

THE EFFECTIVENESS AND EFFICIENCY OF OVULATION INDUCTION AGENTS IN  
MARES

BY

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THESIS

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## ABSTRACT

Study 1: A retrospective analysis of the effects of hCG and deslorelin on the reproductive efficiency on two commercial horse farms.

Reproductive data from two central Illinois horse farms was analyzed to compare the effectiveness of hCG (Chlorulon™) and a sustained release implant formulation of deslorelin (Ovuplant™) for inducing ovulation and their overall effect on reproductive efficiency. Data were collected over 3 consecutive years, from 1999-2001; a total of 1422 cycles were examined from 658 mares. Of the 1422 cycles examined, 383 were treated with hCG, 451 with deslorelin and 583 cycles were untreated. Mares in this study were over 2.1 times more likely to become pregnant if ovulation was induced ( $p < .001$ ). Mean days to ovulation was significantly shortened by using hCG or deslorelin compared to no treatment for all follicle sizes except those  $>45$  mm ( $p = .001$ ). Time from treatment to ovulation was affected by follicle size at time of treatment and by treatment given. When treatment was given at follicle sizes from 35 mm-39 mm, time to ovulation was shorter with deslorelin (2.02 days) than with hCG (2.68 days) ( $p = .000$ ). The number of palpations was not decreased by the use of ovulation induction but follicle size at administration and day of administration showed a positive effect on reducing the number of palpations. Administration of either agent at the first breeding examination when follicles were less than 35 mm in diameter decreased the number of palpations per cycle by one in comparison to non-treated mares. ( $p = .009$ ) Fewer artificial inseminations per cycle were performed using deslorelin for follicles between 35 and 44 mm compared no treatment [35-39 mm follicles ( $p = .000$ ): deslorelin 1.21 AI, untreated 1.39 AI; 40-44 mm follicles ( $p = .000$ ): deslorelin 1.16 AI,

untreated 1.48 AI] . Administration of either hCG or deslorelin to mares possessing a <35 mm follicle at the first breeding exam decreased the number of artificial inseminations required per cycle by 1 (p=.001). Both agents performed equally well at inducing ovulation within 48 hours of administration. Deslorelin appeared more consistent in decreasing the days to ovulation in comparison to hCG . Deslorelin decreased the days to ovulation at <35 mm follicles (p=.001)[deslorelin, 2.34 days, hCG 2.57days, untreated 3.54days], 35-39 mm follicles (p=.000) [deslorelin 2.02 days, hCG 2.68 days, untreated 3.87 days] and 40-44mm follicles (p=.000) [deslorelin 2.10 days, hCG 2.47 days, untreated 3.49 days] , Human chorionic gonadotropin only decreased the days significantly on follicles sized between 35-39 and 40-44 mm. Deslorelin also significantly decreased the days to ovulation over hCG at follicles sized between 35-39 mm. Use of these agents in a commercial breeding setting appears to be of value for improving pregnancy rate and decreasing the time to ovulation for improved timing of insemination. Management of the estrus cycle of the mare will determine if ovulation induction decreases the number of palpations and artificial inseminations per cycle. Management schemes must be considered in evaluating effectiveness of ovulation induction drugs since time of administration within the cycle and size of follicle at administration appear to affect reproductive efficiency.

Study 2: Effect of deslorelin sustained release implants on the interovulatory period and response to PGF2 $\alpha$  administration 6 days after ovulation

Ovuplant<sup>TM</sup> is a sustained release implant that contains the gonadotropin releasing hormone (GnRH) agonist deslorelin. Subcutaneous administration to a mare during estrus will

induce ovulation within 48 hours. Clinical evidence suggests that Ovuplant™ causes an increase in the interovulatory period of mares not conceiving on the treated cycle. A down regulation of the hypothalamic pituitary axis is thought to be the main cause of the increased interovulatory interval but no investigation has occurred concerning the function of the corpus luteum formed by ovulation induction by Ovuplant™. This study was performed using six teaching mares at the University of Illinois Veterinary Teaching Hospital in a cross over design clinical trial with four treatment cycles: a control (untreated cycle), a natural cycle with luteolysis induced with prostaglandin F2 alpha (PGF2 $\alpha$ ), a cycle with ovulation induced with Ovuplant™, and a final cycle with ovulation induced with Ovuplant™ and luteolysis induced with prostaglandinF2alpha (PGF2 $\alpha$ ). Progesterone levels for assessment of corpus luteum function were determined every three days during the diestrus period and days between ovulations on each treatment cycle determined the interovulatory period. The goals of the study were to determine the effect of Ovuplant™ administration on the interovulatory period, to examine the progesterone production of the corpus luteum formed after ovulation induction with Ovuplant™, and to determine the response of the corpus luteum formed by Ovuplant™ to induced luteolysis.

Progesterone levels differed between control mares and mares induced to ovulate with Ovuplant and administered prostaglandin 6 days after corpus luteum formation ( $p=.02$ ). The interovulatory periods of mares treated with Ovuplant™ (26.00d) did not differ significantly from untreated mares(21.67 days) ( $p=.01$ ). The interovulatory periods of untreated mares administered prostaglandin (11.8 days) differed significantly from those treated with Ovuplant™ (26.00 days) ( $p=.01$ ). Four mares treated with Ovuplant™ experienced delayed returns to estrus of 3-25 days. Ovuplant™ did not induce a corpus luteum which differed in progesterone

production or its ability to respond to PGF2 $\alpha$ . Ovuplant™ appears to extend the interovulatory period of sensitive mares.

*To my mother, father and two sisters,  
for their lessons and support*

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CHAPTER 1  
INTRODUCTION

A major objective for horse breeders is to inseminate a desired number of mares in a minimum amount of time with the majority of mares becoming pregnant and birthing a live foal the following spring (Pickett and Voss, 1999). Economic considerations require this to be accomplished as inexpensively as possible. Synchronization of ovulation with the placement of an adequate amount of viable spermatozoa is critical to obtain both of these objectives. Ovulation induction agents can assist in attaining this synchrony and may also decrease the cost and physical effect of breeding especially when considering the use of transported semen and natural breeding.

There are currently three types of ovulation induction agents available for use in the mare: human chorionic gonadotropin (hCG), gonadotropin releasing hormone (GnRH) agonists, and recombinant luteinizing hormone (LH). The two commercially available, commonly used agents today are hCG (Chorulon™) and deslorelin, a GnRH agonist, available in an injectable form (SucroMate™, Bioniche Life Sciences Inc.) or a slow release implant (Ovuplant™, Fort Dodge Animal Health/Peptec). Ovuplant™ was the first available preparation of deslorelin and was released for commercial use in 1998 by Fort Dodge Animal Health.

Both agents have their limitations. Human chorionic gonadotropin has often failed to induce ovulation after being administered to the same mare multiple times. This is especially noticeable when given to the same mare within the same breeding season (Roser et al, 1979; Voss et al, 1975; McCue et al, 2004). Being a large protein, human derived hormone, it has also induced hypersensitivity reactions in mares. (Roser et al, 1979; Voss et al, 1975) In the search

for a more reliable agent, deslorelin was developed and first released in the form of a slow release implant called Ovuplant™. (Jochle and Trigg 1994) Ovuplant™ was well received in the 1998 breeding season as it displayed consistent ovulation induction even when administered repeatedly to the same mare. (McKinnon et al, 1993; Ganheim et al, 1995; Samper et al, 2002) By the end of the 1998 breeding season and into the 1999 breeding season, increased interovulatory intervals in mares treated were being observed. (Vanderwall et al, 2001; Morehead et al, 2000) Mares that were slow to return to heat when not pregnant was considered a costly inconvenience especially if the mare foaled late in the year (further delaying or disallowing her rebreeding for that season) or was an embryo donor mare (decreasing the number of embryos that could be retrieved leading to a decrease in profitability). Many mares failed to return to estrus for months. (McCue et al, 2002)

Control of gonadal function arises at the level of the hypothalamus and pituitary within the brain although other factors, such as light stimulus and melatonin, affect cycling in mares. Reasons for a delayed return to estrus could be attributed to anything affecting this hypothalamic-pituitary-gonadal axis. Research into the cause of the delayed return to estrus induced by Ovuplant™ was pursued and it centered on the effect of this GnRH agonist on the hypothalamic-pituitary axis of the mare. (Johnson and Thompson Jr. 2000; Porter and Sharp, 2002) Earlier clinical trials using Ovuplant™ noted clinical symptoms indicative of receptor down-regulation at the level of the pituitary (increases in the duration of estrus due to the delayed growth of large follicles, increased interovulatory intervals, increasing LH levels with decreasing FSH levels) when the amount of deslorelin administered was increased to 5-10 times the minimally effective dose. (Jochle and Trigg 1994) No research evaluated the effect of Ovuplant™ on the gonad and specifically the possibility of prolonged or abnormal function of

the corpus luteum (CL) formed upon induction of ovulation by Ovuplant™, as a cause of the increased interovulatory interval. (Ginther, 1990)

The goals of this project were to examine the effectiveness and efficiency of using ovulation induction agents when breeding mares in an uncontrolled, clinical setting and to examine the effect the Ovuplant™ form of deslorelin may have on luteal retention and function in a controlled clinical trial.

For Study 1, reproductive data was collected from 2 central Illinois horse farms and analyzed retrospectively to compare the effect of the two most commonly used ovulation induction agents, hCG (Chlorulon™) and deslorelin (Ovuplant™) on mare reproduction when compared to no treatment. The data was analyzed to determine if pregnancy rate, number of palpations, and number of artificial inseminations (AIs) were affected by giving either of the two induction agents. The two agents were assessed for their effectiveness at different follicle sizes to reduce the number of days to ovulation in comparison to untreated mares. Each drug's ability to induce ovulation within 48 h of administration in a field setting as per label claim was assessed. The data was also explored to define if early administration of an induction agent within the breeding cycle may be beneficial for reducing the days to ovulation.

For the controlled study examining the effect of deslorelin, specifically the Ovuplant™ formulation, on the function of the CL post-ovulation induction, six teaching mares housed at the University of Illinois Veterinary Teaching Hospital were utilized. A cross over clinical trial design was used to examine the mares through an untreated estrus, an estrus induced with Ovuplant™, and an estrus induced with Ovuplant™ followed by prostaglandinF2 alpha (PGF2α). Days between ovulations were counted to determine the interovulatory interval and

progesterone sampling allowed for assessment of CL function. An increased interovulatory interval was determined by the number of days from one ovulation to the next exceeding the expected number of days determined by the untreated cycle. CL function was examined by determining the progesterone levels during the diestrus phase of the cycle for each mare and for each cycle studied. Both experiments are detailed in the Materials and Methods section.

The mare's estrous cycle is controlled by several extraneous factors, most notably light, and is subject to aberrations from both internal and external influences. The normal estrous cycle as well as follicle growth, ovulation and luteolysis and the factors which can influence normalcy are explored in the literature search. Detailed descriptions of pertinent hormones involved in this study and the available ovulation induction agents will be imparted. A description of the GnRH receptor of the mare and a discussion of its function will finalize the literature search.

Study 1 will show that ovulation induction agents do appear to improve reproduction efficiency when breeding mares especially in regards to pregnancy rate and both the agents discussed appear equal in their effect at inducing ovulation and shortening the estrus period. Both agents appear to have their advantages and the results allude to the conclusion that there are more opportune times within the estrus period to utilize ovulation induction. An elaboration of the concluded points, the best utilization of ovulation induction agents, and the limitations of this study are found within the discussion.

Study 2 will show that deslorelin in the sustained release formulation (Ovuplant™) did prolong the interovulatory interval of some of our study mares but there appeared no influence

on the function of the corpus luteum formed by Ovuplant™. The conclusions drawn and the shortcomings of the study will be deliberated in the discussion section.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Unique Anatomy of the Mare's Ovary

In comparison to other domestic mammals, the mare's reproductive tract has some unusual features, which may influence our ability to control her cycle and improve reproductive efficiency. Two of the most striking differences are the shape and structure of the ovary. The ovaries of the mare are kidney shaped (reniform) and have a distinctive ovulation fossa. (Witherspoon, 1971) At birth, the equine ovary's structure is similar to other domestic species. At or near puberty, the ovary changes from a symmetrical bulbous structure to the more kidney shaped mass of the sexually mature adult. The germinal tissue (cortical tissue), originally exterior, assumes an interior position with a peripheral collagenous connective tissue casing (medullary tissue) where the vascular supply is found. The cortex of the ovary only reaches the surface of the ovary at the ovulation fossa and ovulation can only occur at this area. (Adams et al., 1988; Stabenfeldt et al, 1975) This inverted structure can become confusing during rectal exams as developing follicles are difficult to palpate manually and a mature corpus luteum (CL) is non-palpable. The ovaries of the mare are also very large in comparison to many other domestic mammals measuring 5-8 cm in length, 3-5 cm in height, and 2-3 cm in width for most light horse breeds. With these large ovaries come uniquely large pre-ovulatory follicles measuring on average 45 mm at ovulation. (Witherspoon, 1971) This is much larger than the preovulatory follicles of other domestic hoof stock such as the cow (11-16mm) and ewe (5-8mm). (Driancourt, 1991)

## 2.2 Normal Estrous Cycle of the Mare

The estrous cycle of the mare can be divided easily into a follicular phase and a luteal phase as per other domestic species and is typically defined as the period from one ovulation to the subsequent ovulation when accompanied by signs of estrus and /or progesterone levels below 1ng/ml of plasma. (Hughes et al, 1975; Stabenfeldt et al., 1971) During the “true”, or physiologic, breeding season, the estrous cycle averages 21-22 days with an average duration of estrus of 5-7 days and diestrus 15-16 days. Unlike other domestic species such as the cow, ewe and doe which display much more consistency in cycle length, estrous cycle length in the mare can vary considerably ranging from 13-34 days depending on the mare and season (Hughes et al, 1977). In the northern hemisphere, most mares will be cycling normally by the month of May and will continue to cycle for approximately 155 days. (Adams et al, 1988; Witherspoon, 1971; Ginther et al, 1972)

Mares are seasonally polyestrous with regulation of cyclicity coming primarily from photoperiod, with other factors such as nutrition and climate (mainly temperature) contributing to a lesser degree (Adams, 1988; Ginther et al, 1974; Hughes et al, 1977). These factors also contribute to the variability in the length of the estrous cycle noted between mares. As a mare progresses into the physiologic breeding season with increasing daylight, the length of estrus significantly decreases while the length of diestrus increases while the overall length of the estrous cycle remains unaltered. (Ginther et al, 1972) Considerable variation though is noted in the length of estrus between mares, between different breeds and between different seasons of the year. (Stabenfeldt et al, 1975) Much of the variation is noted in the length of the follicular phase and is related to season (primarily the photoperiod effect). Genetic factors (breed) appear also to play a minor role in influencing the length of the follicular phase. The diestrus phase of



the estrous cycle appears to remain most constant in duration amongst mares with photoperiod and season imparting minor effects. (Adams et al, 1988; Witherspoon, 1971; Ginther et al, 1972) Interestingly though, individual mares consistently either display a short or long estrus (Hughes, 1980) with the length of estrus more repeatable within mares than the length of diestrus.

### 2.3 Control of the Estrous Cycle: The Hypothalamic-Pituitary-Gonadal Axis

Regulation of reproduction in the mare is mainly via photoperiodic control of the hypothalamic-pituitary-gonadal axis although temperature and nutrition also play a role. (Adams, 1988; Ginther et al, 1974; Hughes et al, 1977) This is similar to other domestic species such as the ewe except ewes cycle during times of short photoperiod and mares cycle during times of long photoperiod. The exact mechanisms of control for the mare estrous cycle have not been fully established. A unique interaction between the nervous system and the endocrine system, the hypothalamus and the pituitary gland, provides the platform for regulation. (Ginther, 2012) Control begins with perception of light by the retina of the eye. Via the nervous system, this signal is transmitted to the pineal gland within the brain. The pineal gland transforms the nervous signal into an endocrine signal with the production of melatonin. Melatonin is synthesized and released during dark periods. Melatonin acts upon the hypothalamus to produce and stimulate the release of gonadotropin releasing hormone (GnRH). The release of GnRH initiates cyclicity in the short day breeders. In the mare, the opposite is true: GnRH is released and cyclicity occurs in response to low levels of melatonin.

