age > 19 years, had the smallest mean diameter (33.30 ± 4.66 mm). The study also showed that mares ovulated larger follicles during winter months than summer months and that mares with multiple pre-ovulatory follicles ovulated follicles of a smaller size than those with a single follicle [185].

Other prerequisites for induction include a endometrial edema score of 2 or greater and cervical softening. Edema is often considered a bioassay to determine follicular maturity and its ability to respond to an ovulation induction agent. Edema is formed in the endometrium secondary to estrogen production and secretion by large follicles. Endometrial edema is best evaluated via transrectal ultrasonography. A high degree of edema, when visualized on the cross section of a uterine horn is similar to a pinwheel or orange slice in appearance, while edema of the uterine body takes on a herring-bone pattern [27]. Multiple grading systems for scoring endometrial edema exist. One system scores edema on a scale from 0 - 5, where 0 is indicative of a uterus with no edema and a homogenous appearance, and 5 indicates hyperedema, or a pathologic thickening of endometrial folds with excessive uterine fluid [27]. Most mares begin to show behavioral signs of estrus when they reach an edema score of 2, while scores of 4 and 5

tend to be associated with pathological uterine conditions [186]. Other scoring systems use a scale of 0 - 3, where 0 indicates no edema and 3 indicates marked edema [187]. During estrus, edema gradually rises, peaks at the time of formation of the pre-ovulatory follicle and dissipates prior to ovulation [27]. Recent studies have shown that endometrial edema is important in the production of uterocalin, a protein responsible for providing lipids to an embryo during pregnancy, and the ideal amount of exposure of the uterus to edema is 6 days [122,123].

Multiple ovulations

Warmblood, Thoroughbred, and Standardbred horses are breeds known to more frequently have double ovulations. Arabians and Quarter Horses have lower incidence of multiple ovulations [188,189]. Also, older mares have higher incidence of multiple ovulations due to variations in FSH concentrations around follicle deviation [190]. One study found that mares aged 5 - 10 and 13 - 25 years were more likely to have double ovulations than young mares [191]. Multiple ovulations tend to occur more commonly bilaterally [188,192] than unilaterally. In unilateral multiple ovulations, about 71.3% were found to be synchronous (ovulating within a few hours timespan) and 28.7% asynchronous (ovulating within days of each other) [188]. Synchrony greatly affects fertility as the second ovulation in asynchronous ovulations is blocked by the clot formed after the first ovulation in the ovulation fossa.

The occurrence of multiple ovulations is considered favorable in the reproductive management of embryo donor mares as it greatly increases reproductive efficiency. Divergent opinions exist whether multiple ovulations are favorable for mares bred to carry a foal to term. For practitioners looking at multiple ovulations as an unfavorable event in broodmares, the concern is the occurrence of twin pregnancies, which invariably result in abortions; conversely,

practitioners looking at multiple ovulations as a favorable event see this as an opportunity for enhancing the chance of a mare getting pregnant and that if twins are present those can be manually reduced around the time of fixation (i.e., 15 d post-ovulation). Pregnancy rates are higher for double ovulations 0 or 2 days apart (84%) than for single ovulations (54%) [189]. In addition, twins are diagnosed more frequently when double ovulations occur [7]. The use of GnRH agonists can result in a higher incidence of multiple ovulations in mares [162]. In mares, follicles with a size of 30 mm and greater can respond to GnRH agonists. Conversely, hCG, the most popular induction drug worldwide, requires that the pre-ovulatory follicle be at least 35 mm [137].

2.6 Conclusions

To maximize production of economically valuable mares, much effort is placed on breeding mares outside of the physiological breeding season. Methods of circumventing seasonality in the mare, such as altering photoperiod, providing temperature-controlled environments, offering high-quality nutrition, and administering pharmacological agents are commonly utilized. However, despite all that is known about the mare's estrous cycle and the physiology of seasonal breeder, no methods have been found to reliably bypass anestrus. There is evidence to suggest that mares are able to be bred late in fall transition. However, there is a strong belief that mares will not respond to GnRH induction agents, the most popular and efficacious group of induction agents available in the U.S., because it has been suggested that pituitary LH stores are depleted during this time. In addition, it has been theorized that corpus lutea formed during the fall may not produce sufficient amounts of progesterone for pregnancy maintenance. Further work investigating this area could lead to a greater output in embryos and foals produced during the fall, thereby maximizing reproductive output of valuable mares.

CHAPTER 3: Ovulatory response to GnRH agonist during early and late fall in mares¹

3.1 Abstract

This study aimed to assess the ovulatory response of deslorelin acetate during the fall and the response to PGF2 α 8 d post-ovulation. One hundred estrous cycles from 22 mares kept in 40° latitude were evaluated. Mares were checked by transrectal ultrasonography until a preovulatory follicle was detected and ovulation induced with deslorelin acetate. Ovulation was confirmed by ultrasonography performed at 24, 36 h post-induction and then repeated at 2-h intervals post-induction. Serum progesterone concentrations and luteal tissue area were determined daily to assess CL function. A dose of PGF2 α was administered 8 d post-ovulation and interval to the subsequent ovulation was observed. Each mare repeated this cycle up to five times. The effects

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of local climate on endpoints were analyzed. Cycles were grouped as early (Sept. 13, 2020-Oct. 31, 2020; n=55) and late fall (Nov. 1, 2020- Dec. 31, 2020; n=45) based on the date of induction. The overall number of mares responding to deslorelin with ovulations between 24-48 h was 90%. Multiple ovulations were similar between early (n=5) and late (n=4) fall (P=0.87). There were no differences in spontaneous ovulations occurring before 24 h between early (n=6) and late (n=2) fall (P=0.29). Two failures to respond to deslorelin by 48 h were recorded in early fall and none in the late fall. The interval from induction to ovulation was similar in early (40.6 ± 0.4 h) and late (41.2 ± 0.5 h) fall (P=0.55). The percentage of mares ovulating between 36 and 48 h post-deslorelin did not vary between early and late fall (91 vs. 95 %, P=0.21), as did ovulation occurring between 38 h and 44 h (62 vs. 60%, P=0.69). Follicle size at induction tended to be smaller in early (36.4 ± 0.4 mm) than late (37.4 ± 0.4 mm) fall (P=0.07). Edema scores varied with time relative to ovulation (P<0.001) and were lower in late fall (P=0.01). Progesterone concentrations varied with time (P<0.001) but did not differ between early and late fall (P=0.73) and correlated weakly with the luteal area (r=0.13; P=0.031). Follicles < 35mm at the PGF 2α had a shorter interval to the next ovulation than follicles ≥ 35 mm (9.2 ± 0.5 d vs. 10.6 ± 1.2 d) (P=0.03). Lower temperature led to a smaller follicle size at induction (P=0.0021) and ovulation (P=0.009) and lower relative humidity yielded a larger follicle size at ovulation (P=0.032). In conclusion, cycling mares displayed a highly efficacious response to deslorelin acetate and apparently normal luteal function during the fall, despite lower edema scores in late fall.

Keywords: ovulation, deslorelin acetate, progesterone, luteal function

3.2 Introduction

Racing, sport and show horse foals born early in the calendar year have an advantage over those born later in the season in terms of yearling sales price and futurity performance. Due to the economic pressure to have early born foals, major efforts have been placed on maximizing pregnancy rates during late winter and early spring when mares are physiologically not cycling or transitioning [8]. The horse breeding industry currently uses strategies that include single or combinatorial use of extended artificial light exposure, pharmacological interventions, and nutritional modulation, that have been shown to circumvent this physiological limitation [8,10,11].