The hypothalamus, designated the control center for reproductive hormones, lies within the brain and is comprised of groups of nerve cell bodies. These neurons produce the hormone called GnRH which is the primary releasing hormone of reproduction. Neurons from the

hypothalamus communicate with the pituitary gland, specifically the anterior pituitary gland, via a unique aspect of the circulatory system called the hypothalamo-hypophyseal portal system. (Ginther, 2012) GnRH, via this portal system, stimulates the release of the anterior pituitary hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH act upon the gonads of the mare to stimulate follicular growth and ovulation. FSH stimulates follicular growth and maturation leading to the production of the steroid hormone estrogen. (Donadeu and Watson, 2007) LH induces ovulation of the dominant follicle and formation of the corpus luteum (CL) leading to the production of the steroid hormone progesterone. These two steroid hormones, along with several other peptide, protein and glycoprotein hormones, act to further control reproduction via positive and negative feedback loops to the hypothalamus, pituitary, and the gonads themselves.

Onset of the mare estrous cycle is associated with the stimulus of long light periods and artificial manipulation of light has for many years been utilized by the equine industry to advance the physiologic breeding season. A transitional period from anestrus to estrus cycling, characterized by irregular and anovulatory estrus cycles, occurs in mares whether manipulating the cycle with artificial lighting or not. Artificially manipulating the mare's estrous cycle does not negate transition or the effect it has on the hormone and receptor levels within the hypothalamus and anterior pituitary. (Hart et al, 1984; Silvia et al, 1986; Donadeu and Watson, 2007) Understanding that there are changes in the hormones and their receptors during the spring transition phase as well as the physiologic breeding season is important when managing mares for maximal reproductive efficiency. Follicular development patterns are different in the early spring months and these follicles respond differently to ovulation induction agents partially

due to the incompetence of the hypothalamic-pituitary axis. (Silvia et al, 1986; Donadeu and Watson, 2007)

#### 2.4 Follicular Growth

Mares have a very effective follicle selection mechanism. They, along with cows, have the lowest incidence of multiple ovulations, and hence multiple fetuses, of the farm animal species. (Ginther, 2000) Follicular waves develop in the mare during the second half of an estrous cycle and typically culminate in the ovulation of one follicle. Double ovulations can occur and are affected by season, age of the mare, breed, and pharmacologic manipulation of the estrous cycle. Near the peak of the physiologic breeding season, double ovulations can occur in approximately 25-26% of estrous cycles. (Hughes et al, 1977) The incidence of double ovulations in the pony mare is reportedly rare. (Ginther et al, 1972)

Mares develop both major and minor follicular waves, as in other farm animal species, but a single major wave is most commonly associated with the estrous cycle of the mare. A major wave is characterized by the divergence of a follicular wave into subordinate and a dominant follicle(s), which may ovulate while the subordinate follicles regress. A minor wave is characterized by a follicular wave which never forms a designated dominant follicle. Considerable breed variation exists in wave patterns. Pony mares and quarter horses usually develop one major wave in late diestrus culminating in an ovulation. In contrast, thoroughbreds, related warm-blood breeds, and Standardbred mares are considered to have a higher double ovulation rate as they often develop a secondary major wave in early diestrus with the dominant follicle regressing or ovulating. (Hughes et al, 1980; Ginther, 2000)

Mares that have a major secondary wave develop in late estrus or early diestrus can produce a dominant follicle, which can ovulate, resulting in a diestral or secondary ovulation, or produce an anovulatory dominant follicle, which regresses. Diestrus ovulations are defined as ovulations occurring under the influence of high progesterone. This phenomenon of diestrus ovulations is unique to the mare. (Hughes et al, 1977; Ginther, 1974; Stabenfeldt et al, 1975) These ovulations produce fertilizable oocytes but typically do not result in pregnancy unless the ovulation closely follows an ovulation of the primary major wave when semen may still be present in the track. These secondary major waves can affect reproductive efficiency since a functional CL formed by the secondary ovulation may affect the mare's expected return to estrus increasing the interovulatory interval. (Ginther, 1990)

### 2.5 Follicular Dynamics, Ovulation, & Luteolysis in the Mare

Stimulation of a follicular wave occurs via a surge of FSH in the mare just as in other domestic farm animal species although the resulting sizes of the follicles are notably larger. (Witherspoon, 1971) Wave emergence begins with the appearance of one of two 6mm follicles with numerous others joining the wave over about a 3-4 day period. One of these initial follicles will typically remain slightly larger and end as the dominant or ovulatory follicle. These emerging follicles undergo a period of simultaneous growth, under the influence of FSH, growing at a common rate of about 2-3mm/day until the largest follicles reach a size of about 22mm, typically about 7 days prior to ovulation. At the same time, FSH levels peak and LH levels begin to increase and three days after the peak, deviation occurs. Deviation occurs when one or two of the largest follicles continue to grow at the 2-3mm/day rate while all the smaller,

subordinate follicles become static in growth and regress. The largest or dominant follicle(s) continue to grow until an LH surge initiates ovulation. (Ginther, 1992; Ginther, 2000)

Follicles control deviation through the inhibition of FSH release. During wave emergence, FSH drives the growth of the follicles and peaks when the follicles are about 13mm in diameter. The decline in the concentration of plasma FSH at this time is a function of the larger follicles of the wave producing inhibin within their granulosa cells which suppresses FSH release from the hypothalamus. (Gastal, Gastal, Wiltbank et al, 1999) Receptors within the larger follicle(s) switch to become more LH responsive for growth (vs. only FSH responsive) making LH critical to the continued growth of the dominant follicle. After deviation, continued suppression of FSH activity becomes the sole responsibility of the dominant follicle. (Donadeu et al, 2001) With deviation comes a rapid increase in the production of estradiol by the granulosa cells of future dominant follicle. (Donadeu et al., 2001; Gastal, Gastal, Wiltbank et al, 1999) Uterine edema is noted on ultrasound examination as the estrogen level raises. Behavioral signs of estrus will characteristically begin shortly after this point as will a greater responsiveness to FSH and LH by the developing dominant follicle. (Gastal et al, 1999; Ginther, 2000; Ginther, 2003) LH suppression at this point in the cycle will cause restricted growth of the dominant follicle(s). (Ginther et al, 2003) It is an interesting point that LH plays a critical role in the both the growth and maturation of the dominant follicle as well as its ovulation and luteinization.

The pre-ovulatory dominant follicle grows at a rate of approximately 3mm in diameter a day up to 35mm about four days before ovulation. At this point it enters the pre-ovulatory period when the follicle begins a more rapid growth rate plateauing approximately two days before ovulation at about 40-50mm in diameter. As estrogen levels reach higher concentrations near the end of the follicular phase, follicles acquire the LH receptors needed for ovulation to occur.

(Ginther, 1992) At two days prior to ovulation, follicular growth becomes static and estradiol peaks. As estradiol peaks and begins to drop, any negative effects on LH secretion are removed allowing for a more rapid release of LH. However, the rise of LH is anything but rapid in the mare as she is unique in her prolonged LH surge which occurs over a period of several days. (Geschwind et al, 1975) At the time of estradiol peak two days prior to ovulation, the negative influence on FSH is removed causing FSH secretion to resume and a new cohort of follicles begins to grow. (Ginther, 1992) As ovulation and luteinization of the once dominant follicle occurs, progesterone levels rise having a negative influence on LH levels leading to the peak and drop of LH one day post ovulation. (Ginther, Gastal et al., 2008) This is also unique to the mare.

After the induction of ovulation has occurred, formation of the corpus luteum (CL) begins with morphologic changes within the granulosa cells of the collapsed follicle which allow for a switch from producing estrogen to producing progesterone. This process is called luteinization and involves the transformation of the theca interna cells and granulosa cells of the ovulating follicle into luteal tissue. Granulosa cells become large luteal cells which contain receptors for  $\text{PGF}_2\alpha$  hence mediating the luteolytic cascade and the theca interna cells become small luteal cells which are responsive to LH in their production of progesterone. Both types of cells contribute to the production of progesterone from the corpus luteum. (Niswender et al, 2000) Production of progesterone rises significantly in 12 hours (Plotka et al, 1975) and usually measure greater than 2 ng/ml in the plasma by 48 hours post ovulation (Townson et al, 1989; Koskinen et al, 1990). Morphogenesis becomes complete in about 5-6 days post-ovulation and the corpus luteum is fully functional in its production of progesterone at that time. The progesterone levels begin to drop around day 14 post ovulation if the mare fails to become pregnant. (Short, 1959)

## 2.6 Hormonal Control of the Peri-ovulatory Period and Luteolysis

Near the end of the luteal phase ( $13.0 \pm 3d$ ), (Ginther and Beg, 2011), provided there is no embryo within the uterine lumen, another hormonal cascade ensues to cause lysis of the corpus luteum with subsequent functional regression. The complete mechanism by which luteolysis occurs in the mare is as yet undefined but likely begins in a similar fashion to the ruminant model although an increase in estrogen from growing follicles is not required. (Ginther, 2012) Initially, in the mare, there is a small transitional pulse of PGFM (a prostaglandin metabolite) with a concurrent increase in pulsatile release of oxytocin. The source of the oxytocin is thought the posterior pituitary in the cow but is yet undetermined in the mare. Oxytocin has been identified in the CL of the mare but could also be released from the endometrium or posterior pituitary. (Ginther and Beg, 2011) Oxytocin triggers the episodic release of  $PGF2\alpha$  from the uterine endometrium. A positive feedback loop furthering the release of both hormones has been identified in the cow but all the components of a feedback loop have not been identified in the mare. In the mare, PGFM stimulates increased levels of oxytocin and PGMF stimulates increased release of oxytocin. (Shand et al, 2000, Ginther and Beg, 2011) Progesterone, prolactin and cortisol may also play some part in luteolysis in the mare but estradiol and cortisol are not the initiators of the cascade. (Ginther and Beg, 2011) Progesterone drops rapidly within 1-2 hours after luteolysis to levels around 2ng/ml or less after which a more moderate ensues. (Ginther and Beg, 2011) Loss of progesterone action in other species studied occurs due to down regulation of its own receptors, both in the hypothalamus and the endometrium of the uterus, allowing estrogen to begin to affect these tissues. (McCracken et al, 1999) Estrogen has

a positive feedback effect on the hypothalamic pituitary gonadal axis allowing follicles to grow and another cycle to ensue.

## 2.7 GnRH, LH, and Prostaglandin F2 $\alpha$

### GnRH (gonadotropin releasing hormone):

Hypothalamic GnRH is a decapeptide (contains 10 amino acids) neural hormone with a short half-life (5 to 10 minutes). The sequence of amino acids is (1) glutamine (2) histidine (3) tryptophan (4) serine (5) tyrosine (6) glycine (7) leucine (8) arginine (9) proline (10) glycine. Its composition remains conserved across species.

Synthesis occurs in the neurosecretory cells of the hypothalamus and in the horse, this production does not appear to be restricted to a discrete area of the hypothalamus as in other species, but rather appears evenly distributed throughout the hypothalamus. GnRH axons from the hypothalamus pass mainly to the median eminence where the secretory product is stored in terminal granules. Neuron depolarization releases the stored GnRH to enter capillaries leading to the portal vessels that transport the hormone to the pituitary where it binds to receptors on the gonadotrophs. Release occurs in picomolar concentrations in the portal blood and even smaller values are found (subfemtomolar) in the peripheral blood. (Ginther, 1992)

GnRH release occurs in a pulsatile nature in the periovulatory period with secretion actually appearing as a continuum with broad pulses found superimposed over a tonic background. Pituitary concentrations of the gonadotropes FSH and LH appear pulsatile and coincident with the GnRH pulses. As determined by frequent collection of blood from the intercavernous sinus (allowing collection of blood relatively undiluted from the pituitary venous blood), 93% of the LH pulses occurred within 5 minutes after a GnRH pulse. (Alexander and



Irvine, 1987). FSH and LH pulses coincide in a nearly opposite relationship through the periovulatory period except near ovulation when concentrations reach similar levels. (Miller et al, 1980.; Alexander and Irvine, 1987)

### LH:

Luteinizing hormone is the primary hormone involved in ovulation of the dominant follicle. LH is a glycoprotein hormones consisting of two subunits, termed alpha and a beta. Different genes encode for each subunit and synthesis of each subunit occurs separately within the gonadotroph cell and each subunit later spontaneously associates to form the entire molecule. Synthesis of the beta subunit appears the rate-limiting step. The alpha subunit appears in excess within the cells. (Ginther, 1992). The alpha subunit is species specific and has essentially the same structure as the other glycoprotein hormones found in the horse, thyroid stimulating hormone (TSH) and equine chorionic gonadotropin (eCG). The equine alpha subunit's amino acid sequence differs from that of other species and this may dictate the equine gonadotropin's unusual receptor binding ability when administered to other species. The beta subunit appears to confer each hormones specific biologic activity.

Synthesis and release of LH is a regulated process and is controlled mainly by GnRH. Estradiol and testosterone influence secretion. LH beta gene expression, leading to biosynthesis, is highly dependent on the dose of GnRH administered. The frequency of GnRH pulses has a major effect on gonadotropin gene expression. Rapid frequency elicits maximal stimulation of alpha and LH beta mRNA; lower frequencies signal expression of the FSH beta mRNA. This unique biosynthetic mechanism allows the production of two hormones, preferentially, within one cell type by one signaling hormone. In addition, the same signaling hormone stimulating

synthesis also signals differential release. The magnitude of FSH release from GnRH stimulation is less than LH (Ursula et al, 1997).

Equine gonadotropins have significant sialic acid content, which is unique in comparison to other species. The terminal sugar on the majority of the carbohydrate side chains of both LH and FSH is sialic acid. With its strongly negative charge, sialic acid imparts particular receptor binding abilities to the individual hormones and influences both biologic activity and circulatory half-life. Sialic acid appears to decrease the degradation of LH in the mare hence increasing its half-life and conferring increased biologic potency. (Irvine, 1979) Mares are unique among other domestic farm animals in that the LH surge leading to ovulation is prolonged beginning several days prior to ovulation. This longer half-life of LH associated with the sialic acid content helps to explain the persistence of high concentrations of LH up to three days post-ovulation. (Geschwind et al, 1975)

#### Prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>):

Prostaglandins consist of a chain of 20-carbon fatty acids which are derived from arachidonic acid and control the length of the mare's estrus cycle by determining the functional lifespan of the corpus luteum. (Niswender et al, 2000) Prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) is a very potent hormone with mares being 18 times more responsive to its effects than other farm species. (Ginther, 1992) The endometrium of the uterus produces and secretes PGF<sub>2α</sub> which, in the mare, reaches the ovary via the systemic circulation. Other species have a more direct path by which PGF<sub>2α</sub> reaches the ovary i.e. the counter current mechanism of the ruminant. The path through the systemic circulation subjects the hormone to more rapid metabolism in the lungs, liver, and kidneys. The luteal cell membranes of mares though have a much greater affinity for

prostaglandin in comparison to cows. Mares actually require a much smaller exogenous dose of prostaglandin for clinical induction of luteolysis than cows. (Ginther, 2012)

Prostaglandin is typically present in the blood of non-pregnant mares beginning on day 13 post-ovulation. The pulses of  $\text{PGF}_2\alpha$  precede the first measurable decline of systemic progesterone by about 3-4 hours. Progesterone takes only 24-48 hours to decline to baseline ( $<1\text{ng/ml}$ ). (Ginther, 2012) On occasion, non-pregnant mares will retain function of their CL past this 14-17 day period of normal function. Such mares may have depressed or absent pulsatile release of  $\text{PGF}_2\alpha$  from the endometrium or they may have an insensitivity to oxytocin stimulation causing the diestrus phase of the cycle to continue beyond a normal period. This persistent luteal tissue is responsive to exogenous prostaglandins. (Kindahl et al, 2000)

Two exogenous prostaglandin products are available for use in the mare to induce luteolysis. Dinoprost tromethamine (Lutalyse™, Pfizer Animal Health, New York City, New York, USA) and cloprostenol sodium (Estrumate™, Merck Animal Health, Summit, New Jersey, USA) Dinoprost tromethamine (5mg/ml) is a natural prostaglandin approved for use in the mare in the USA. A dosage of 10mg IM/1000 pounds of body weight administered at 5 days or greater post-ovulation ( Blanchard et al, 2003) is recommended although smaller doses have been found effective at inducing luteolysis. (Nie et al, 2004) Cloprostenol (250 $\mu\text{g/ml}$ ) is a more potent analogue of prostaglandin approved for use in cattle but commonly used off label for estrus induction in the mare. A dosage of 250 $\mu\text{g}$  IM/1000 pounds body weight is recommended but smaller doses have also been found effective in the mare. (Nie et al, 2004) Both products appear equally effective. Cloprostenol costs more but induces fewer side effects in mares and is hence a better choice for sensitive mares. The equine CL does not become responsive to the effect of exogenous prostaglandin until around day 5 post-ovulation. After terminating the

production of progesterone by the CL, a follicle wave must ensue for the mare to return to estrus. This return to estrus usually occurs in 3-7 days with ovulation occurring in from 6-10 days post administration.