Mares are seasonal polyestrous long-day breeders, meaning that the natural reproductive season in the Northern Hemisphere typically takes place from March to October [6]. Before and after the natural reproductive season, mares undergo transitional periods leading to cyclicity (spring transition) and anestrus (fall transition), respectively [6]. As fall progresses in the Northern Hemisphere, daylight length and temperature decrease, and the nutritional value of pasture begins to depreciate. For these reasons, it is suspected that mares not only stop cycling in the fall, but also display a poor response to GnRH agonists when this class of drugs is administered to induce ovulation while they are still cycling.

Induction of ovulation is an important tool for maximizing reproductive efficiency in the mare. The multitude of benefits of using an induction agent include decreasing the interval from breeding to ovulation, facilitating the use of timed artificial insemination protocols, decreasing the number of breeding and reproductive examinations per estrous cycle, controlling the timing of breeding to facilitate semen and personnel availability, insuring at least one ovulation for each cycle, enhancing the progesterone production capabilities of subsequent corpora lutea and allowing for the estrous synchronization between embryo donors and recipients [12,13].

Multiple classes of induction agents used worldwide include luteinizing hormone (LH)-agonists, PGF2 α , native GnRH, and its agonists [12,193]. However, GnRH agonists and human chorionic gonadotropin (hCG) are the primary agents used in the United States [159]. While hCG is the oldest and one of the most popular drugs used globally to induce ovulation in mares, it lost its popularity in the USA after the introduction of an FDA-approved GnRH agonist product. This product consists of deslorelin acetate combined with sucrose acetate isobutyrate (SAIB)/propylene carbonate (70:30 v:v) to increase the half-life of this molecule [165]. The reasons for this popularity include a more consistent response regarding time and percentage of ovulations after deslorelin administration in older multiparous mares, the increased reliability of availability of deslorelin acetate as compared with hCG, and the decreased risk of anaphylactic reaction at the time of administration [160,194]. Several years after the introduction of this product, other sources of compounded non-FDA-approved deslorelin acetate became available in the United States but the efficacy of these products is not known. Despite well-documented efficacy of the FDA-approved deslorelin acetate in inducing ovulation in mares during the spring and summer [169,170,195], there is much speculation among practitioners that this product is not effective to induce ovulation in the fall. However, this speculation has not been critically tested in a controlled study.

Within some segments of the equine industry such as Warmbloods and breeds with futurity competitions like Quarter Horses, there is a substantial interest in breeding mares for fall pregnancy or collecting and cryopreserving embryos for transfer early in the subsequent spring, respectively; however, this new trend clashes with the seasonal fall transition for most mares [13]. Equine embryo flushing is typically performed 7 to 9 d post-ovulation and most breeding farms and clinical practices perform this procedure 8 d post-ovulation. Immediately after embryo

flushing, PGF2 α is administered to the mare, so that she can return into estrus for rebreeding, and prevent uterine infection or establishing of a pregnancy if the mare failed to yield one embryo per each ovulation. While there is a plethora of studies assessing response to PGF2 α on 5, 6 and 7 d post-ovulation [137,196], there is no data on the administration on 8 d post-ovulation in mares.

The present study sought to assess the ovulatory response of the GnRH agonist deslorelin acetate in mares during the fall by comparing success in inducing ovulation, interval to ovulation, progesterone concentration and parameters of the resultant corpus luteum in early fall and late fall. In addition, the present study aimed to assess the response to PGF2 α when it is administered 8 d post-ovulation in mares. We hypothesized that deslorelin acetate would have a better ovulatory response during early than late fall and that interval from PGF2 α administration to ovulation would be affected by the follicle size at the time of administration and that the interval would increase in late fall compared to early.

3.3 Materials and methods

This study was completed in the fall 2020 at the University of Illinois Urbana-Champaign in Urbana, Illinois. The Institutional Animal Care Unit Committee approved all procedures carried out in the present study under protocol #19141.

Animals and husbandry

Twenty-two mares with ages ranging from 3 to 23 years of various light breeds (ten Arabians, four Standardbreds, three American Quarter Horses, two Warmbloods, one American Paint Horse, one Thoroughbred and one Tennessee Walking Horse) were enrolled in the study. All mares were housed at the University of Illinois Veterinary Teaching Hospital, Urbana,

Illinois and were kept on pasture and free-choice mixed hay. Mares were supplemented once daily with 1.5 kg of grain (Strategy, Purina Animal Nutrition, L.L.C., St. Louis, MO) each top-dressed with 50 mL of a commercially available multivitamin and fatty acid product (Broodmare Plus, Botupharma USA, Phoenix, AZ).

Study design

Mares were restrained in stocks in a climate-controlled barn and checked by transrectal palpation and ultrasonography every other day until a dominant follicle with a follicle size of 30-33 mm was detected. Once a dominant follicle was identified, mares were checked daily until a pre-ovulatory follicle was detected. A pre-ovulatory follicle was defined as having a size of ~35 mm in the presence of endometrial edema (score of 2 or greater) [186]. After detection of a pre-ovulatory follicle, each mare received a single dose of a commercially available, FDA-approved, deslorelin acetate (1.8 mg, intramuscularly; SucroMate™, Thorn BioScience, LLC, Louisville, KY) to induce ovulation. The GnRH agonist was given ~6 am, in conjunction with the ultrasonographic examination.

The time of ovulation was determined by transrectal ultrasonography performed at 24 h post-induction and then repeated every 2 h starting at 36 h post-induction. Ovulations occurring before 24 h and after 48 h post-induction were considered as spontaneous and failures to respond to deslorelin acetate, respectively. If a mare failed to develop a pre-ovulatory follicle after two consecutive weeks, she was excluded from the remainder of the study.

After 200 h post-ovulation transrectal ultrasonography was performed to assess follicle size and luteal tissue area. A single dose of PGF2 α (dinoprost tromethamine, 7.5 mg, intramuscularly; Lutalyse®, Zoetis Inc., Kalamazoo, MI) was administered to each mare. Mares were checked as previously described until the next pre-ovulatory follicle was detected. The

number of days between PGF2 α administration and the subsequent ovulation was recorded. The cycle was repeated five times for each mare during the study period if the mare displayed regular ovarian cyclic activity.

The data for interval from induction to ovulation was grouped according to perceived relevance to clinical practice. The intervals considered were 24 to 48 h, 36 to 48 h, and 38 to 44 h of interval. The second and third interval included the timeframe where ovulation is expected after the use of deslorelin acetate and where the majority of ovulations actually take place, respectively [169].

Blood collection and determination of immunoreactive progesterone

Serum was collected in 10 mL tubes with no anticoagulant (Covidien Monoject, Covidien LLC, Mansfield, MA) once daily starting at the time of deslorelin acetate administration for 120 h at 24 h intervals and then once at 200 h (i.e., 8d) post-ovulation. Blood was kept at room temperature to let clot formation and serum separation for less than 1 h. Thereafter, each sample was centrifuged at 1000 $\times$ g for 20 min, and serum was harvested and stored at -20°C until assessment.