## 2.8 Ovulation Induction in Mares

The purpose of ovulation induction is to better synchronize ovulation in the mare with mating by the stallion or insemination of semen. It is advantageous to have ovulations occurring at a predictable time and increased pregnancy rates are found when semen is deposited within the fertile mares tract as close to ovulation as possible.(Newcombe and Cuervo-Arango, 2011)

When using ovulation induction agents, considerations are given to sperm longevity within the mare's reproductive tract. Sperm survival within the mare's reproductive tract averages 2.6 days using fresh semen (Clement et al, 2000) for healthy stallions and mares, however, this may vary based on the individual stallion and mare.

Pregnancy rates for fresh semen insemination, by either artificial insemination or natural breeding are best from 0 to 48 hours of ovulation. For cooled, shipped semen in which sperm longevity is reduced from cooling, storing, and rewarming, the optimal time is 12-24 hours prior to ovulation up to six hours post-ovulation. For frozen semen, which has further reduced longevity and viability, the best pregnancy rates occur with deposition as close to ovulation as possible but ideally less than 12 hours prior to ovulation and no more than 6 hours post-ovulation. (Woods et al, 1990, Sieme et al, 2003)

## 2.9 Ovulation Induction Agents Used In the Mare

Currently the commercially available and useful agents for ovulation induction in the mare are hCG (human chorionic gonadotropin) (Chorulon®, Intervet-Schering Plough Animal Health, USA), deslorelin (a gonadotropin releasing hormone agonist, Ovuplant™, Fort Dodge Animal Health, 1998) and SucroMate™, (Bioniche Life Sciences Inc., 2010 ), and r-LH (recombinant luteinizing hormone, EquiPure LH™, Aspenbio Pharma Inc., 2005). Recent studies have also shown that the GnRH agonist histrelin (historelin) is a useful ovulation induction agent in the mare but no FDA approved product is yet available. (Lindholm et al, 2011; Voge et al, 2012)

Human chorionic gonadotropin (hCG) was the first drug used to induce ovulation in the mare in 1939. It is a large protein hormone and bears LH like activity when used in the mare. Dosages used range from 1500 IU to 10,000 IU intravenously (IV), subcutaneously (SC), or intramuscular (IM) with the typical administered effective dose of 2000-2500 units IV or IM. (Berezowski et al, 2004; Barbacini et al, 2000) The study by Barbacini and colleagues in 2000 looked at 1040 estrous cycles during which hCG was used to induce ovulation in mares bred with frozen semen. The results showed approximately 75% of mares ovulated within the expected time frame of 25-48 hours after injection using a dose of 2000 IU when the follicle measured greater than or equal to 35 mm in diameter and uterine edema was present. Older mares in this study definitely showed a reduced ability to respond to the hCG and this could be due to antibody formation after repeated injections (Roser, 1979) or a down regulation of the hypothalamus, pituitary gland, and/or ovaries of older mares. (Barbacini, 2000; Clark et al, 2005) Hypersensitivity reactions occasionally occur after administration of hCG to mares due to its large size and human origin. Another concern with Chorulon™ is its stability after

reconstitution. (Meyers et al, 1997) After reconstitution with ten milliliters of sterile water (10,000 units total in 10ml), a vial typically contains 3-4 doses depending on the amount used per mare. Manufacturer recommendations are to store cooled between +2°C to +8°C for no more than 24 hours and then discard. (Chorulon™ Data Sheet, MSD Animal Health-United Kingdom, Merck Animal Health, Summit, New Jersey, USA) No clinical trials have been published reporting stability beyond 24 hours.

Native GnRH products are ineffective in the mare to induce ovulation. Early work using gonadorelin, the native form of GnRH commonly used for induction of ovulation in cattle, proved unsuccessful in inducing consistent and useful ovulation in the mare even over a multiple dosing period. (Squires, 1981) This short acting GnRH failed to provide the necessary prolonged period of LH influence on the dominant follicle required to induce ovulation in the mare even with the use of multiple doses. This knowledge led to the investigation and development of GnRH analogues called “super agonists” for ovulation induction in the mare. (Squires et al. 1994; Meyers et al. 1997; McKinnon et al. 1993; Ganhiem et al. 1995) Potent synthetic GnRH agonists created by altering the ten amino acid composition of native GnRH proved to provide the necessary stimulation required for ovulation induction in the mare. The potency of an agonist relates to its receptor affinity, in vivo absorption, distribution, and resistance to degradation and elimination. Deslorelin, buserelin, and histrelin have all proven efficacious in inducing ovulation in the mare (Lindholm et al, 2011; Voge et al, 2012; Barrier-Battut et al, 2000) Histrelin is the most potent of these three being about 210 times more potent than native GnRH while busrelin is only 20 times more potent. Deslorelin is 114 times more potent. (Conn, 1994; Padul, 2005) Deslorelin and histrelin are termed super agonists because of their

potency and prolonged effect on the hypothalamus and pituitary gland. Presently, the most commonly used GnRH agonist for mares is deslorelin acetate.

Deslorelin acetate has been released in many successful formulations: slow release implant (Ovuplant™ Peptech/ Fort Dodge Animal Health, 1998), short term biodegradable liquid (Biorelease Deslorelin, BET Pharm, Lexington, KY), aqueous, lyophilized liquid (Ferris et al, 2011), and a recently released control released liquid (SucroMate™, Bioniche Life Sciences Inc., 2010). All formulations have proven similar and successful at inducing ovulation in field and controlled studies. (Ferris et al, 2011, Ferris, et al, 2012; Berezowski et al, 2004; Jochle et al, 2004) Many of these formulations derive from compounding pharmacies. SucroMate™ is currently the only FDA approved GnRH agonist available for use in the USA.

Deslorelin in the slow release implant formulation (Ovuplant™) was first studied as a viable option for a commercially available ovulation induction agent in the mare from 1990-1994. (Jochle et al, 1994) This implant formulation which is injected subcutaneously in the tissues of the neck or vulva (McCue et al, 2002) fulfilled the need for a more consistently effective agent, which would shorten the interval between breeding and ovulation to 48 hours or less, hence increasing reproductive efficiency and without unwanted side effects. Jochle found that the drug consistently accelerated the ovulation of a follicle of 30mm or greater in diameter and that 80% or greater of the mares in the study ovulated within 48 hours after treatment, reducing the time to ovulation by 5%. No adverse effects on pregnancy rates, early embryonic loss, abortion rates or foal viability were noted. No tolerance to the drug was found in individual mares and Ovuplant™ was found to reliably trigger LH and FSH release from the pituitary, lasting at least 24 hours. Factors found to adversely influence response to treatment were follicles treated too early and not ready for ovulation or follicles treated too late and already

committed to ovulation. Jochle did note that increasing the Ovuplant™ dose to five or ten times the recommended dose caused the occurrence of endocrine and clinical symptoms indicative of receptor down regulation at the level of the pituitary. With a five times greater dose, mares had an increase in the duration of estrus, an increase in the time for follicles to reach the 30 mm size (dominance), and an increase in the inter-ovulatory interval. These signs were accompanied by increasing LH but decreasing FSH blood concentrations. Follicles in these mares were smaller at ovulation but fertility was not affected. At ten times the dose, follicular development and growth at the ovarian level disappeared within 15 days after treatment. Meyers and his group also confirmed the findings of Jochle in two separated controlled clinical trials using a 2.2 mg implant vs. the 2.1 mg used by Jochle. (Meyers et al, 1997) Both implants were formulated by Peptide Technology Ltd.

The FDA removed the Ovuplant™ product from the market due to manufacturing concerns in 2004. Ovuplant™ is still approved for use in mares in the USA but, currently, must be imported from other countries. In the 1998 breeding season, breeders and veterinarians found it quite effective in inducing ovulation in mares possessing a dominant follicle greater than 30-35mm in size with prominent uterine edema. Ovulation occurred consistently in approximately 42 hours and no decrease in efficacy occurred even after multiple doses to a single mare because of lack of conception and rebreeding (McKinnon, et al, 1993; Ganheim, et al. 1995; Samper, et al. 2002). This made Ovuplant™ quite attractive to equine reproductive veterinarians and breeders. Although the expense of the deslorelin implant was significantly greater than the hCG product Chorulon™, the benefit of time and dollars saved from avoiding the need for multiple vet exams, multiple breedings in mares susceptible to post mating endometritis, and multiple expensive semen shipments was a welcome trade-off. (Meyers, et al,



1997) Owners of mares enrolled as embryo donor mares were especially receptive to its use. Embryo donor mares are routinely given a prostaglandin injection seven days after breeding, immediately after the embryo flush, to return them to estrus for rebreeding and possibly subsequent embryo flushes. Such mares must respond to an ovulation induction agent repeatedly in a shorter period of time than typical to make the endeavor successful and profitable for the owner.

Recombinant equine luteinizing hormone (reLH or r-LH) is a single chain gonadotropin while hCG is a two chain gonadotropin (has an alpha and beta subunit). Hence, recombinant equine LH is of lower molecular weight and hence less antigenic than hCG. When studied for use as an ovulation induction agent in 2007 by Yoon, et al, it was found to induce ovulation about 80-90% of the time depending on the dosage given. The results were similar to the hCG treated mares (85.7%). The study concluded that reLH was a reliable and effective ovulatory induction agent that did not alter endogenous hormone profiles or affect interovulatory intervals. (Yoon et al, 2007) Currently it is not in active use to induce ovulation in the mare due to limited availability in the marketplace.

#### 2.10 The Induction of a Prolonged Interovulatory Period by Ovuplant™

During the second breeding season in 1999, anecdotal reports of mares slow to return to estrus after Ovuplant™ administration started to appear. (Vanderwall et al, 2001; Morehead et al. 2000) Confounding these reports were the realization that mares given prostaglandin F2α after induction of ovulation by the deslorelin implant appeared to have an even more significant delay to the next ovulation. (McCue et al, 2002) To study these questions, research ensued looking at the effect of deslorelin, and specifically Ovuplant™, on the hypothalamic-pituitary axis to

determine if the cause could be related to desensitization of the hypothalamus or down-regulation of the pituitary. Mares, though, appear rather insensitive to continuous GnRH signaling. (Porter et al, 1997; Porter and Sharp, 2002)

Other possible causes of a prolonged inter-estrous interval in a mare involve the gonad itself: retention of function of the corpus luteum (CL) on the ovary formed at the prior estrus, the development of sequential secondary CLs after a primary CL is established, severe damage to the endometrium causing an inability to produce and release prostaglandin F<sub>2</sub>alpha (PGF<sub>2</sub>α) hence inhibiting lysis of the CL, the loss of an embryo past the time of maternal recognition of pregnancy hence prolonging the function of the CL formed at the prior estrus, or a late diestrus ovulation leading to prolonged progesterone influence. (Ginther, 1990) The unique anatomy of the ovary of the mare has also led to difficulty in developing superovulation protocols and attaining good embryo recovery rates in mares having multiple ovulations on a single ovary. (Logan et al, 2007; Carmo et al, 2006; Riera et al, 2006)

### 2.11 The Equine GnRH Receptor:

The interaction of GnRH with its receptor is pivotal in the control of reproduction in all species. In veterinary medicine as well as in human medicine, GnRH is studied more and more for use in pro- and anti-fertility roles. Numerous agonists and antagonists have now been discovered to assist in these roles. With the development of these analogues, we now understand that the GnRH receptor is not a unique structure unto itself. (Schneider et al, 2006)

In 2002, the equine GnRH receptor was cloned and sequenced by M.B. Porter and colleagues. The cloned equine GnRH receptor consisted of a protein of 328 amino acids and showed high homology (>85%) with other mammalian GnRH receptor sequences. This protein

based receptor displayed the typical conservation of key amino acids believed important for membrane receptor binding, trans-membrane existence, G-protein association, and phosphorylation. As with most other mammalian receptors, it lacked the intracellular carboxy (C)-terminal tail. The equine receptor proved unique in its amino acid sequencing: Ser<sup>17</sup> and Ala<sup>26</sup> both reside within the N-terminus, His<sup>61</sup> and Asn<sup>69</sup> reside in the first intracellular loop, and Phe<sup>226</sup> resides in the fifth trans-membrane domain. (Porter et al, 2002)

### 2.12 The Equine GnRH Receptor Function:

The GnRH receptor functions as a G- protein-coupled receptor (GPCR). It is found primarily on the plasma membrane of pituitary gonadotroph cells. (Clayton R, 1989) GnRH released from the hypothalamus associates with G-proteins of the receptor and activates a phosphatidylinositol-calcium second messenger system allowing for release of FSH and LH from the pituitary. There are two types of GnRH and two types of GnRH receptors (GnRH-R Types 1 and 2). Type 1 GnRH-R is found in most mammalian vertebrate species whereas the Type 2 GnRH-R has not been identified in all mammals. Type 1 receptors have great affinity for GnRH I and are unique from other GPCRs in that they do not possess C-terminal tails. This is the most striking difference between the two types of receptors although there are other structural differences.

Continuous stimulation of GnRH receptors leads to refractoriness, which can result from down regulation of the receptor or desensitization of the receptor. Down regulation occurs when receptors for a hormone are internalized into the cell and then degraded hence disallowing a response. Desensitization is defined as a diminishing of a response, in this case the release of gonadotrophs from the pituitary, in the face of sustained or repetitive GCPR

stimulation. In the horse, gonadotropin secretion is much less easily suppressed by continuous GnRH signaling. (Porter et al, 1997; Porter and Sharp, 2002) Sustained stimulation of G-protein coupled receptors typically results in desensitization that is mediated by phosphorylation, commonly within this C terminal tail. (McArdle et al, 2002; McArdle et al, 1999) Since the Type 1 receptor (as in the horse) lack a C-terminal tail, they are unique in that do not undergo agonist induced phosphorylation and desensitization. Desensitization occurs by some other unique mechanism in these receptors, mostly likely distal to the receptor, but other possibilities are depletion of releasable gonadotropin pools, down-regulation of GnRH-R itself and inhibition of gonadotrophin synthesis. (McArdle et al., 2002; McArdle et al, 1999)

Although the GnRH-R is relatively resistant to desensitization due to its structure, sustained exposure to GnRH can cause desensitization of GnRH-stimulated gonadotropin secretion. This is an important consideration when using GnRH analogues clinically. The exact mechanism of this phenomenon is not known although it is known that, upon agonist stimulation, the GnRH-R undergoes internalization and recycling much like other GPCRs albeit by an apparently different mechanism and at a reduced rate. A contributing factor in the mechanism may lie in yet another atypical characteristic of this receptor. It appears to possess the ability to efficiently provoke a novel form of post-receptor desensitization that inhibits a signaling component within the pituitary cell membrane itself to inhibit functional release of gonadotrophin. (McArdle et al, 2002) This effect appears slow and modest hence may not easily explain long term desensitization.