Serum progesterone concentrations were measured using an automated system based on solid-phase, competitive chemiluminescence enzyme immunoassay (Immulite 2000 XPi Platform, Siemens Medical Solutions, Inc.). Reported cross-reactivities were as follows: androstenedione 0.076 %, corticosterone 0.417 %, cortisol 0.003 %, DHEA sulfate 0.01, 11-deoxycorticosterone 1.82 %, 17 α -hydroxyprogesterone 0.444 %, medroxyprogesterone 0.029%, pregnenolone 0.047 %, testosterone 0.119 %. Intra-assay coefficient of variations was 6.12 %. Inter-assay coefficient of variations was 5.08% for progesterone. The calibrated sensitivity of the progesterone assay was 0.01 ng/mL, and the detection limits were 0.2 to 40 ng/mL. Samples with

values below the lower detection limit were deemed as 0 ng/mL for statistical analyses. There were no samples above the upper limit of the detection for the assay.

Ultrasonographic parameters

A portable ultrasound machine (Ibex Evo[®]II, E.I. Medical Imaging®, Loveland, CO) coupled with a 7.5 MHz linear transducer was used for all ultrasonographic examinations performed throughout the study. From 24 h before to 48 h after induction all mares had endometrial edema scored (0-3). Score 0 was given when no edema was visible in the endometrial folds, score 1, 2 and 3 were given when a very small, a moderate and a large amount of edema was visible, respectively [187].

The luteal tissue area was assessed daily via transrectal ultrasonography from 24 h to 96 h post-ovulation and then at 200 h post-ovulation. Images were taken when the corpus luteum appeared at its largest size. A digital image of each corpus luteum was opened in ImageJ (ImageJ 1.53k, National Institute of Health, USA). The scale was calibrated using the gridlines. The boundary of each corpus luteum was traced using the freehand selection tool and then area was calculated using the measurement analysis tool. One corpus luteum or multiple corpora lutea were combined into one luteal tissue area for data analyses.

The dominant follicle size was assessed at the induction of ovulation, immediately before ovulation and at 200 h post-ovulation. The follicle size was the mean value of two largest diameters measured by transrectal ultrasonography using the digital caliper/measurement function on the ultrasound. Since this variable has been reported to affect response to PGF2 α and fertility [196], the follicle sizes at the time of PGF2 α administration was compiled in three different ways to further evaluate the clinical responses. Follicle sizes were split into groups as follows: first 30 mm; 30 - 34 mm; and $\geq$ 35 mm. Second, follicle data was compiled as < 30 mm

and ≥ 30 mm. Lastly, the follicle sizes were compiled into a third arrangement of follicle < 35 mm and ≥ 35 mm.

Climate of the region

Urbana, Illinois is located at 40.109665 latitude and -88.222766 longitude with a Köppen classification of dfa, i.e., humid continental climate with well-defined seasons; cold, windy, snowy winters, mild springs, hot, humid summers, and crisp and relatively short falls.

Descriptive statistics of climate in the region regarding minimum and maximum daily temperatures, and humidity were obtained from National Weather Service and analyzed. Then the daily minimum and maximum temperatures and relative humidity were grouped into weeks and averaged. The first seven weeks of the study (Sept. 13, 2020 - Oct. 31, 2020) were considered in the early fall, conversely the last nine weeks (Nov. 1, 2020 - Dec. 31, 2022) were considered part of the late fall.

Statistical analyses

Statistical analyses were performed with R Version 1.4.1717 (R Studio, PBC). Normal distribution was assessed with histograms and Shapiro-Wilk test and non-normally distributed data were log-transformed. Continuous variables such as follicle size at time of induction and just before ovulation, follicle size at the time of PGF2 α administration, interval from induction to ovulation, interval from PGF2 α administration to the subsequent ovulation, luteal tissue area, and progesterone concentration were analyzed using mixed model, and when significant effects were found, post-hoc comparisons were made with Tukey's test. The mare and cycle order were accounted as random effects, whereas fall (early vs. late), time, number of ovulations, climate parameters as fixed effects. One-way analysis of variance was used to compare daily minimum temperature, daily maximum temperature, and relative humidity between early and late fall.

Fisher's exact test was used to compare the occurrence of spontaneous ovulations, ovulation failure, and ovulatory response in specific timelines relevant to clinical practice (e.g., percentage of mares ovulating from 36 to 48 h and percentage of mares ovulating from 38 to 44 h) and occurrence of multiple ovulations with regards to early and late fall. Kruskal-Wallis rank sum test was used to analyze edema scores at time of ovulation in comparison with time (early vs. late fall) and follicle size at induction and Dunn's test was used for post-hoc comparison of significant effects. Pearson's coefficient of correlation was used to analyze the associations between i) progesterone concentration and luteal tissue area; ii) progesterone concentration and number of ovulations; iii) time and weekly average minimum temperature, maximum temperature and relative humidity iv) number of ovulation and weekly average minimum temperature, maximum temperature and relative humidity. Significance was defined as $P \leq 0.05$ and statistical tendency was defined as $0.05 < P \leq 0.10$. Data were presented as mean $\pm$ SEM.

3.4 Results

Number of ovulations and ovulatory response

A total number of 100 estrous cycles was observed. Out of 22 mares, five mares stopped displaying regular ovarian cyclic activity prior completion. Two mares (a three-year old Arabian and a twenty-three-year old Standardbred) completed two cycles in early fall before ceasing cyclicity. One mare (a seventeen year-old Standardbred) completed only three estrous cycles (two in early fall and one in late fall) and two mares completed only four estrous cycles (a fourteen year-old Arabian and a twenty-one year-old Warmblood) in both early and late fall.

The first induction of ovulation was performed on September 13, 2020 and the last one on December 29, 2020; the first and last ovulation were recorded on September 14, 2020 and

December 31, 2020, respectively. Throughout the study, 55 estrous cycles were completed in the early fall from 22 mares and 45 cycles from 20 mares in the late fall (Figure 1).

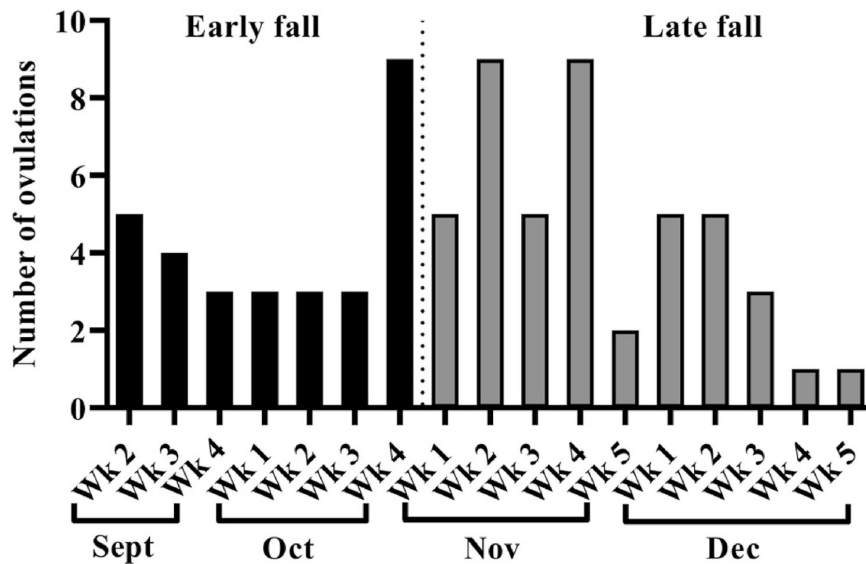


Fig. 1. Number of ovulations in light-breed mares ($n = 22$) induced with deslorelin acetate in the fall. Ovulations were grouped by date of induction and classified by week as early fall (Sept 13, 2020–Oct 31, 2020; 55 cycles from 22 mares) and late fall (Nov 1, 2020–Dec 31, 2020; 45 cycles from 20 mares). The first ovulation occurred on Sept 14 and the last on Dec 31, 2020.

Eight spontaneous ovulations were detected throughout the study. There was no difference in number of spontaneous ovulations in early fall ($n = 6/55$ cycles) when compared with late fall ($n = 2/45$ cycles) ($P = 0.29$). Two estrous cycles of one Arabian mare were considered failures of ovulation, as this mare ovulated at 55 and 72 h after deslorelin acetate administration in early fall. The remaining ovulations ($n = 90$) occurred at 40.8 ± 0.3 h (Figure

2), which is within the expected time for spring-induced mares [169]

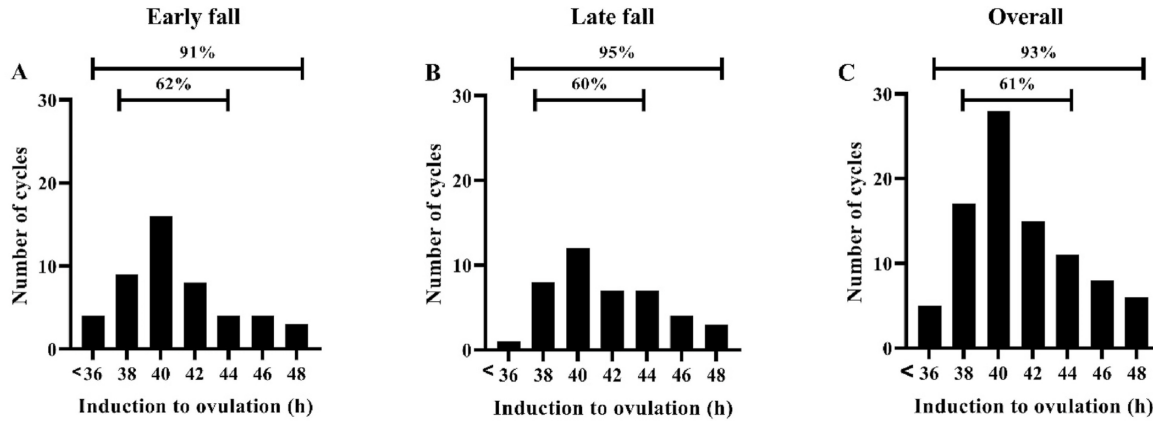


Fig. 2. Frequency distribution of intervals from administration of deslorelin acetate to detection of ovulation in light breed mares (n = 22) during the fall. The ovulation intervals were divided into (A) early fall (Sept 13, 2020–Oct 31, 2020; n = 55 cycles) and (B) late fall (Nov 1, 2020–Dec 31, 2020; n = 45 cycles) and overall (C) Cycles which were considered to have spontaneous ovulations (≤ 24 h post-deslorelin; n = 8 cycles) and ovulation failures (>48 h post-deslorelin; n = 2cycles) were excluded. There were no differences in number of ovulations within the intervals of detection 36–48 h ($P = 0.10$) or 38–44 h ($P = 0.69$).

There was no difference in the interval from induction to ovulation in early fall (40.6 ± 0.4 h) vs. late fall (41.2 ± 0.5 h) ($P = 0.55$). The percentage of ovulations which occurred between 36 and 48 h did not vary between early (91%, n=43) and late fall (95%, n=41) ($P = 0.21$) (Figure 2). In addition, there were no differences in ovulations between 38 and 44 h post-administration of deslorelin acetate early (62%, n=29) and late fall (60%, n=26) ($P = 0.69$).

Nine cycles of multiple ovulations occurred throughout the study, five in early fall and four in late fall (Table 1). Of these cycles, one mare, a 21 year-old Warmblood, ovulated three follicles in early fall. In addition, this mare and the other 16 year-old Warmblood mare had two cycles of two ovulations, one cycle in early fall and one cycle in late fall for each of these mares. Four other mares (two Standardbreds 6 and 20 year-old, one 18 year-old Thoroughbred and one 14 year-old Arabian) had one cycle of two ovulations.

Table 1. Number of single, double, and multiple ovulations in light breed mares receiving [deslorelin](#) acetate early (Sept 13, 2020–Oct 31, 2020) and late fall (Nov 1, 2020–Dec 31, 2020). Data is expressed as percentages, with proportion of cycles in parentheses.

Fall	Number of ovulations		
	One	Two	Three
Early	91% (50/55)	7% (4/55)	2% (1/55)
Late	91% (41/45)	9% (4/45)	0% (0/45)

There were no differences in the number of multiple ovulations between early and late fall ($P = 0.87$).

Follicle size at induction and immediately before ovulation

The overall follicle size at induction of ovulation was 36.9 ± 0.2 mm. Early fall, mares tended to have smaller follicle size at induction of ovulation (36.4 ± 0.4 mm) than late fall (37.4 ± 0.4 mm) ($P = 0.034$) (Figure 3A). The follicle size immediately before ovulation was 39.2 ± 0.3 mm (Figure 3B). There was no effect of time on follicle size prior to ovulation (early 39.3 ± 0.4 mm vs. late 39.1 ± 0.4 mm, $P = 0.54$).

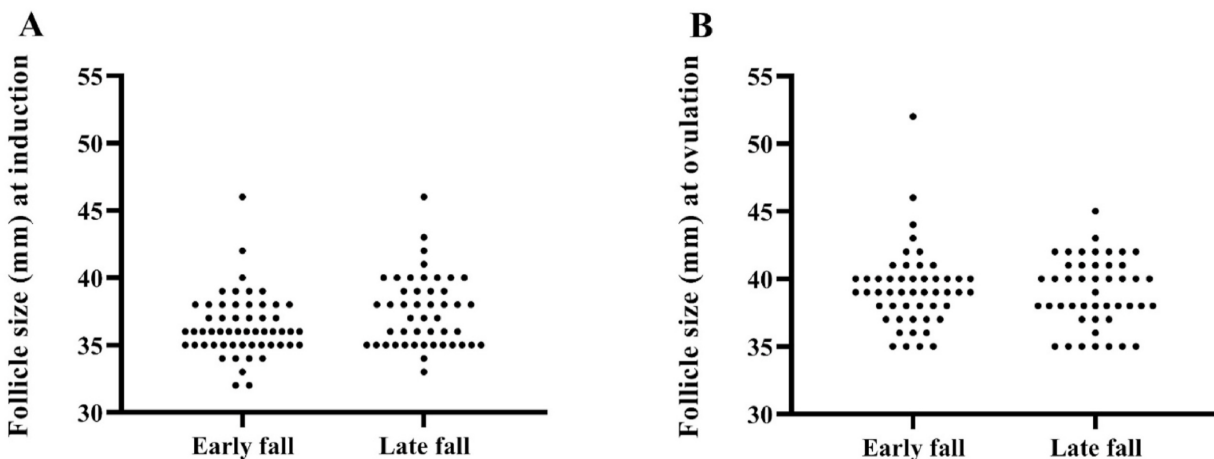


Fig. 3. Follicular size in light breed mares ($n = 22$) at the time of induction of ovulation with deslorelin acetate (A) and immediately before ovulation (B). The data was grouped as early fall (Sept 13, 2020–Oct 31, 2020; $n = 55$ cycles) and late fall (Nov 1, 2020–Dec 31, 2020; $n = 45$

Figure 3 (cont). cycles). Follicular size tended to be greater at the time of induction of ovulation in late fall ($P = 0.07$) but it was not different immediately before ovulation ($P = 0.54$).