Much of the study of GnRH agonist and antagonist activity has occurred in human medicine, the results finding application primarily in the areas of assisted reproduction and treatment of gonadal-related hormone disorders. A dose- and time- dependent down-regulation

of promoter activity occurs in the human GnRH-R after stimulation with an agonist compound indicating that activation of a signaling pathway (specifically the PKC pathway) by GnRH is important in controlling human GnRH-R gene expression and, more specifically, plays a role in receptor down-regulation. (Cheng et al, 2000) The number of receptors in the anterior pituitary correlates with the various stages of the reproductive cycle in rats (Clayton et al, 1980) and appear regulated primarily by estradiol and GnRH (a self-priming effect) . In the rat, GnRH receptors are minimal on the day of estrus, double in number on diestrus day two, and remain elevated on the day of proestrus, leading to the conclusion that estrogen may stimulate this increase in receptors for GnRH. Similar increases in receptor numbers occur in estrogen primed animals. (Clayton et al, 1980; Duval et al, 2000)

### 2.13 Current Knowledge In Breeding Mares

Reportedly the horse was the first domestic species to undergo successful artificial insemination but the benefits of artificial insemination and other assisted reproductive technologies seem to have lagged significantly in the species. (Allen, 2005; Pickett and Voss, 1999) Much of the lack of early progress related to breed registry constrictions. Over the last 10-15 years, techniques such as artificial insemination with shipped chilled semen and frozen thawed semen and embryo transfer have gained approval from breed registries worldwide and exploration into ways improve the techniques have literally exploded. The procedures have become economically feasible for horse breeders wanting to produce top progeny from their elite mares and stallions.

Success of artificial insemination requires the precise timing of insemination in relation to ovulation. Hence, ovulation is often pharmacologically induced to achieve this synchrony.

For optimal pregnancy rates, semen deposition must occur 12 hours prior to six hours after ovulation. (Woods et al, 1990; Sieme et al, 2003) Embryo transfer requires this same timing not only to achieve pregnancy in the donor mare but also to ensure synchrony of the donor and recipient mare. (Allen, 2005; Allen and Rowson, 1975) The first ovulation induction agent used was human chorionic gonadotropin (hCG) in 1939. A GnRH agonist, deslorelin, came into popular use in the late 1990's due to its more consistent effect of inducing ovulation.

Both ovulation induction agents have their shortcomings but both also have their advantages. hCG can often fail to induce ovulation in some mares and can invoke a hypersensitivity reaction but it is inexpensive. Use of deslorelin, especially in the form of a slow release implant, can produce a slow return of many mares to estrus (Vanderwall et al, 2001) and has a much higher price tag in comparison to hCG. Its ability to induce ovulation though proves much more consistent than hCG. (McKinnon et al, 1993; Ganheim et al, 1995; Samper et al, 2002)

One purpose of this project was to analyze the effect of inducing ovulation in a field setting on reproduction efficiency and determine the value of the procedure. The project looked at a large number of cycles induced to ovulate with either deslorelin (Ovuplant™) or hCG (Chorulon™) comparing them to estrus cycles where ovulation was not induced. The purpose was not to prove one product more effective than the other but rather determine the performance of each product in a clinical setting in hopes of producing useful information for field veterinarians and breeding managers when choosing between the two agents or no ovulation induction. No studies found in the literature were designed to convey this information although several studies completed retrospectively have examined different measures of management which can affect reproductive efficiency. (Morris and Allen, 2002; Allen and Brown, 2007;

Bosh et al, 2009; Nath et al, 2010) Bosh specifically looked at farm management factors which could influence reproductive performance but did not look at the use of ovulation induction in breeding management. Nath, et al, mentions that the use of ovulation induction “was encouraged” by the managing veterinarian of the farms but he did specifically gather data on their effect.

Deslorelin does affect the interovulatory interval of some treated mares and this has been well described. (Morehead et al, 2000; Vanderwall et al, 2001; Johnson et al, 2000a and b; Johnson et al, 2002; McCue et al, 2002) Although the effect is most likely related to a down-regulation of pituitary gonadotropin secretion leading to suppression of ovarian follicular development (Johnson et al, 2000a) and of FSH and LH (Johnson et al, 2000b), the exact mechanism has not come to full clarification in the literature. The mare is unique in her ovarian conformation and ovulation mechanism and research has not fully explored the option of a gonadal effect, specifically the function of the corpus luteum formed after the induction of ovulation by deslorelin (Ovuplant™).

CHAPTER 3  
MATERIALS AND METHODS

3. 1 Study 1: The effect of the ovulation induction agents hCG and deslorelin sustained release implants (Ovuplant™) on reproductive efficiency in mares

Objective: To analyze reproductive data collected from two central Illinois horse farms to determine if the use of ovulation induction agents improves reproductive efficiency in an uncontrolled, clinical setting.

Data were collected over three consecutive years, 1999-2001. A total of 1422 cycles were examined from 658 mares (some mares bred in consecutive years). Five cycles from Farm 2 were discarded from the analysis comparing hCG with deslorelin due to the confounding treatment of both hCG and deslorelin in the same cycle.

Farm 1 was located within 100 miles of Farm 2. Both were similar in management styles. Farm 1 bred a majority of Standardbred mares; Farm 2 bred a majority of American Quarter Horse type mares. Both farms had other breeds represented within the data set as a consequence of providing management of chilled semen inseminations but were of insignificant numbers. Mares on both farms had rectal palpation with ultrasound performed at least every other day and the majority of time palpations were performed on an every other day schedule as is typical for most large horse breeding farms. Inseminations for mares occurred as needed at least every other day. Mares bred with chilled semen had palpations and inseminations performed daily or as needed. A 37 cycles from embryo donor mares were included in the data. These mares would have been palpated daily after breeding to ensure the day of ovulation was



determined. The ovulation induction agents used at each farm were either hCG (Chorulon™, Intervet-Schering Plough Animal Health, USA) or deslorelin in the form of a short term release implant (Ovuplant™, Peptech Virbac Group, New South Wales, Australia).

Data collected from records on each farm included: stallion used for breeding, month and year in which the estrus cycle occurred, the number of the cycle in years one, two or three, the number of palpations per cycle, the number of artificial inseminations per cycle, treatment given (hCG, deslorelin, no treatment), follicle size in millimeters at treatment, days to ovulation after treatment, days to ovulation from the first day of estrus, the incidence of pregnancy, and the farm identification (Farm 1 or Farm 2). The first day of estrus was defined as the first day a follicle of at least 25mm in diameter was diagnosed via trans-rectal palpation or ultrasound along with obvious uterine edema and a softening cervix. (Samper, et al, 1997)

To determine the effect that ovulation induction agents have on reproductive efficiency and management schemes, the following hypotheses were retested:

- 1.) There is a significant difference in mare pregnancy rates associated with the use of ovulation induction agents.
- 2.) Mares treated with an ovulation induction agent have a significant reduction in the number of artificial inseminations administered per cycle.
- 3.) Mares treated with an ovulation induction agent have a significant reduction in the number of rectal palpations administered per cycle.
- 4.) Mares treated with an ovulation induction agent have are significantly more likely to ovulate within 48 hours of eligibility for treatment compared to mares that are not treated with an ovulation induction agent.

- 5.) There is a statistically significant association between follicle size at ovulation induction and the number of days from treatment to ovulation when ovulation is induced on the first breeding examination.
- 6.) There is a statistically significant association between follicle size at ovulation induction and the number of days from treatment to ovulation.

To determine differences between follicle sizes, recorded follicle sizes were stratified into 4 groups: <35 mm, 35-39 mm, 40-44 mm and >45 mm. The first breeding exam day was determined to be when a dominant follicle over 25 mm was first noted on at least one ovary with a uterine edema score of 1 or greater (score of 0-3 with 0 = no obvious edema and 3=prominent edema) and/or a cervical score of 1 or greater (score of 0-3 with 0 = tightly closed and 3 = fully relaxed). For each follicle size, the number of AIs and palpations were recorded for each of the three treatment groups. Days to ovulation was examined for each individual agent to determine the value of each agent at different follicle sizes and when administered on the day of the first breeding exam. Each agent's influence on the overall length of the cycle at varying follicle sizes was used to ascertain the most effective time to induce ovulation.

The data were analyzed using the IBM SPSS Statistics 20 software package. Chi Square and multivariable logistic regression using a forward stepping model building approach were used to determine the association between probability of pregnancy and treatment. The association between ovulation within two days of treatment eligibility and the treatment itself were determined using multivariable logistic regression. One-Way ANOVA and multivariable regression were used to determine the mean number of artificial inseminations and palpations associated with treatment. The Wilcoxon-Signed Ranks Test for pairwise comparisons was used

for post hoc analysis. Kruskal-Wallis One Way Analysis of Variance using mean ranks was used to compare treatment with the number of palpations, number of AI, and days to ovulation at the follicle size stratifications of <35 mm, 35-39 mm, 40-44 mm, and >45 mm. Post hoc analysis was performed between groups using Kruskal-Wallis All-Pairwise Comparisons Tests.

### 3.2 Study 2: Effect of deslorelin sustained release implants on the interovulatory period and response to PGF<sub>2</sub> $\alpha$ administration 6 days after ovulation

Objective: To determine if deslorelin in the form of a sustained release implant called Ovuplant™ will increase the interovulatory period of the treated mares and to determine whether the change in interovulatory period is associated with the function of the corpus luteum formed at the Ovuplant™-induced ovulation.

Ovuplant™ is a sustained release implant that contains the gonadotropin releasing hormone (GnRH) agonist deslorelin and which is given to mares subcutaneously during estrus to induce ovulation. Clinical evidence suggests that Ovuplant™ causes an increase in the interovulatory period of mares not conceiving on the treated cycle. (Vanderwall et al, 2001; Morehead et al. 2000) To test the hypothesis that treatment with Ovuplant™ induces corpus luteum persistence beyond the normal lifespan of 12-14 days (Ginther and Beg, 2011), a convenience sample of six regularly cycling teaching mares housed in the University of Illinois Veterinary Teaching Hospital was used. The group consisted of five Trakehner and one American Quarter Horse. A crossover design clinical trial was utilized to optimize use of the mares and control variability. (Figure 1, Tables & Figures) The study was conducted during the

months of April, May June, July, August, and September. Prior to beginning the study, the mares were examined for reproductive normalcy by transrectal palpation with a 5.0 MHz linear array probe and a Medison Sonovet 600 ultrasound. A normal cycle was defined as the presence of uterine edema, a follicle greater than 25 mm in diameter and subsequent ovulation. Once normal cycling was determined, the mares were randomly assigned to a control cycle with no treatment, a cycle with the diestrus phase shortened with PGF2 $\alpha$ , or an Ovuplant<sup>TM</sup> treated cycle. The final cycle for all mares was a cycle with ovulation induced with Ovuplant<sup>TM</sup> followed in six days by an IM injection of dinoprost tromethamine (Lutalyse<sup>TM</sup>).

Mares in estrus and assigned to a control cycle underwent daily trans-rectal palpation with ultrasound until ovulation occurred. An example is found in Figure 2. After ovulation, examinations were reduced to every 3 days allowing for determination of the interovulatory interval while decreasing the manipulation of the mares. The mares were monitored for physical signs of a return to estrus. Criteria for determining a return to estrus were: a uterine edema score greater than 0, a growing follicle over 25 mm, and a softening cervix. At the return of estrus, mares were palpated daily until a second spontaneous ovulation occurred. This second ovulation ended the control cycle. Counting the days between the two ovulations determined the baseline interovulatory interval for the mare. Blood for progesterone levels were taken every three days during the interovulatory period. The progesterone sampling determined the function of the naturally induced CL for each mare. Five to seven days after the second ovulation, an injection of prostaglandin F2 alpha, dinoprost tromethamine (Lutalyse<sup>TM</sup>), was given IM to cause luteolysis and return the mare to estrus. The beginning of the next heat cycle was determined by trans-rectal palpations every three days. Once the mare displayed signs of a return to estrus, she was assigned to an untreated estrus with the diestrus phase of the cycle shortened by the

administration of dinoprost tromethamine (Lutalyse™) six days after ovulation, the Ovuplant™ treatment group, or, if it was her final treatment cycle, to the Ovuplant™ followed by an injection of dinoprost tromethamine (Lutalyse™) treatment group.

Mares assigned to the untreated cycle with diestrus shortened by the injection of the prostaglandin, dinoprost tromethamine (Lutalyse™) cycled through estrus in a natural manner. Six days after ovulation was detected, an injection of prostaglandin was administered with the intent to shorten the diestrus phase and return the mare to estrus earlier than a natural cycle. Rectal palpations with ultrasound were performed daily during estrus until ovulation was detected and then every three days until physical signs of a return to estrus were noted. Exams continued daily until ovulation was detected. The interovulatory period determined for this cycle gave the baseline for comparison for the Ovuplant™/prostaglandin treatment cycle. No progesterone sampling was completed for this cycle.

Mares assigned to the Ovuplant™ treatment group were examined daily until a 35 mm follicle was noted in conjunction with uterine edema and cervical softening. At that time, Ovuplant was administered as per the manufacturer's directions. Daily monitoring continued until ovulation. During diestrus, examinations were performed every 3 days and blood samples were drawn for progesterone analysis at each of these exams. Upon noting signs of returning estrus, daily monitoring resumed until the day of the second ovulation of the treatment cycle was recorded. This determined the interovulatory period for the Ovuplant™ treatment cycle. Six days after the second ovulation of the treatment period, dinoprost tromethamine was administered to shorten the interestrous interval preparing the mare for another treatment cycle. Mares were then examined every three days until they returned to estrus and were designated to another treatment group- either a control cycle or, if it was her final treatment cycle, the

Ovuplant™ followed by dinoprost tromethamine (Lutalyse™). The purpose of the Ovuplant™ cycle was to determine the effect that Ovuplant™ alone had on the interovulatory period and functioning of the CL for each mare. An example of this treatment cycle is found in Figure 3.

The final cycle for all the mares was a cycle with ovulation induced with Ovuplant™ followed on the fifth day post-ovulation by an injection of dinoprost tromethamine to induce luteolysis. As previously, the monitoring of mares occurred daily through estrus. When a 35mm diameter follicle was first noted, an Ovuplant™ implant was administered to induce ovulation. Once ovulation was detected, rectal exams were reduced to every three days through the diestrals phase and blood samples were drawn for progesterone determination at each exam. Five days post ovulation (Day 1 equals the first day after ovulation was detected), an injection of dinoprost tromethamine was administered IM. Upon detection of physical signs of estrus, daily examinations resumed until the second ovulation of the treatment cycle was detected. The interovulatory period was examined for a lengthening secondary to Ovuplant™ administration. Blood progesterone levels assisted in determining if the prostaglandin injection had induced luteolysis. The second ovulation on this treatment cycle ended the study. An example of this treatment cycle is found in Figure 4.

Blood samples were drawn during the diestrals phase on all treatment cycles for progesterone testing to evaluate the function of the CL. Blood collection occurred by venipuncture of the jugular vein on the day of ovulation and every three days after until a return to estrus was detected. Blood was drawn into a 10ml red topped clot tube. Samples were allowed to clot for 30 minutes and centrifuged for 10 minutes to separate the serum. Serum samples collected were frozen in a refrigerator freezer at 0°F (-18°C) for preservation. The samples were submitted together for analysis one month after the study ended.

Progesterone concentrations were determined by a modified double-antibody ELISA procedure of Kesler, et al (Kesler D, et al., 1990) Modifications included use of anti-rabbit IgG linked to 96 well polystyrene microtiter plates vs. polystyrene tubes, use of progesterone antiserum diluted at 1:50,000, use of the substrate 3,3',5,5'-tetramethylbenzidine (TMB), and evaluation of plates by spectrophotometry at an absorbance of 630nm. Anti-rabbit IgG was linked to polystyrene microtiter plates via an enhanced linking procedure. Progesterone in serum samples was extracted with ethyl ether. Ether extracted hormone was reconstituted with 0.1% gelatin-phosphate buffered saline. For assaying, addition of three components (100µl each) to the plates occurred in the following order: 1.) standards or extracted samples, 2.) conjugate, 3.) primary antibody (rabbit anti-goat). Standard concentration of progesterone were 0.125, 0.75, 1.5, 3, 6 ng/ml. Conjugate was linked to horseradish peroxidase. Secondary antibody was goat anti-progesterone. Plates were incubated for 0.5 hour at room temperature and then the plates were washed with distilled water removing the unbound and free progesterone. The substrate 330 µl/well TMB was added to the plates and incubated for 30 minutes at room temperature. Evaluation of plates by spectrophotometry at an absorbance of 630nm occurred as the final step. High conjugate binding denoted by a dark blue coloration indicated low progesterone levels. Low conjugate binding denoted by a clear coloration indicated high levels of progesterone.