Endometrial edema scores

Overall, mares in early fall received higher edema scores than late fall ($P = 0.01$). There was a temporal effect of edema score from 24 h before to 48 h after induction ($P < 0.001$) (Figure 4). Pairwise comparisons showed a significant change in edema score every 24 h before and after induction of ovulation: - 24 to 0 h ($P < 0.001$), 0 to 24 h ($P = 0.006$), and 24 to 48 h ($P < 0.001$). There was a negative correlation between edema score at the time of induction of ovulation on interval to ovulation ($r = -0.88$, $P = 0.05$). Follicle size at induction did not affect edema score at the time of induction ($P = 0.23$).

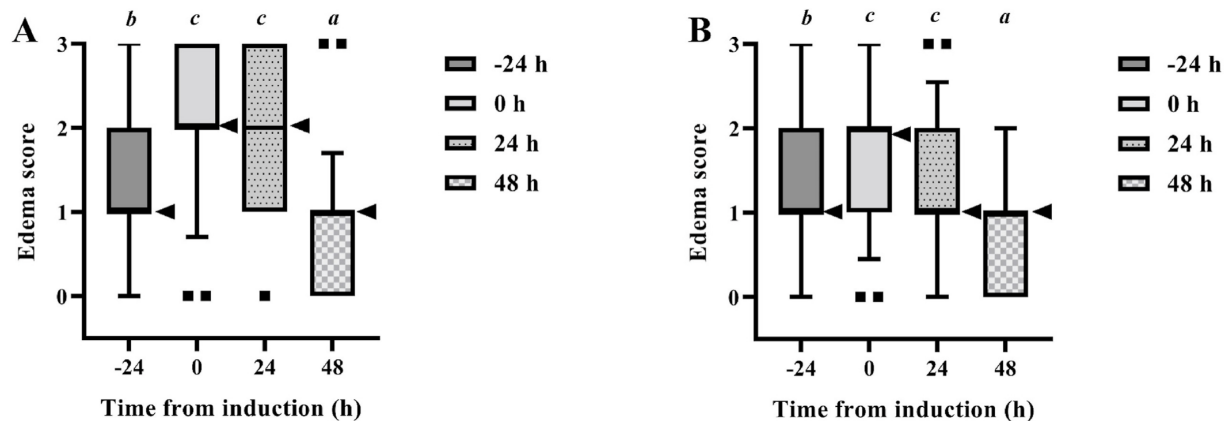


Fig. 4. Median and interquartile ranges of endometrial edema scores (0–3) in estrus of light breed mares ($n = 22$) 24 h before to 48 h post-induction of ovulation during early (A) (Sept 13, 2020–Oct 31, 2020; $n = 55$ cycles) and (B) late fall (Nov 1, 2020–Dec 31, 2020; $n = 45$ cycles). Lower and upper limits of whiskers are 5% and 95%, respectively of data range and outliers are shown using squares. Edema score changed over time in both early ($P < 0.001$) and late ($P < 0.001$) fall. Letters (*a*, *b*, *c*) denote changes in edema score between early and late fall ($P < 0.05$). The arrows denote the median for each timepoint.

Progesterone concentrations and luteal tissue area

There was an effect of time ($P < 0.001$) but no effect of the period between early vs. late fall ($P = 0.73$) or of interactions between time and early or late fall ($P = 0.14$). Progesterone

concentration increased after 72 h post-ovulation ($P < 0.001$) (Figure 5A). There was a significant positive association between the luteal tissue area and progesterone concentration ($r = 0.13$; $P = 0.031$). There was no association between multiple ovulations and early vs. late fall ($P = 0.87$). The number of ovulations had no effect on progesterone concentration ($P = 0.62$) (Figure 5B). No interactions were found between luteal area and number of ovulations or early vs. late fall ($P = 0.27$). There were no effects of early vs. late fall ($P = 0.69$), number of ovulations ($P = 0.97$) or time from ovulations ($P = 0.91$) and no interactions ($P = 0.50$) on the luteal tissue area (Table 2).

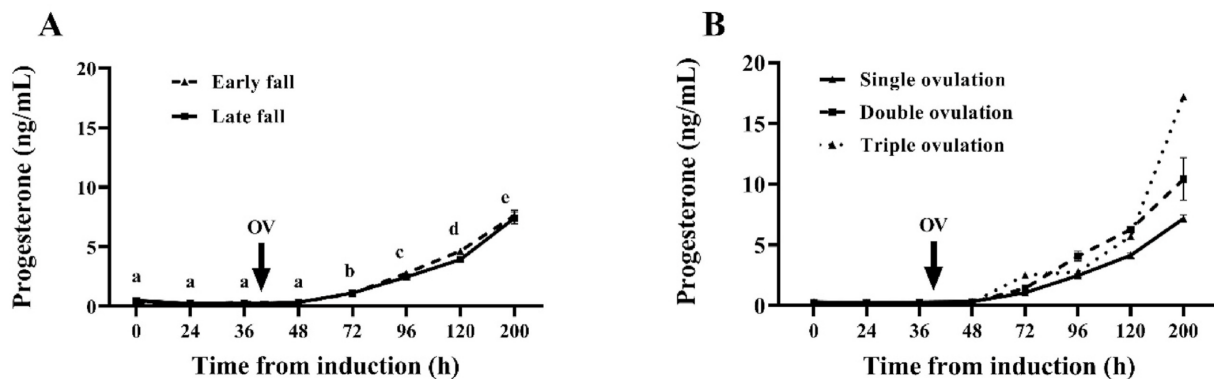


Fig. 5. (A) Progesterone concentration (mean $\pm$ SEM) in 100 estrous cycles from light breed mares ($n = 22$) after administration of deslorelin acetate in the fall. Time is represented as 0 h–120 h from induction and 200 h post-ovulation. The ovulation intervals were divided into early fall (Sept 13, 2020–Oct 31, 2020, $n = 55$ cycles) and late fall (Nov 1, 2020–Dec 31, 2020, $n = 45$ cycles). Mean time of ovulation is shown with an arrow. There was no effect of early and late fall. There was a positive correlation between progesterone concentration and time. *Different superscripts denote differences across timepoints ($P < 0.05$); (B) Comparison of progesterone concentration (mean $\pm$ SEM) with number of ovulations per cycle in light breed mares. Time is represented as 0 h–120 h from induction and 200 h post-ovulation. Cycles were divided by number of ovulations and split into three groups-single ovulation ($n = 91$), double ovulation ($n = 8$), and triple ovulation ($n = 1$). Mean time of ovulation is shown with an arrow. There was no significant effect of number of ovulations on progesterone concentration.

Table 2. Luteal tissue area (cm²) from one 24 h–96 h and 200 h after ovulation in 100 estrous cycles of light breed mares (n = 22) during the fall. Data is expressed as mean ± SEM.

Fall	24 h	48 h	72 h	96 h	200 h
Early (n = 55)	5.2 ± 0.4	5.5 ± 0.5	5.9 ± 0.5	5.5 ± 0.4	4.9 ± 0.4
Late (n = 45)	4.9 ± 0.3	5.8 ± 0.4	6.1 ± 0.5	6.4 ± 0.7	5.2 ± 0.5
Overall (n = 100)	5.1 ± 0.3	5.6 ± 0.4	6.0 ± 0.3	5.6 ± 0.4	5.0 ± 0.3
Number Ovulations					
One (n = 91)	5.0 ± 0.3	5.6 ± 0.4	5.9 ± 0.3	5.9 ± 0.4	5.0 ± 0.3
Two (n = 8)	5.7 ± 0.5	5.9 ± 1.2	7.8 ± 1.6	6.4 ± 1.3	5.1 ± 0.7
Three (n = 1)	5.2	5.2	5.7	6.0	6.9

There was no temporal difference of luteal area over time ($P = 0.91$) and no difference when comparing early and late fall ($P = 0.69$). There was no association between number of ovulations and luteal area ($P = 0.37$).