Trans-rectal ultrasound was used to score uterine edema (0-3 with 0 = no obvious edema), determine follicle size in millimeters (mm), and cervical score (0-3 with 0 = tightly closed). An experienced evaluator was used to determine these values. Progesterone levels were determined in ng/ml.

The descriptive and logistic regression components of the statistical analysis were completed using the SAS 9.2 statistical software package and other testing was completed using

the IBM SPSS Statistic20 software. Friedman's two-way analysis of variance and Kruskal-Wallis one-way nonparametric analysis of variance using mean ranks was used to compare treatment with interovulatory period, follicle size at treatment for the four treatment cycles, follicle size at ovulation for the four treatment cycles, and days to ovulation for the four treatment cycles. Kruskal-Wallis all-pairwise comparisons test compared significant results. Progesterone levels were analyzed by treatment group using Kruskal-Wallis nonparametric analysis of variance and Kruskal-Wallis all-pairwise comparison test.



## CHAPTER 4

### RESULTS

#### 4.1 Study 1: The effect of the ovulation induction agents hCG and deslorelin sustained release implants (Ovuplant™) on reproductive efficiency in mares

##### Comparison of reproduction management practices between study farms

Across both study farms, estrus cycles treated with hCG numbered 383, cycles treated with deslorelin numbered 451, and 583 cycles were untreated. See Table 2 for a detailed comparison of the study farms. There were statistically significant differences between the two farms with respect to treatments used and timing of administration of the treatments. Farm 1 treated 255 mares (48.39%) with hCG while Farm 2 treated 128 (14.38%). (Table 2) Farm 1 treated 4 mares (0.76%) with deslorelin and Farm 2 treated 447 mares (50.22%). Farm 1 left 268 cycles (50.85%) untreated while Farm 2 left 315 (35.39%) untreated.

The average days from the first reproductive exam to ovulation for all cycles were  $3.72 \pm 1.96$  for Farm 1,  $4.41 \pm 2.07$  for Farm 2 with an average for both farms of  $4.16 \pm 2.05$  days. Farm 1 treated mares 0.58 days after the first sign of estrus while Farm 2 treated 1.35 days with the average day of treatment from first day of estrus being 1.07 days for both farms. Treatment to ovulation (time from administration of an ovulation induction agent to confirmation of ovulation by a rectal ultrasound examination) for mares treated averaged  $3.09 \pm 1.86$  days with Farm 1 having an interval of  $3.14 \pm 1.72$  days and Farm 2 having an interval of  $3.06 \pm 1.95$  days. Average number of artificial insemination per mare per cycle on Farm 1 was 1.69 and Farm 2 was 1.08. Average number of palpations on Farm 1 was 3.59 and Farm 2 was 2.69. (Table 3)

The average diameter of a follicle treated with an ovulation induction agent (hCG or deslorelin) was 40.45 for hCG, 36.94 for deslorelin, and 40.17 for untreated. Average day of administration for hCG was 1.5 +/- 1.81 and 2.10 +/- 1.73 for deslorelin. Average number of inseminations for hCG 1.59 +/- 0.88, for deslorelin 1.21 +/- 1.06 and for untreated cycles 1.18 +/- 0.48. (Table 1) Average number of palpations was 2.94 +/- 1.15 for hCG, 3.58 +/- 1.35 for Ovuplant and 3.20 +/- 1.36 for untreated cycles. (Table 1) Average time of treatment with an ovulation induction agent (hCG or deslorelin) to ovulation was 2.49 +/- 1.32 for hCG, 2.11 +/- 0.74 for deslorelin, and 4.22 +/- 2.12 for untreated cycles. (Table 1)

There is a significant difference in mare pregnancy rates associated with the use of ovulation induction agents:

The multivariable logistic regression model indicated that mares treated with either deslorelin (Ovuplant™) or hCG (Chorulon™) were over twice as likely to become pregnant as those left untreated (O.R. =2.187, 95% CI 1.700, 2.814, p<0.001). The model found two variables of significance: treatment (p<0.001) and an interaction term for Month\*AI\*DaysO (p<0.01). (Table 4) There was no difference between the effect of the two treatments on pregnancy rate

Mares treated with an ovulation induction agent have a significant reduction in the number of artificial inseminations administered per cycle:

No significant difference was found between treatment and the number of artificial inseminations. (Table 5) Deslorelin treated mares were inseminated a mean of 1.20 times per

cycle, mares treated with hCG were inseminated 1.59 per cycle, and control mares a mean of 1.21 times. The median number of AI for all agents was 1.00.

To further assess the effect of ovulation induction on the number of AI, the effect of a treatment when given at a particular follicle size on the number of AIs was examined using Kruskal –Wallis one way nonparametric analysis of variance and Kruskal-Wallis all-pairwise comparison testing. An effect was found for the follicle size grouping 40-44 mm; ( $p=.000$ ). (Histogram 1) Deslorelin treatment alone decreased the number of AI at this follicle size (1.16 AI) in comparison to hCG (1.61 AI) and untreated mares (1.48 AI). Overall, less than two AI occurred on all mares for all treatment groups at all follicle sizes. (Table 1)

Administering an ovulation induction agent on the day of the first breeding exam significantly decreased the number of AI for treated mares vs. untreated mares at all follicle sizes except those 45mm or greater in diameter ( $p=.0005$ ). (Histogram 2) Pairwise comparison testing determined that with follicles <35mm, untreated mares were inseminated a greater number of times (2.47 AI) in comparison to deslorelin treated mares (1.36 AI) and hCG treated mare (1.17 AI) ( $p=.001$ ). The same occurred at 35-39 mm follicles (untreated=1.83, deslorelin treated 1.13, and hCG treated= 1.38) ( $p=.000$ ) and 40-44 mm follicles (untreated=1.62, deslorelin treated=1.14, and hCG treated=1.23) ( $p=.000$ ).

Mares treated with an ovulation induction agent have a significant reduction in the number of rectal palpations administered per cycle:

Across all follicle sizes, ovulation induction did not decrease the number of rectal palpations in this study. ANOVA showed significance ( $P=.000$ ) but post hoc comparisons using Wilcoxon-Signed Ranks showed deslorelin treated mares had a significantly increased number of

palpations performed in comparison to untreated mares ( $p=.000$ ) and hCG treated mares ( $p=.000$ ). The mean number of palpations for the control group was 3.2, the deslorelin treated group was 3.6 and the hCG treated group was 2.9. The median number of palpations for treated and untreated mares was 3.00. Data is presented in the appendix A. However, a decrease in the number of palpations was found for mares treated when possessing a follicle less than 35 mm in diameter ( $p=.009$ ) (Histogram 3); pairwise comparison testing showed deslorelin treated mares (3.51 palpations) and hCG treated mares (3.19 palpations) were palpated less than untreated mares (3.92 palpations)

Mares induced to ovulate on their first examination were palpated one to two times less than mares not treated at the follicle size groupings of <35 mm ( $p=.009$ ) and 35-39 mm ( $p=.000$ ) (Histogram 4). The greatest effect was found for the smaller follicle grouping of <35mm (deslorelin 2.55 palpations vs. hCG 2.17 palpations vs. untreated 4.24 palpations) when compared to the 35-39mm follicle grouping (deslorelin 2.33 vs. hCG 2.39 palpations vs. untreated 2.82 palpations) ( $p=.000$ ).

Mares treated with an ovulation induction agent have are significantly more likely to ovulate within 48 hours of eligibility for treatment compared to mares that are not treated with an ovulation induction agent:

No difference was found by multiple logistic regression (Table 6i) between the ability of hCG and deslorelin to induce ovulation in  $\leq 48$  hours ( $p=.073$ ). Neither agent had an average time to ovulation of less than forty-eight hours. The mean time to ovulation recorded for hCG was 2.55 days and for deslorelin, 2.10 days. (Table 6) The median time to ovulation was 2.0 days for both agents. Most mares treated ovulated within 2 days of treatment (77%). Mares

which ovulated within 2 days of eligibility for treatment were 13.7 times more likely to have been treated with hCG or deslorelin than not treated (O.R.=13.73; 95% CI:7.40, 25.85; p,0.001). (Table 6ii)

There is a statistically significant association between follicle size at ovulation induction and the number of days from treatment to ovulation when ovulation induction occurs on the first breeding examination:

Human chorionic gonadotropin (hCG) decreased the days from treatment or eligibility for treatment to ovulation over untreated mares at follicles sized between 35-39 mm (p=.000) and 40-44 mm (p=.000). (Histogram 5) Administration on the first breeding exam showed no significant effect of decreasing the days to ovulation over administering it after multiple exams. Ovulation was consistently reported in less than three days. For follicle sizes 35-39 mm (p=.000): hCG on Day 0 was 2.72 days and on Day $\geq$ 1 was 2.63days while untreated mares ovulated in 3.87 days. For follicle sizes 40-44 mm(p=.000): hCG on Day 0 was 2.62 days to ovulation and on Day $\geq$ 1 was 2.34 days while untreated mares ovulated in 3.49 days.

The ovulation response for deslorelin by day of administration for different follicle sizes also showed a decrease in the days from treatment or eligibility for treatment to ovulation for follicles between 35-39 mm (p=.000) and 40-44 mm (p=.000). (Histogram 6) This relationship existed whether administration was on the first exam (2.29 days or 2.67 days) or a subsequent exam (1.96 days, 1.97days). Days from first eligibility to ovulation (first breeding date) for non-treated mares were 3.87 days for follicles 35-40 mm (p=.000) and 3.49 days for follicles 40-44 mm (p=.000). A significant association was also noted for deslorelin at the smallest follicle sizes

(<35 mm follicles) when the drug was given subsequent to the time of the first exam (2.19 days; untreated mares 3.54 days) (p=.002).

There is a statistically significant association between follicle size at ovulation induction and the number of days from treatment to ovulation.

Mares not induced to ovulate were compared to those induced to ovulate with either hCG or deslorelin. Mares possessing a dominant follicle on at least one ovary with a uterine edema score of 1 or greater and a cervical score of 1 or greater were considered eligible for ovulation induction. Comparison between the three groups was based on follicle size at administration of each drug or first eligibility to be induced to ovulate and the number of days to ovulation.

(Histogram 7) Overall, mean days from treatment or eligibility for treatment to ovulation were decreased for mares treated with either hCG or deslorelin in comparison to mares left untreated at all follicle sizes except the follicles >45 mm. At the follicle size groupings of <35 mm (p=.001) and 40-44 mm (p=.000), hCG and deslorelin decreased the days to ovulation by approximately one day in comparison to no treatment. For follicle sizes 35-39 mm (p=.000), deslorelin (2.02 days) decreased the days to ovulation compared to both hCG (2.68 days) and no treatment (3.87days).

#### 4.2 Study 2: Effect of deslorelin sustained release implants on the interovulatory period and response to PGF<sub>2</sub>α administration 6 days after ovulation

1. Hypothesis: The interovulatory period of mare reproductive cycles treated with Ovuplant™ will be significantly different from those not treated with Ovuplant™.

2. Hypothesis: The interovulatory period of mare reproductive cycles treated with Ovuplant™ will be significantly different from those not treated with Ovuplant™ when luteolysis is induced at 6 days post ovulation.
3. Hypothesis: The serum progesterone levels of mares whose reproductive cycles were treated with Ovuplant™ will be significantly different from those not treated with Ovuplant™ when PGF2 $\alpha$  is administered to induce luteolysis.

The treatment titles, sequence of treatments for each mare, and the collected data are found in Table 7. The longest interovulatory period noted was for Mare 5 at 42 days when treated with Ovuplant™. The shortest period was found for Mare 2 and Mare 6 during their natural cycle followed by prostaglandin (8 days). Ovuplant™ treated mares prove to have an increased interovulatory period with a mean number of days between ovulations of 26. The mean days between ovulations for untreated mares (control cycle) was 21.67 days ( $p=.0062$ ). (Table 8)

No effect was found for follicle size, follicle size at ovulation, or days to ovulation. Kruskal-Wallis one-way nonparametric analysis of variance comparing treatment ranks found a difference between the treatment groups. The interovulatory period for treatment 4 (natural cycle w/PGF2 $\alpha$ ) differed from that of both treatment 1 (control cycle) and treatment 2 (Ovuplant™ cycle) ( $p=.0112$ ). The interovulatory period for Treatment 3 (Ovuplant™ followed by PGF2 $\alpha$ ) did not differ from the other 3 treatment groups ( $p=.0112$ )

Table 9 presents the differences in length of interovulatory period for each treatment. The difference in days between the Ovuplant™ cycle and the control cycle is lengthened except for Mare 3 and Mare 8 where the interovulatory period was actually decreased in length compared to the expected average of 21 days. The interovulatory periods for the Ovuplant™

followed by prostaglandin treatment cycle and the control followed by prostaglandin cycle was also increased for all mares except Mare 3 for which there was no difference.

Mean progesterone levels for each group are found in Table 10. Three sample values were missing for the Ovuplant<sup>TM</sup>/PGF group and were discarded from analysis. Mean progesterone levels were highest for the Ovuplant<sup>TM</sup> group (8.4ng/ml) in comparison to the control group (4.42ng/ml) and the Ovuplant<sup>TM</sup>/PGF group (3.06 ng/ml). Kruskal- Wallis one-way nonparametric analysis of variance was used to compare progesterone levels by treatment group. The progesterone levels for the Ovuplant<sup>TM</sup> treatment group differed from the Ovuplant<sup>TM</sup>/PGF group ( $p=.0223$ ). The intermediate progesterone levels of the control group were not found to differ from the Ovuplant<sup>TM</sup> and Ovuplant<sup>TM</sup>/PGF group.



## CHAPTER 5

### DISCUSSION

Changing economic times have altered the horse industry and challenged horse producers to breed fewer foals but foals of higher quality and value. Many horse breeders have hence moved to assisted reproductive techniques and transported semen to assist in attaining their goals. Control of the estrous cycle through management of ovulation has played an important role in the development of assisted reproductive techniques (Squires, et al, 1998), has allowed the success of transported semen in the horse breeding industry (Voss et al, 1975, Barbacini et al, 2000). It has also opened the door for successfully managing subfertile breeding animals. (Solomon, 1991; Blanchard et al, 2003) An essential part of reproductive efficiency is the timely induction of ovulation with agents such as hCG (Sieme et al, 2003) and deslorelin in coordination with deposition of good quality, fertile semen.

To achieve optimal pregnancy rates, deposition of semen must occur 12 hours prior or six hours after ovulation. (Woods et al, 1990; Sieme et al, 2003) Two strategies can be employed to accomplish this time frame: multiple frequent palpations per rectum until ovulation appears imminent (or has recently occurred) or induce ovulation with a reliable induction agent near the time of semen deposition. For maximal reproductive efficiency, the goal for breeding a mare is one insemination per estrus cycle with a pregnancy resulting. (Sieme et al, 2003) With economic constraints an important issue for many breeders, accurate timing of inseminations to avoid multiple inseminations, multiple veterinarian exams, multiple shipments of semen, and the overuse of aged or physically compromised stallions is important. (Blanchard et al, 2003)

The retrospective study was conducted to investigate whether ovulation induction improved reproductive efficiency in mares and how an ovulation induction agent might be used to maximize this efficiency. This study examined data that was collected on a large number of estrous cycles, (n=1422), from two similar farms spanning period of three years. The most striking and interesting conclusion found in this study was that a mare induced to ovulate with either deslorelin or hCG was over twice as likely to become pregnant as a mare left untreated (Table 4). The individual ovulation induction agents themselves, deslorelin and hCG, did not show a difference in their effectiveness on pregnancy rate in comparison to each other. This lack of difference between the two agents concurs with other studies comparing the two agents (Blanchard et al, 2002, Vanderwall et al, 2001). One limitation of the study findings may be attributed to differences in the management practices at the two farms. Although the two farms were similar, there were several factors which differed between the farms which could have affected results. Farm management teams, including veterinarians providing care, were different and farm philosophy on managing mares may have varied. Farm 1 and Farm 2 did treat a similar number of mares with ovulation induction agents hence there appears a similar management strategy to increase efficiency by decreasing the numbers of inseminations and palpations required to achieve pregnancy. (Table 2) However, Farm 2 treated more mares with deslorelin and Farm 1 treated more mares with hCG. This most likely represents a difference in economic viewpoints between each farm's veterinarians, managers, and/or the owners of the mares. (Squires, 2008)

The major breeds on each farm differed but a diversity of breeds is actually represented in the study. A previous comparison of the reproductive efficiency of two breeds, Thoroughbreds and Standardbreds, on several farms in the north east Victoria area of Australia

determined the disparity between reproductive parameters was more related to management decisions and not the breeds themselves (Nath et al, 2010). The farms were located within 125 miles of each other, potentially limiting the impact of environmental effects on reproduction.