Follicle size at PGF2 α administration

There was no effect of the early vs. late fall or interaction effects on the follicle size at the PGF2 α administration ($P = 0.23$) (Table 3). When follicle sizes were not grouped, early fall had shorter interval from PGF2 α to the subsequent ovulation than late fall ($P = 0.05$), but there was no effect of the follicle size ($P = 0.68$) or interactions ($P > 0.05$)

Follicle sizes at the PGF2 α administration were compiled in different ways to determine its effects on until a subsequent ovulation. There were no effects or interaction of the follicle size and fall (early vs. late) when follicle sizes were grouped in three size-ranges (<30 mm, 30-34 mm, ≥ 35 mm; $P > 0.05$); or two sizes (<30 mm or ≥ 30 mm; $P > 0.05$). When the follicle sizes were grouped as < 35mm and ≥ 35 mm, there was an effect of the follicle size but not early vs.

late fall ($P = 0.91$) or interactions ($P > 0.05$) on the interval to the next ovulation. Cycles with follicles $\geq 35\text{mm}$ had shorter interval until the next ovulation vs. follicles $< 35\text{mm}$ (9.2 ± 0.5 d vs. 10.6 ± 1.2 d) ($P = 0.03$) (Figure 7).

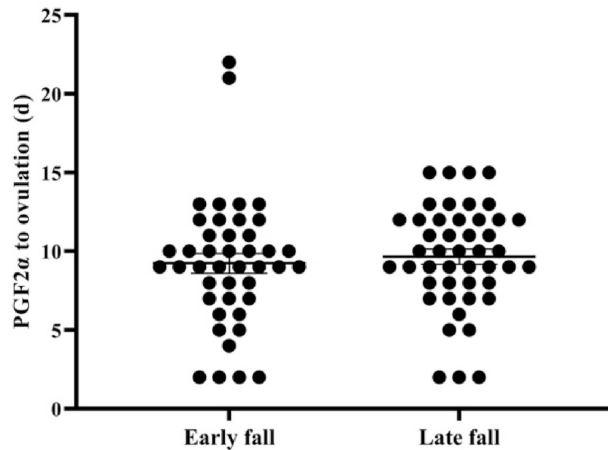


Fig. 6. Intervals from PGF2 α administration on day 8 of the estrous cycle until subsequent ovulation in twenty-two light breed mares during early and late fall. Cycles early fall ovulated sooner than late fall (9.1 ± 0.7 vs. 9.5 ± 0.5 d) ($P = 0.035$).

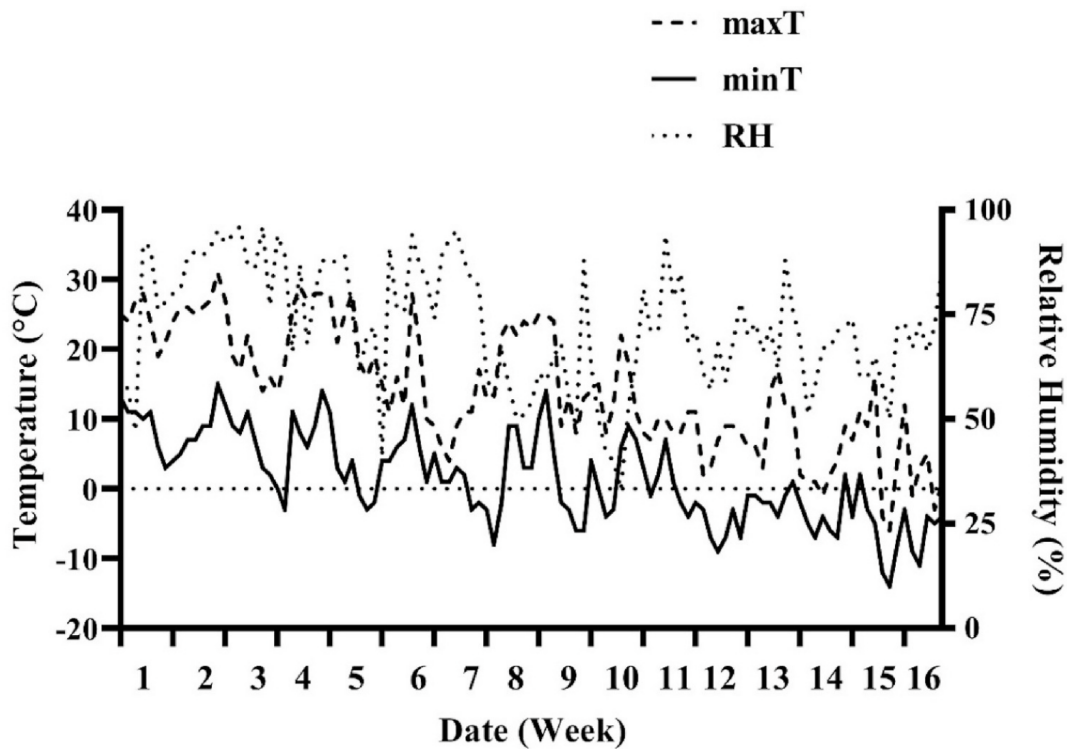


Fig 7.

Fig. 7 (cont). Daily maximum (maxT) and minimum (minT) temperature and relative humidity (RH) in Urbana, Illinois during early and late fall 2020. All three parameters decreased over time ($r_{\min T} = -0.93$, $P_{\min T} < 0.0001$; $r_{\max T} = -0.9$, $P_{\max T} < 0.001$; $r_{\min RH} = -0.43$, $P_{\max RH} < 0.001$).

Climate of the region

The daily minimum temperature, daily maximum temperature and relative humidity were different between early and late fall ($P < 0.001$) (Table 4). When evaluating time on a weekly basis, there was a negative reduction observed for minimum ($r = -0.93$, $P < 0.0001$) and maximum ($r = -0.9$, $P < 0.001$) temperature and a weakly negative correlation with relative humidity ($r = -0.43$, $P < 0.001$) over time.

Table 3. Mean daily values for minimum temperature, maximum temperature, and relative humidity in early (Sept 13, 2020–Oct 31, 2020) and late fall (Nov 1, 2020–Dec 31, 2020) in Urbana, IL. Data is expressed as mean $\pm$ SEM and CI: Confidence interval 95%.

Fall	Minimum Temp (°C)			Maximum Temp (°C)			Relative Humidity (%)		
	Mean $\pm$ SEM	CI 95%		Mean $\pm$ SEM	CI 95%		Mean $\pm$ SEM	CI 95%	
		Min	Max		Min	Max		Min	Max
Early	5.8 $\pm$ 1.1 ^a	4.3	7.0	20.2 $\pm$ 2.2 ^a	18.0	22.0	81.6 $\pm$ 2.4 ^a	78.0	85.0
Late	-1.8 $\pm$ 1.2 ^b	-3.2	-0.3	9.7 $\pm$ 6.1 ^b	7.9	12.0	65.1 $\pm$ 3.0 ^b	62.0	68.0
Overall	1.5 $\pm$ 1.3	0.3	2.8	14.3 $\pm$ 2.0	12.7	16.0	72.4 $\pm$ 2.8	69.7	75.2

Different superscripts within columns denote differences between early and late fall ($P < 0.05$).