Farm 2 tended to induce ovulation later in the heat cycle, (Table 3) which may have indicated a management decision to improve conception rates by ensuring that breeding occurred as close to ovulation as possible. This could be for several reasons: stallion management (subfertility, high demand for semen, physical issue), availability of semen, timing of the ovulatory event as close to insemination as possible (secondary to poor sperm longevity secondary to chilling, freezing, or just poor quality) or management of mares with uterine pathology (acute and chronic endometritis). (Crowe et al, 2008; Sieme et al, 2003) Per record analysis, Farm 2 managed more chilled semen inseminations, embryo donor mares, and treated more mares post breeding than did Farm 1.

Both farms minimized the number of palpations and artificial inseminations even on mares which were not treated. (Table 1 and Table 3) Farm 2 averaged slightly over one insemination per mare. Both farms managed all mares in a manner in which, on average, less than four palpations were required per mare per cycle and the median number of rectal palpations was three. Farm 2 averaged less than 3 palpations per mare. Optimal observation of a mare's reproductive tract for breeding would require a minimum of two palpations-one prior to breeding to determine the optimal time of insemination and one to confirm ovulation and uterine health after breeding. The management on both farms appeared to be of high quality and intensive. Farm 2 may have managed slightly more intensively than Farm 1 as indicated by the higher percentage of mares induced to ovulate (Farm 2= 64.6% vs. Farm 1= 49.1%, Table 2).

Previous studies evaluating reproductive efficiency that noted the use of ovulation induction agents reported a use rate of approximately 51%. (Morris et al, 2002)

For this study, inducing ovulation appeared to have a strong positive effect on pregnancy outcome. This is contrary to the results of other similar large retrospective studies evaluating reproductive efficiency, which found that ovulation induction had neither a significantly positive nor negative effect on pregnancy outcome (Allen et al, 2004; Morris et al, 2002; Vanderwall, et al, 2001) In these studies and other similar studies, other factors such as mare age (Morris et al, 2002; Allen et al, 2007; Bosh et al, 2009) and mare status (maiden, barren, foaling, aborted or rested) had the most significant effect on pregnancy and foaling rate (Morris et al, 2002). Mare age and reproductive status were not specifically evaluated in this study. Stepwise regression analysis using a forward approach was used to determine this outcome and the model evaluated treatment, mare, month of insemination, number of AI, size of follicle at treatment, day of ovulation, and farm plus all possible interactions. Only treatment ( $p=.000$ ) and an interaction term of month, days to ovulation, and number of AI ( $p=.004$ ) appeared significant and there was no significant effect of the individual mare (Table 4).

Both randomized clinical trials and retrospective studies have been utilized to examine the effect of deslorelin in comparison to placebo treated mares or non-treated mares on pregnancy outcome (Meyers et al, 1997; Ganheim et al, 1995; Morehead et al, 1999). No study has shown a positive effect. Studies have also failed to establish an effect of hCG (Voss et al, 1975) except for one large retrospective study out of New Zealand in 2001. This study determined that administering hCG 24 hours prior to insemination tended to improve the odds of pregnancy at 14 days by approximately 21%. (Perkins et al, 2001) Although the p value from multiple logistic regression for this association failed to show significance ( $p=.06$ ), the authors

reasoned their finding as biologically meaningful by demonstrating a large sample size (2119 ovulatory cycles) with an odds ratio of 1.21 which had a 95% confidence interval of 0.99 to 1.48 which was primarily in the positive direction of an effect of hCG. They also noted that the p value was only marginally greater than the accepted value of  $p=.05$ .

A major advantage to induction of ovulation by deslorelin or hCG should be the reduction in the artificial inseminations per cycle and per pregnancy. (Meyers et al, 1997; Squires, 2008, Jochle et al, 1994) This study did not determine a decrease in artificial inseminations (AIs) for either the inclusive group of mares or the subset which became pregnant based on inducing mares to ovulate alone. (Table 5 and Appendix B) Overall, the mean number of inseminations per mare was low for all groups (Ovuplant =  $1.19\pm 0.52$ ; hCG =  $1.59\pm 0.878$ ; untreated =  $1.21\pm 1.06$ ) (Table 5). The median number of artificial inseminations per cycle was one. This minimal insemination rate may have contributed to this result.

Evaluation of the effectiveness of each ovulation agent at different follicle sizes to reduce the number of inseminations per cycle was used to determine if there was an optimal follicle size when induction of ovulation proved most beneficial to reducing the number of inseminations. (Histogram 1) Both drugs were compared to no treatment at 4 follicle size groupings- $<35$ mm, 35-39 mm, 40-44 mm, and  $\geq 45$  mm. Induction with deslorelin decreased the number of inseminations for follicles between 40 and 44 mm in diameter in comparison to both hCG and no treatment. Deslorelin appeared more consistent in its ability to decrease the number of AIs over hCG for all follicle sizes except those less than 35mm in diameter. No other follicle sizes demonstrated a significant effect of treatment over no treatment. Again, the overall number of inseminations on both farms were minimal for both treated and untreated mares (Sieme H, et al, 2003) and comparable to other retrospective studies on intensively managed farms (Morris et al,

2002). Hence, it appears there was no follicle size at which induction of ovulation was more beneficial than any other for decreasing inseminations and improving semen usage but deslorelin did appear to perform better than hCG at reducing artificial inseminations. Noting that performance of both ovulation induction agents was poor at follicles less than 35mm in diameter could have related to induction of ovulation before minimum qualifications had been met (a 30-35mm follicle with uterine edema and a softening cervix). Hence, ovulation failed requiring more inseminations be performed. (Barbacini S, et al, 2000; McKinnon et al, 1993)

Analysis of the timing of administration of the induction agent showed that inducing ovulation at the first breeding exam when follicles were less than 45 mm in diameter proved most beneficial in decreasing the number of artificial inseminations. (Histogram 2) HCG and deslorelin proved equally effective at this time point for reducing the number of inseminations for all follicle sizes except those greater than or equal to 45 mm. There was no sparing effect at this follicle size in comparison to no treatment as expected since these follicles are destined for natural ovulation. (Ginther O, 1992; McKinnon A and Voss J, 1993) The most notable outcome was the obvious difference between the number of inseminations performed on untreated mares possessing a follicle size <35 mm (2.47 AI) and treated mares. Deslorelin treated mares were inseminated a mean number of 1.36 times and hCG treated mares were inseminated a mean number of only 1.17 times at this follicle size when given on the first breeding exam. There was no apparent difference in this effect between hCG or deslorelin. As follicles became larger, hence moving closer to natural ovulation, no difference between outcomes was significant. One may conclude from the analysis that treating mares on the first breeding exam, even when follicles are quite small but meet the minimum requirements for ovulation induction, may prove most beneficial to decreasing the number of inseminations.

Decreasing the number of artificial inseminations would be advantageous in numerous regards especially if inseminations could be reduced to one per cycle with pregnancy resulting. Decreased inseminations prove labor saving for the farm personnel and aid in stallion management. Financial advantages may be gained by clients using shipped chilled semen if only one shipment is required for pregnancy in a valuable mare. (Sieme et al., 2003) Mares susceptible to post mating endometritis could benefit from the reduced amount of semen placed within the uterus. (Pycocock, 2006) In addition, a financial reward may be obtained since an improved uterine environment in these mares may lead to less need for post mating uterine treatments and an increased likelihood of pregnancy after one mating.

Reducing the number of palpations per cycle would have similar labor and financial advantages. In this study we did not note a significant decrease in the overall number of palpations performed on the mares after inducing ovulation. (Appendix A) There are numerous factors which could affect the number of palpations required per cycle including season of the year, status of the mare as an embryo donor or recipient, breeding with semen fresh on farm or shipped, and the reproductive health of the mare. No studies were found that looked at the effect of ovulation induction on the number of palpations performed.

An effect of ovulation induction on the number of palpations was found when consideration was given to both the follicle size at administration and if administration occurred on the first breeding examination. When either of the ovulation induction agents were administered to a mare possessing a small follicle, defined as <35 mm in diameter, the number of palpations were significantly decreased in comparison to untreated mares. (Histogram 4) The total number of palpations was also decreased by one to two palpations per cycle if a mare, ready for breeding on her first rectal ultrasound exam and possessing a follicle less than 40mm in

diameter, was induced to ovulate with either agent. This indicates that induction agents should be given early in the heat cycle, as soon as a follicle appears of appropriate maturity for ovulation induction, rather than waiting, if a goal is to palpate the mare less than three times. A significant savings in time and money could be obtained by following this practice.

A final objective of this study was to determine the effectiveness of hCG and deslorelin in this clinical setting to induce ovulation within 48 hours of administration. (Table 8) This was a difficult association to make since the mares in this study were typically only palpated on an every other day basis and the time of drug administration was not recorded by the farm personnel. No significant association was found using multivariable regression analysis but the median days to ovulation were determined to be two. Examining mares more often throughout a day or daily would have given a more timely determination of ovulation but this is not practical in a field situation. Meyers, evaluating the ability deslorelin to induce ovulation, found an overall time to ovulation of 54.1 hours when examining mares on a daily basis after induction. No record of the exact time of induction appeared recorded in this study and the mares did not appear to be check at the same time daily. Overall, the study determined that 80.9% had ovulated within 48 hours. (Meyers et al., 1997) Approximately 77% of the mares treated in this study had ovulated by a second breeding exam occurring two days later. Approximately 80% of deslorelin treated mares had ovulated by a second breeding exam occurring two days later and approximately 74% of hCG treated mares had. Another retrospective study determined the ovulation rate within two days of administration to be higher for both deslorelin implants (92%) and hCG (83%). (Berezowski et al., 2004). These mares were also examined daily after induction.



The number of days to ovulation was notably decreased by ovulation induction for all follicles sizes except those follicles which were destined for natural ovulation (greater than 45 mm). (Histogram 7) To compare treated and untreated mares at a similar point in their cycle, mares not treated (controls) were considered eligible for ovulation induction when they were bred for the first time and possessed a dominant follicle with uterine edema. Mares not induced to ovulate consistently took over 3.5 days to ovulate except when possessing a follicle over 45 mm in diameter. Treated mares consistently ovulated in less than 3 days. As expected, small follicles less than 35mm did not respond as quickly to ovulation induction with hCG (Samper et al, 2002) but the days to ovulation still appeared slightly decreased in comparison to non-treated mares. Deslorelin appeared superior to hCG when used with follicles sized between 35-39 mm. Both agents performed similarly on follicles between 40 and 44 mm. This again appears to reaffirm the fact that hCG may need more mature follicles to consistently induce ovulation in the most timely fashion.

Analysis of both induction agents and their ability to shorten the time to ovulation when administered at different follicle sizes on the first breeding day was conducted to determine if there was a “best” time for an ovulation induction agent to be administered within the estrus cycle. Administering the agent hCG when a follicle was between 35 and 44 mm in diameter decreased the time to ovulation by approximately one day over non-treated mares. (Histogram 5) Time of administration had no effect. There appears some benefit to waiting to give hCG until a follicle has matured appropriately as has been previously suggested (Samper et al, 2002). Most likely, a mature follicle is required for consistent ovulation induction by hCG.

Deslorelin appeared to perform better than hCG at smaller follicle sizes. (Histogram 6)

Deslorelin given to follicles less than 45 mm in diameter decreased the days to ovulation in

comparison to untreated mares and by almost a day and a half for follicles measuring 35-39 mm. This result was found whether given on the first day a mare was examined for breeding or on a subsequent exam.

For the smallest follicles (< 35 mm in diameter) there was no difference between no treatment and ovulation induction on the first examination even with deslorelin administration. These small follicles most likely require more hormonal priming and a final maturation period before ovulation can occur (Ginther et al., 2003; Ginther, 2000; McKinnon A and Voss J, 1993). Very small dominant follicles cannot be compelled to ovulate in a shorter period than forty eight hours if they are not appropriately mature. The benefit of a decreased time to ovulation cannot be expected from inducing ovulation on small dominant follicles less than 35mm in diameter. (Glazar et al., 2004; Farquhar et al., 2000; Meinert et al., 1993) Placement of the sperm within the reproductive tract needs considered when inducing ovulation at small follicle sizes if synchronization of ovulation and insemination are critical and the amount of sperm is limited.

Data for this study was collected retrospectively. Such studies utilize data previously collected for another reason and hence the data may prove incomplete or inaccurate. Incomplete or inaccurate data can confound the statistical analysis leading to inaccurate conclusions. (Mantel and Haenszel, 1959; Hess, 2004) Confounders may bias data and hinder the ability to make accurate associations during analysis. Retrospective analysis of data however can serve as a valuable and inexpensive way of analyzing multiple outcomes from existing records containing desired information. A retrospective study cannot yield solid conclusions but can give us leads to follow from which more controlled, prospective studies can be developed. (Mantel and Haenszel, 1959)

The goal of performing this retrospective study was to analyze the use of the ovulation induction agents by veterinarians and breeding managers in a routine clinical setting to see if they improve reproductive efficiency, performed as proven in controlled settings, and to optimistically demonstrate best use practices. The desired outcome was to determine the worth of using ovulation induction agents for breeding mares and how to capitalize on their use when managing a breeding. The results of this study are quite intriguing and have allowed us to make some interesting presumptions as to the worth of ovulation induction in mare breeding. The study has also allowed us to deduce protocols to best utilize the drugs when managing a breeding. Proving these protocols conclusively in randomized controlled trials may prove of significant value to horse breeders.

From this study we can presume the relative worth of ovulation induction agents as high. Ovulation induction appeared to allow for an increased pregnancy rate, a decreased number of artificial inseminations per conception (especially when using deslorelin and administering the agent on the first breeding exam day), and a shortening of the estrus period of mares. A decreased in the number of palpations also appears possible when the agents are used earlier in the estrus period on small follicles, less than 35 mm, in diameter provided the follicles are mature enough to respond appropriately to induction. An increased pregnancy rate would hopefully improve foaling rate hence increasing the number of foals produced and available for sale or performance. Decreasing inseminations per pregnancy would decrease cost of semen and should decrease time and labor costs of gathering semen and inseminating. The same should be seen with a decrease in the number of palpations per mare and per pregnancy. Both agents appear to perform equally well for ovulation induction although deslorelin may perform the most consistently over all follicle sizes and throughout the estrus period. If cost is secondary and

reliability a main concern, deslorelin may prove the best choice for ovulation induction. If finances are considered when breeding a mare and reliability is secondary, hCG may be concluded as the best choice over deslorelin. Ovulation induction does appear economically valuable when breeding mares. A controlled study, specifically evaluating this hypothesis would be both relevant and desirable for the equine breeding industry.