There was an effect of minimum temperature ($P = 0.0021$) and of the interaction between minimum temperature and relative humidity ($P = 0.02$) on the follicle size at induction, but no effect of maximum temperature ($P = 0.69$), relative humidity ($P = 0.70$) or their interactions ($P > 0.05$). There was an effect of minimum temperature ($P = 0.009$) and relative humidity ($P = 0.032$) and their interaction ($P = 0.006$) on the follicle size at ovulation, but no effect of maximum temperature ($P = 0.39$). There was no effect of minimum temperature ($P = 0.30$),

maximum temperature ($P = 0.74$) and relative humidity ($P = 0.22$) on follicle size at $\text{PGF2}\alpha$ administration; there was an effect of the interaction between minimum and maximum temperature on follicle size at $\text{PGF2}\alpha$ administration ($P = 0.04$). There was no effect of minimum temperature, maximum temperature and relative humidity and their interactions on the interval from induction of ovulation to ovulation and $\text{PGF2}\alpha$ administration to the subsequent ovulation ($P > 0.05$).

There were no correlations between the number of ovulations and weekly average maximum temperature ($r = -0.003$, $P = 0.97$), minimum temperature ($r = 0.24$, $P = 0.81$) and relative humidity ($r = 0.032$, $P = 0.75$).

3.5 Discussion

This study was set-forth to determine the efficacy of deslorelin acetate, a GnRH agonist classified as superagonist, in inducing ovulation during the fall in light breed mares kept in the Northern hemisphere in a 40° latitude. In addition, the fall was subdivided in early and late to determine the potential influence of time of season on main endpoints assessed in the study. Time from induction to ovulation, luteal tissue area, follicle size at ovulation, and serum progesterone concentration were not affected by time of season. Our findings collectively demonstrate that deslorelin acetate is efficacious at inducing ovulation in mares early and late fall.

While there were no direct comparisons between spring and fall seasons, the findings of the present study suggest that administration of deslorelin acetate in the fall has a similar efficacy to that reported for the spring [165,169,170]. In the present study, 90% of ovulations took place within 48 h after administration of deslorelin acetate, which is consistent with studies showing an efficacy of ~90% when the same GnRH agonist product was given to Quarter Horse mares

during the spring [169]. It is recognized that, because there was no control group for comparison, an unknown number of cycles would have resulted in spontaneous ovulations during this time frame in the absence of an induction agent. In addition, there was a similar efficacy of deslorelin acetate administration and a similarity between intervals from induction to ovulation between early and late fall. This study provides the first description of response to estrous cycle manipulation in the fall.

Ninety-three percent of ovulations occurred from 36 to 48 h after induction and 61% of those occurred within 38 to 44 h. This information is of clinical relevance to practitioners using timed artificial insemination protocols and to those trying to reduce the number of pre-breeding examinations by reducing the cost per breeding [160] and allowing the practitioner to manage more mares per season. Breeding a mare in proximity to ovulation can be beneficial not only for frozen semen, but when quantity and quality of fresh semen are subject of concerns.

Follicle size is an important criterium for induction of ovulation in mares and can be used as an indicator of responsiveness to GnRH agonists [17,19]. However, pre-ovulatory follicle dynamics at induction of ovulation have not been well studied in light breed mares during the fall. Overall, follicle size at induction of ovulation was smaller than previously reported for light breed mares in the spring [169,172]. The findings may have been influenced by breed distribution in the study population. Ten mares were Arabians and three were Quarter Horses out of twenty-two mares. These breeds are known to ovulate smaller follicles (around 35 mm) in comparison with larger light breeds like Thoroughbred and Standardbred [20-21].

In this study, mean follicle size at the time of induction tended to increase between late and early fall cycles. A study with Thoroughbred mares showed that follicle size decreased from early spring to summer [185], this reduction in size of the pre-ovulatory follicle is accompanied

by a reduction in the number of days in estrus as the season progresses. It is possible that the smaller follicle size in the early fall period reflects the summer influences on the estrous cycle, and that the increase in pre-ovulatory size late fall, signals proximity to the fall transitional period. There is also a possibility that this difference is due to an unintentional bias of the research team making the decision of when to induce ovulation based on a mixture of subjective and objective characteristics, as previously described.

Endometrial edema is an important clinical parameter that is used to determine a follicle's potential readiness to respond to an induction agent [27,186,197]. Resulting from an increase in estrogen secreted by the pre-ovulatory follicle, endometrial edema increases during estrus as the size of the pre-ovulatory follicle increases and then rapidly decreases just prior to ovulation when estrogen concentration decreases [18, 22]. Recent studies have shown that extended time periods of endometrial edema lead to better fertility by increasing uterocalin production, allowing for a more hospitable environment for the embryo entering the uterus [122,123,123]. Anecdotally, in the fall, it has been suggested that endometrial edema is absent and evidence suggests that days of edema per estrus are greatly decreased [198]. The present study showed that mares do have endometrial edema in the fall, however, there is a relationship between endometrial edema score and time of the season, as the scores decreased in the late fall vs. early fall. There is a chance that edema score was lower in late fall due to an unintentional bias of the researchers, however, the decision to administer the induction agent was made based on a combination of factors, such as pre-ovulatory follicle characteristics, not solely edema score. Additionally, there was a noticeable change in edema scores over time, as edema increased during the 24 h period before induction and then decreased thereafter. It seemed that edema seems to increase slowly in late fall as compared with early fall. Retrospectively, on

could have held off on administration of deslorelin acetate until edema in this period increased, however, based on characteristics of the pre-ovulatory follicle, this delay may increase the odds of spontaneous ovulation. Conversely, this delay could also result in follicles becoming anovulatory and undergoing atresia. Higher edema score at the time of induction was related to decreased intervals to ovulation, which was expected as more mature follicles produce greater amounts of estrogen. However, this effect did not seem to be related to follicle size itself.

Progesterone is a key hormone for pregnancy maintenance and luteal insufficiency is a cause of early pregnancy loss in the mare [133]. Progesterone concentration was assessed herein following induction of ovulation with deslorelin acetate as studies have suggested that mares experience poor luteal function during fall as evidenced by lower peripheral progesterone concentrations than the spring and summer [50,199]. As these comparisons were not carried out in the present study and differences in assays combined with inter-animal variability across studies prevents us from drawing direct conclusions but it is reasonable to suggest that progesterone concentrations obtained in this study were consistent with reported concentrations of progesterone in diestrus mares in the spring and summer [45]. The present study showed the expected increase in progesterone concentrations with time following ovulation and there was no difference in luteal tissue area or progesterone concentrations when comparing early and late fall.