The clinical trial portion of this study was the first documented research on the effect of deslorelin on the equine ovary, specifically the CL formed at ovulation. It was conducted to evaluate the effect of the deslorelin sustained release formulation called Ovuplant™ on the interovulatory interval of mares administered the drug for ovulation induction and to examine progesterone production of the corpus luteum formed. The study was small with only six mares used but a crossover type design allowed a total of 24 cycles to be examined. Each mare was examined through four cycles (an untreated, natural control cycle, a control cycle with prostaglandin given to shorten the diestrus period, a cycle with ovulation induced with Ovuplant™, and a cycle with ovulation induced with Ovuplant™ and the diestrus phase shortened with prostaglandin). A cross over clinical trial design was utilized so each mare would serve as her own control hence making comparison of interovulatory periods less subject to natural and individual mare variability. (Adams et al, 1988; Witherspoon et al, 1971; Ginther et al, 1972; Stabenfelt et al, 1975) Mares were randomly assigned to different treatment cycles with the exception of the last cycle which for every mare was the Ovuplant™ cycle followed by prostaglandin in six days post-ovulation. Reportedly, this protocol may cause an even further delay of return to estrus for some mares. (McCue et al, 2002)

A difference between the interovulatory periods of the four treatment groups was found ( $p=.0062$ ). Treatment with Ovuplant™ alone displayed the highest mean interovulatory period

of 26 days followed by the control cycle (21.67 days), the Ovuplant™ w/PGF2α cycle (17.33 days), and the control w/PGF2α cycle (11.83 days). This result for the Ovuplant™ treatment was expected and agrees with current literature. (Vanderwall et al, 2001; Morehead et al. 2000) The control cycle followed by PGF2α displayed the shortest interovulatory period as expected. The Ovuplant™ cycle with the diestrus phase shortened by PGF2α did not show a difference in the length of the interovulatory period from the three other treatments. Determining no difference between the control cycle with the shortened diestrus and the Ovuplant™ cycle with a shortened diestrus indicates that the prostaglandin likely lyse the corpus luteum formed at the Ovuplant™ induced ovulation and the mare returned to estrus prematurely. Examination of progesterone levels confirmed that the progesterone did indeed drop after prostaglandin administration on the Ovuplant™/PGF cycle. (Table 10) and the corpus luteum did respond as expected to prostaglandin administration.

Examining the lengths of the interovulatory periods between the control with prostaglandin administration and the Ovuplant™ with prostaglandin administration (Table 10), a comparable shortening of the interovulatory period is not noted as expected. The interovulatory period of the mares treated with Ovuplant™ and given prostaglandin appears longer than the mares undergoing an untreated estrus followed by prostaglandin. Only Mare 3 showed equality in the length of the interovulatory period between the two treatments. This supports the suspicion that there is an effect from the Ovuplant™ causing a prolongation of the return to estrus which is unrelated to the function of the corpus luteum. Our study did not support the findings that the combination of the two drugs, Ovuplant™ and PGF2α, causes a further delay in return to estrus above and beyond that demonstrated by Ovuplant™ alone. (McCue et al, 2002)

No differences for the study were found comparing treatments to the days to ovulation, follicle size at treatment, uterine edema score or cervical score at treatment. When examining the length of control cycles of the six mares, it is obvious that there was individual variation amongst the mares. (Table 7 and 8) Only Mare 2, 3, and 6 displayed an expected, normal length for the estrous cycle of 21-22 days. (Hughes et al, 1997) This could be due to several factors including individual mare variation in estrous cycling (Hughes et al, 1997, Stabenfeldt et al, 1975; Ginther et al, 1972), an inflammation of the endometrium causing abnormal release of prostaglandin (Ginther, 1990), or an effect of environment, both light and temperature, since the mares were primarily housed within the teaching hospital (Adams, 1988; Ginther et al, 1974; Hughes et al, 1977). Employing a crossover type study designed should have controlled for this variation but using more mares for comparison may have been beneficial. Similar variation is noted for individual mares treated with Ovuplant. Mares 1, 4, 5 and 6 displayed prolonged interovulatory periods in comparison to their control cycles. Mare 5 displayed a 25 day difference in her cycles while Mare 1, 4 and 6 displayed the more typically reported 3-6 day lengthening. (Johnson et al, 2002; Mumford et al, 1995) There appears a pool of mares which are especially sensitive to the effects of deslorelin and Mare 5 may be one of these mares. (Johnson et al, 2003)

Progesterone levels were examined every three days until a dominant follicle reappeared on the ovary to determine if corpus luteum function differed between untreated cycles, cycles with ovulation induced by Ovuplant™, and cycles with ovulation induced with Ovuplant™ and unnaturally shortened with prostaglandin. (Table 10) Progesterone levels after ovulation induction with Ovuplant™ and prostaglandin administration six days after ovulation dropped significantly in comparison to those found with ovulation induction with Ovuplant™ alone

( $p=0.0223$ ). This agrees with other studies which have examined progesterone levels in association with deslorelin sustained implant use. (Johnson et al, 2000b; Mumford et al, 1995)

Progesterone values for the control group were intermediate to the Ovuplant™ treated group and the Ovuplant™/PGF2 $\alpha$  groups. It is expected that the progesterone values for the Ovuplant™ /PGF group would be lower than control and the Ovuplant™ group but it is interesting that the values tended higher in the Ovuplant™ group than the controls. No significant difference was found in the mean values. (Table 10) A previous study found that progesterone production by the corpus luteum formed by Ovuplant™ was lower than untreated mares (Johnson et al, 2000b) but others have noted a similar tendency toward a higher progesterone value (Farquhar et al, 2002) Johnson in a different study found significantly higher values when administering one implant daily to mares for three consecutive days in comparison to mares treated with one implant. (Johnson et al, 2003) This alludes to the possibility that Ovuplant™ induces the formation of a corpus luteum which is higher functioning in its production of progesterone. This theory has been proposed and explored in cattle (Ambrose et al, 1998) but has not been proven consistently in all cows (Santos et al, 2004). Studies in canines has shown no difference in mean progesterone levels for dogs treated with one implant until the second half of gestation (or diestrus), During the second half of gestation values drop in the canine because of the need for LH support of the corpus luteum. (Volkman et al, 2006) No one appears to have explored the theory of an increased production of progesterone by the CL formed by Ovuplant™.

Two inherent problems arise when using a crossover study design and both are a concern with this study. First is the issue of the effect of the order in which treatments are given. Mares in this study were assigned randomly to treatments to dissuade bias in evaluation. It has been

shown conclusively that deslorelin in the form of Ovuplant™ does have a down regulating effect on the hypothalamic-pituitary axis (Johnson et al, 2000a and b) unless promptly removed after ovulation (McCue et al, 2002). The length of the down regulating effect on the production of FSH and LH appears different for individual mares. Undergoing either Ovuplant™ treatment prior to the control cycles could have removed a mare from the study had that mare been especially sensitive to the down regulating effect of the implant and ceased to cycling.

The second problem with the cross over study design which could have affected our outcome is the effect of “carry over” (i.e. the possibility of carryover of the down regulating effect of Ovuplant™ from one cycle to the next). No carryover effect was noted in the study. Johnson in 2002 demonstrated that the down regulation of gonadotroph production appeared diminished by the first signs of a return to estrus. (Johnson et al, 2002). On the first heat post treatment, mares treated with Ovuplant™ do display a smaller follicle size than untreated mares (McCue et al, 2002; Johnson et al, 2002). This could have confounded the determination of a return to heat.



CHAPTER 6  
CONCLUSIONS

6.1 Study 1: The effect of the ovulation induction agents hCG and deslorelin sustained release implants (Ovuplant™) on reproductive efficiency in mares

This study allowed us to conclude:

- The use of the ovulation induction agents, hCG and deslorelin (Ovuplant™), may increase the pregnancy rate on well managed breeding farms. This is likely the result of better synchrony between semen placement in the reproductive tract and ovulation.
- The use of the ovulation induction agents, hCG and deslorelin (Ovuplant™), may not decrease the number of artificial inseminations performed per mare on intensively managed breeding farms. Administering an induction on the first breeding exam day when the dominant follicle meets criteria for ovulation induction will decrease the number of inseminations needed per cycle except for follicles over 45mm in diameter.
- The use of the ovulation induction agents, hCG and deslorelin (Ovuplant™), may not decrease the number of rectal palpations performed on intensively managed breeding farms. If observation of a mare begins early in the estrus period, when follicle size is less than 35mm in diameter, than the number of palpations can be decreased significantly by using either agent.
- Both hCG and deslorelin (Ovuplant™), appear equal in their ability to induce ovulation in a clinical setting.
- Administering hCG or deslorelin (Ovuplant™) increases the likelihood of ovulation within 48 hours.

- Ovulation induction decreases the days to ovulation in comparison to non-treated mares. Days to ovulation appear affected by the follicle size at time of administration and the drug used.
- hCG may perform best on dominant-type follicles over 35mm in diameter.
- Deslorelin (Ovuplant™) appears to perform more consistently over various follicle sizes in comparison to hCG and appears to perform better on dominant follicles less than 35mm in diameter in comparison to hCG.
- The number of days to ovulation is consistently decrease by giving hCG or deslorelin (Ovuplant™) except when inducing large, dominant follicles over 45mm in diameter which are already destined for natural ovulation.
- Ovulation induction improves reproductive efficiency by decreasing palpations, decreasing the number of artificial inseminations per cycle, and reducing the days to ovulation improving the synchrony of semen deposition with ovulation and hence the pregnancy rate.
- Management of mares during breeding need considered when assessing reproductive efficiency

6.2 Study 2: Effect of deslorelin on the interovulatory period and response to PGF<sub>2</sub>α administration 6 days after ovulation

This study allowed us to conclude:

- The prostaglandin dinoprost tromethamine given six days after ovulation does effect the progesterone production of the corpus luteum formed after ovulation induction by the deslorelin sustained release implant, Ovuplant™
- Progesterone production by the corpus luteum formed by deslorelin sustained release implants is similar to untreated mares.
- The interovulatory interval of mares induced to ovulate with deslorelin sustained release implants can be shortened by the administration of the prostaglandin dinoprost tromethamine.
- Deslorelin sustained release implants do affect the interovulatory interval of certain mares and some mares appear more sensitive to their effect than others

CHAPTER 7

FIGURES AND TABLES

**TABLES:**

**Table 1:** Descriptive statistics for Study1 examining the effect of hCG, deslorelin, and no treatment on reproductive parameters.

Cycles	hCG (N=383)	Ovuplant (N=451)	No Treatment (N=583)
Mean Follicle Size at Treatment	40.45±5.33	36.94±4.19	40.17±4.89 (at first sign of estrus)
Mean Day of Treatment	1.50±1.81	2.10±1.73	-
Mean Days to Ovulation (from first sign of estrus)	3.99±2.15	4.21±1.83	4.22±2.12
Mean Days from Treatment to Ovulation	2.49±1.32	2.11±0.74	-
Mean Palpation/Cycle	2.94±1.15	3.58±1.35	3.20±1.36
Mean AI/Cycle	1.59±0.88	1.21±1.06	1.18±0.48

**Table 2:** Comparison of the use of ovulation induction agents on Farm 1 and Farm 2.

	FARM 1	FARM 2
<b>HCG</b>	255 (48.39%)	128 (14.38%)
<b>Deslorelin</b>	4 (0.76%)	447 (50.22%)
<b>No Treatment</b>	268 (50.85%)	315 (35.39%)
<b>Total</b>	527	890

**Table 3:** Comparison of Farm 1 and Farm 2 for average day of treatment, days from treatment to ovulation, average number of AI and average number of palpations performed.

	<b>FARM 1</b>	<b>FARM 2</b>
<b>Ave. Day of Treatment</b> <sup>1</sup>	0.58 days	1.35 days
<b>Days from Treatment to Ovulation</b>	3.14±1.72 days	3.06±1.95 days
<b>Average # of AI</b>	1.69	1.08
<b>Average # of Palpations</b>	3.59	2.69

**Table 4:** Simple Logistic Regression Model and Multivariable Logistic Regression Model for comparing the likelihood of pregnancy to treatment with ovulation induction agents hCG and deslorelin.

i.) Simple Logistic Regression Model

Pregnant	Chi	P Value	Odds Ratio	95% Confidence Interval	
	Square			Lower	Upper
<b>TxH</b>	1.530	1.192	1.192	.902	1.574
<b>TxN</b>	37.064	.000	2.187	1.700	2.814
<b>TxO</b>	-	-	-	-	-

R<sup>2</sup> is .03 (Cox and Snell)

ii.) Multivariable Logistic Regression Model

Effect	-2 Log Likelihood of Reduced Model	Chi Square	P Value
<b>Treatment</b>	<b>1903.731</b>	<b>42.408</b>	<b>.000</b>
<b>Month*AI*DaysO</b>	<b>1869.750</b>	<b>8.427</b>	<b>.004</b>

R<sup>2</sup> is 0.36 (Cox and Snell)

**Table 5:** The effect of ovulation induction (with hCG or deslorelin) and no treatment on the number of AI for all mares in the study.

	Control	Treatment hCG	Treatment Ovuplant
N	584	384	454
Missing	0	0	0
Sum	708	610	543
Lo 95% CI	1.1260	1.5005	1.1483
Mean	1.2123	1.5885	1.1960
Up 95% CI	1.2986	1.6766	1.2438
SD	1.0621	0.8776	0.5180
SE Mean	0.0440	0.0448	0.0243
Min	0.000	0.000	0.0000
Med	1.000	1.000	1.0000
Max	8.000	6.000	5.000

i.) One-Way ANOVA<sup>a</sup> comparing the mean number of AI for all mares in the study across the three treatment groups (hCG, deslorelin, and no treatment) for pregnant mares only

	Sum of Squares	Mean Square	F value	P Value
<b>Regression</b>	0.26	1	0.33	.857 <sup>b</sup>
<b>Residual</b>	1115.446	1420		
<b>Total</b>	1115.446			

**Table 6:** The ability of deslorelin and hCG to induce ovulation within 48 hours.

	Treatment hCG	Treatment Ovuplant
N	384	452
Mean	2.5547	2.1018
SD	1.3411	0.9550
SE Mean	0.0684	0.0449
Med	2.000	2.000

i.) Multivariable Logistic Regression Model comparing the probability of ovulation within 2 days across the two treatment groups (hCG and Ovuplant), controlling for: Size at Treatment, Day of Treatment and Farm.

Variables	P value
<b>Treatment</b>	.659
<b>Size at treatment</b>	.654
<b>Days of treatment</b>	.480
<b>Farm</b>	.253

ii.) Chi square analysis for the association between treatment with hCG or deslorelin and ovulation within 2 days. (OR=13.733; 95% CI: 7.396, 25.850; p<0.001)

	Ovulation within 2 days	Failure to Ovulate within 2 days
Treated	645	191
Control	15	61



**Table 7:** Data collected for each mare in Study 2 including treatment identification, treatment order, interovulatory period in days, follicle size in millimeters at treatment, days to ovulation, uterine edema score, and cervical score.