The occurrence of multiple ovulations is considered favorable in the reproductive management of embryo donor mares. Divergent opinions exist whether multiple ovulations are favorable for mares bred to carry a foal to term. For practitioners looking at multiple ovulations as an unfavorable event in broodmares, the concern is the occurrence of twin pregnancies, which invariably result in abortions; conversely, practitioners looking at multiple ovulations as a favorable event see this as an opportunity for enhancing the chance of a mare getting pregnant

and that if twins are present those can be manually reduced around the time of fixation around (i.e., 15 d post-ovulation). The use of GnRH agonists can result in a higher incidence of multiple ovulations in mares [162]. In mares, follicles with a size of 30 mm and greater can respond to GnRH agonists. Conversely, hCG, the most popular induction drug worldwide, requires that the pre-ovulatory follicle be at least 35 mm [137]. The present study found a 9% incidence of multiple ovulations, with no difference between early and late fall. This is considered a low incidence of multiple ovulations in comparisons with other studies in spring and summer bred mares that reported incidences varying from 16.1 to 27.2 % after deslorelin acetate [188,191]. In the present study, all but one of the mares having double ovulation were Warmblood, Thoroughbred, and Standardbred, which are breeds known to more frequently have double ovulations. Arabians and Quarter Horses have lower incidence of multiple ovulations [188,189]. Also, older mares have higher incidence of multiple ovulations due to variations in FSH concentrations around follicle deviation [190]; and this was not different here as all mares in the study with multiple ovulations were older than 14 > year-old. There was no significant increase in serum progesterone concentrations found between mares with multiple ovulations and increased serum progesterone concentration, however, numerically there is an evident increase. Because the number of cycles with multiple ovulations is a small subset of the total number of cycles, there was likely not enough power to yield a significant effect.

Environmental temperature is a key regulator of cyclicity in mares through the increase of opioids in cold weather which downregulate GnRH production [200]. Retrospective studies have shown that environmental temperature has an effect on time period to the first ovulation of the season as well as fertility [201]. In contrast, a controlled study did not find an effect on controlled changed in temperature with time to the first ovulation of the season [85]. In the

present study, both maximum and minimum temperature decreased over time, which was an expected pattern for the regional climate of Urbana, IL. Neither minimum or maximum temperature or relative humidity had an effect on interval to ovulation or interval from PGF2 α administration to ovulation. There was an effect between minimum temperature and follicle diameter at the time of induction and effects of minimum temperature and relative humidity on follicle diameter at ovulation. It is likely, based on known data, that the effects of temperature occur on a larger scale and in tandem with other environmental factors. Due to limitations of this study and the inability to compare trends over several years, trends comparing climate variables with photoperiod remains a constant that cannot be assessed.

All but five mares completed all cycles throughout the study, as two mares stopped cycling during the early fall and the other three during the late fall. One of the first two mares was a 3-years old Arabian. It has been documented that younger mares stop cycling earlier than older mares [69], which is consistent with the present study. Older mares are expected to start cycling later than younger mares, but stop cycling at the same time as middle-aged mares [69]. Herein these mares stopped growing follicles after early fall and did not experience a classic transitional period as they did not grow follicles beyond 10-20 mm of size for several weeks before going fully into anestrus.

The present study sought to determine the associations between follicle size at the administration of PGF2 α after 8 d post-ovulation to and the subsequent ovulation. This interval is critically important in embryo donor mares in maximizing efficiency and embryo output per breeding season. The luteal response to PGF2 α has been well characterized in mares on 5, 6 and 7 d post-ovulation [137,196] but not well studied at 8 d post-ovulation. For this study, follicle size was assessed as a continuous variable and also split into groups, which divided them based

on pre-ovulatory follicle sizes for induction with GnRH agonists. It was hypothesized that follicle size would determine time until the next ovulation, however, there was no relationship between follicle size and interval to ovulation, except when follicles were grouped as $< 35\text{mm}$ and $\geq 35\text{mm}$. The interval from PGF2 α administration to the subsequent ovulation considerably affects pregnancy rates in mares with ovulations taking place less than < 6 d post- PGF2 α , resulting in lower pregnancy rates than mares ovulating 6 – 8 d or >8 d-post PGF2 α [196]. The mean interval from PGF2 α administration to the subsequent ovulation was 9.3 d, which would be expected to yield satisfactory fertility. Also, late fall resulted in longer interval between PGF2 α and the next ovulation when compared with early fall. This is likely due to the fact that there is a gradual reduction in follicle waves [202].

3.6 Conclusions

The findings of this study support the use of deslorelin acetate as an induction agent in cycling mares during the fall, with 90% of mares responding to treatment. Of the responding mares, most ovulated within the window of 38 - 44 h. There was no effect of time on number of mares responding to the treatment or interval to ovulation. There was no seasonal effect on luteal area or progesterone concentration, and the progesterone concentration was weakly affected by luteal area but not by the number of ovulations. PGF2 α administration at 8 d post-ovulation resulted in an increased interval to the next ovulation later in the fall and which is not expected to yield adverse effects on fertility during the next cycle. Going forward, further work is needed to investigate the responses to GnRH agonists in different seasons, latitudes, and breeds of mares as well as the effect of GnRH agonist administration in the fall on fertility.

Declaration of interest: none

Author contributions

Kianna M. Spencer: investigation, formal analysis, conceptualization, visualization, and writing; **Giorgia Podico:** investigation, formal analysis, conceptualization, visualization, and writing; **Ameer A. Megahed:** investigation, resources, writing; **Kristi L. Jones:** investigation, resources, and writing; **João H. J. Bittar:** investigation, resources, and writing; **Igor F. Canisso:** investigation, conceptualization, formal analysis, methodology, resources, project administration, and writing.

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CHAPTER 4: General conclusions and future directions

As a minor objective, the present study sought to determine the associations between follicle diameter at the administration of PGF2 α on day 8 post-ovulation to and the subsequent ovulation. This interval is critically important in embryo donor mares in maximizing embryo output per breeding season. The mean interval from PGF2 α administration to the next ovulation was (9.3 ± 0.4 h) which is favorable according to other studies which have found that there is a higher percentage of fertility associated with intervals from PGF2 α administration to ovulation beyond 8 days [196]. Fall season and follicular diameter at the time of PGF2 α administration did not seem to have an effect on this interval.

As Fall progresses in the Northern Hemisphere, daylight length and temperature decrease and cyclicity in mares begins to decline. Due to this, it was suspected that mares would not respond to GnRH agonists for the induction of ovulation in the Fall. The present study shows that mares are equally responsive to GnRH agonists in the Fall and the Spring. In addition, there was an agreement in the interval from administration of a GnRH agonist to ovulation in the Fall and the Spring and a slightly longer interval in late Fall in comparison with early Fall. Pre-ovulatory follicle diameter in the Fall is less than what is previously reported in Spring cycles. The follicle diameter decreased in late Fall versus early Fall. In this study, luteal area and progesterone concentrations didn't vary between early and late Fall, indicating that there is no seasonal effect on integrity of the corpus luteum. A strong relationship was found between cycles with multiple ovulations and increased progesterone concentrations. There was also found to be no relationship between follicle diameter size at PGF2 α administration and the interval to the next ovulation.

Going forward, further work is needed to further investigate the differences between cyclicity and response to GnRH agonists in different season. One future direction would be to look at the same group of mares throughout an entire year, evaluating changes in follicle size at induction, interval to ovulation and response to treatment at different timepoints. In addition, comparisons of different latitudes and breeds of mares can be made. It would be important to evaluate fertility following induction of ovulation during the fall to ensure that it is comparable to that of the spring or at a threshold which would make fall breeding economically feasible for owners. As discussed, edema score decreased in late fall. Length of time in which a uterus is exposed to edema prior to ovulation can impact uterine environment prior to arrival of an embryo. Thus, it would be pertinent to study these effects both on embryo recovery rate in the fall and on ability of mares to carry an embryo throughout early gestation. Another interesting endpoint would be the measurement of peripheral LH concentrations. Previous studies have suggested that LH stores become depleted during fall transition and winter anestrus. Based on results of this study and the ability of mares to respond to a GnRH agonist with ovulation, which is primarily driven by LH, this hypothesis seems unlikely.

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