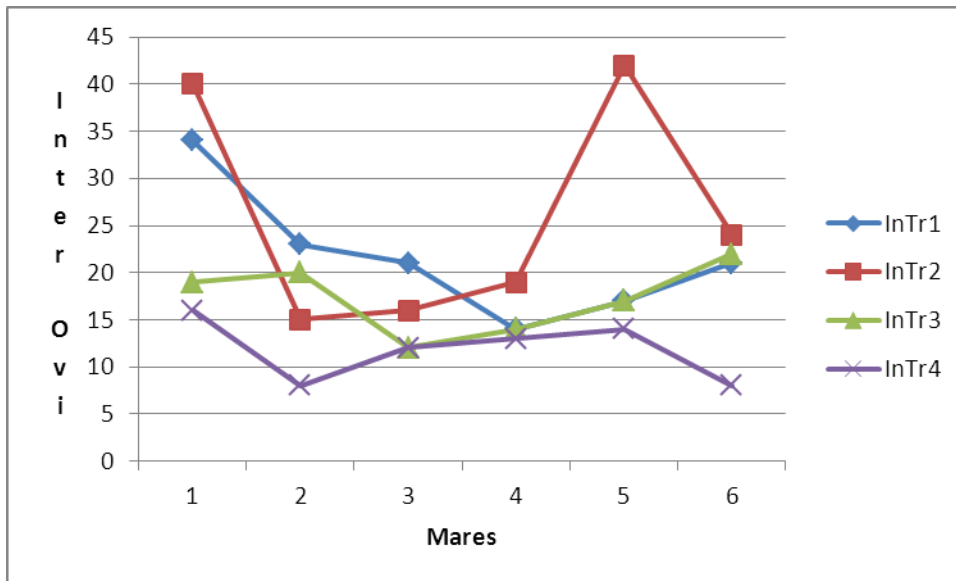
Mare #	Treatment Cycle	Treatment Period	Interovulatory Interval	Follicle Size (mm) at Treatment	Days To Ovulation	Uterine Edema Score	Cervical Score
1	1 (Control Cycle)	3	34	36	3	1	2
	2 (Ovuplant)	1	40	37	3	1	3
	3 (Ovuplant/PFG)	4	19	42	2	2	1
	4 (Control/PGF)	2	16	39	5	2	0
2	1 (Control Cycle)	3	23	37	1	1	0
	2 (Ovuplant)	1	15	39	6	0	0
	3 (Ovuplant/PFG)	4	20	37	2		2
	4 (Control/PGF)	2	8	37	2	1	2
3	1 (Control Cycle)	2	21	47	3	2	2
	2 (Ovuplant)	3	16	32	2	1	0
	3 (Ovuplant/PFG)	4	12	44	2	2	1
	4 (Control/PGF)	1	12	47	2	2	1
4	1 (Control Cycle)	1	14	48	11	1	1
	2 (Ovuplant)	3	19	45	3	1	
	3 (Ovuplant/PFG)	4	14	43	1	3	1
	4 (Control/PGF)	2	13	32	1	1	0
5	1 (Control Cycle)	2	17	40	3	3	2
	2 (Ovuplant)	3	42	37	2	2	1
	3 (Ovuplant/PFG)	4	17	41	2	2	1
	4 (Control/PGF)	1	14	48	4	2.5	2
6	1 (Control Cycle)	3	21	41	3	2	1
	2 (Ovuplant)	2	24	46	3	2	1
	3 (Ovuplant/PFG)	4	22	33	1	1	1
	4 (Control/PGF)	1	8	39	2	2	1

**Table 8:** Friedman Two-Way Non-Parametric Analysis of Variance comparing the association between treatment and the interovulatory period of the six mares in Study 2.

i) Mean rank and sample means for the interovulatory period of the six mares.

Treatment	Mean Rank of Treatment	Mean Interovulatory Period (Days)
1 (Control Cycle)	3.0	21.67
2 (Ovuplant™ Cycle)	3.5	26.00
3 (Ovuplant™/PGF2α)	2.42	17.33
4 (Untreated Cycle/PGF2α)	1.08	11.83

ii.)  $F = 12.368$ ;  $p = .0062$ ; degrees of freedom=3.



**Table 9:** Calculation of the difference in the interovulatory periods in days between treatments for each mare in Study 2.

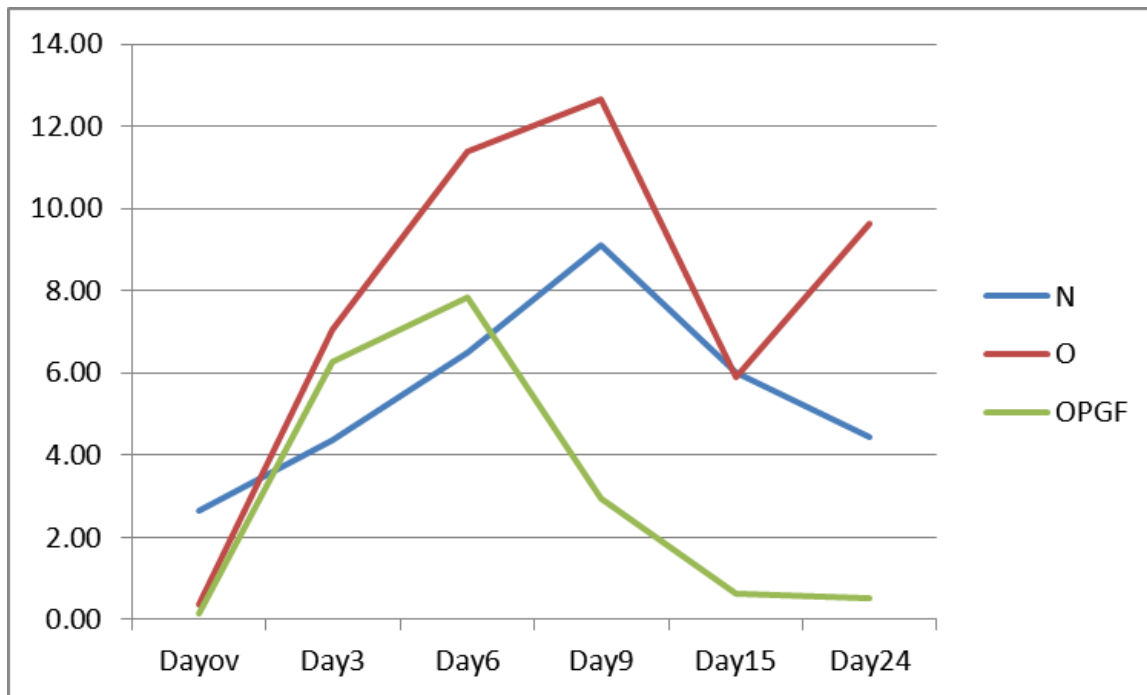
Mare #	Treatment Cycle	Treatment Period	Interovulatory Interval	OV/C	OV/OVPG	C/PG	CPG/OVPG
1	1 (Control Cycle)	3	34	6	21	18	-3
	2 (Ovuplant)	1	40				
	3 (Ovuplant/PFG)	4	19				
	4 (Control/PGF)	2	16				
2	1 (Control Cycle)	3	23	-8	-5	15	-12
	2 (Ovuplant)	1	15				
	3 (Ovuplant/PFG)	4	20				
	4 (Control/PGF)	2	8				
3	1 (Control Cycle)	2	21	-5	4	9	0
	2 (Ovuplant)	3	16				
	3 (Ovuplant/PFG)	4	12				
	4 (Control/PGF)	1	12				
4	1 (Control Cycle)	1	14	5	5	1	-1
	2 (Ovuplant)	3	19				
	3 (Ovuplant/PFG)	4	14				
	4 (Control/PGF)	2	13				
5	1 (Control Cycle)	2	17	25	25	3	-3
	2 (Ovuplant)	3	42				
	3 (Ovuplant/PFG)	4	17				
	4 (Control/PGF)	1	14				
6	1 (Control Cycle)	3	21	3	3	13	-14
	2 (Ovuplant)	2	24				
	3 (Ovuplant/PFG)	4	22				
	4 (Control/PGF)	1	8				

**Table 10:** Progesterone analysis for Study 2 using Kruskal-Wallis One-Way Nonparametric AOV (P=.0223) and Kruskal-Wallis All-Pairwise Comparison Testing.

	Control (Untreated)	Ovuplant Treated	Ovuplant treatment w/PGF2 $\alpha$
N	9	9	6
Missing	0	0	3
Mean	5.42 ng/ml	8.40 ng/ml	3.06 ng/ml

Variable	Mean	Homogenous groups
O	17.33	A
N	11.00	AB
OPGF	7.5	B

A=0.05; Z value=2.394



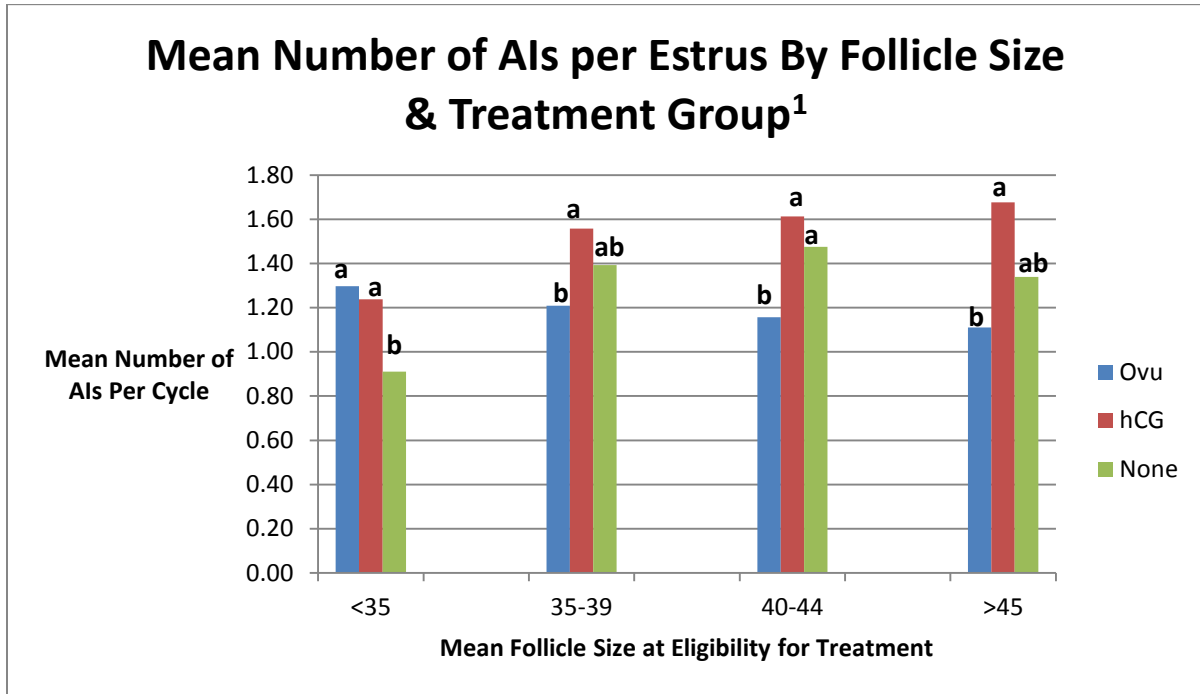
**N**=No treatment (control)

**O**=Ovuplant treatment only

**OPGF**=Ovuplant treatment with prostaglandin

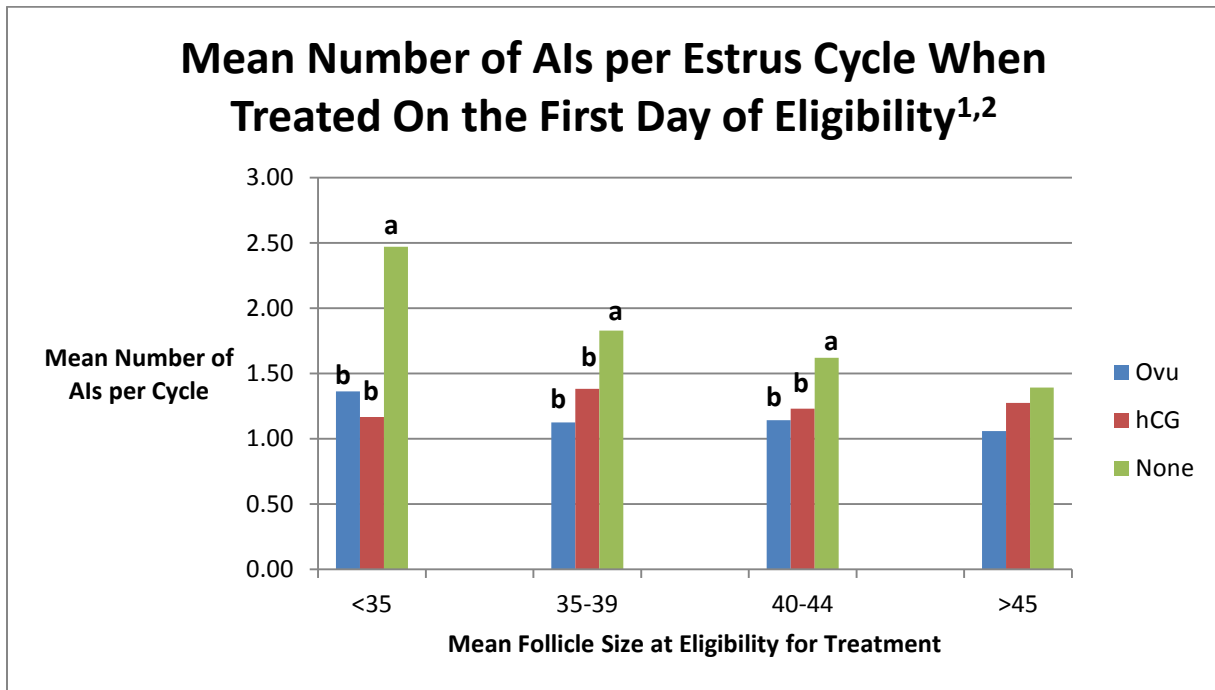
**HISTOGRAMS:**

**Histogram 1:** The mean number of AIs performed during an estrus cycle at a particular follicle size comparing mares treated with hCG, mares treated with deslorelin and mares not treated. [Ovu= deslorelin (Ovuplant™); hCG=human chorionic gonadotropin (Chorulon™); None= untreated mares]



<sup>1</sup>P values: <35mm=.000; 35-39mm=.004; 40-44mm=.000; >45mm=.000

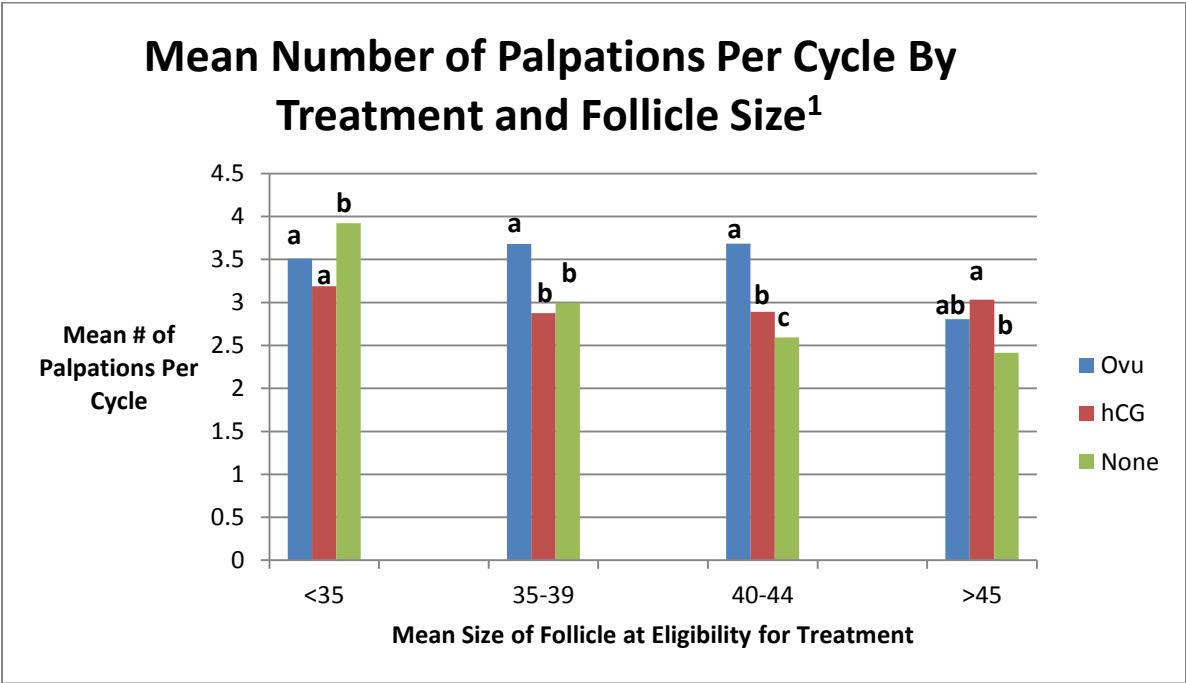
**Histogram 2:** The mean number of AIs performed during an estrus cycle at a particular follicle size when ovulation induction was performed with either hCG or deslorelin on the first day a mare was examined for breeding and met the requirements for ovulation induction. [Ovu= deslorelin (Ovuplant™); hCG=human chorionic gonadotropin (Chorulon™); None= untreated mares]



<sup>1</sup>First day of eligibility for ovulation induction for treated mares=day of ovulation induction; untreated mares first day of eligibility=first breeding date.

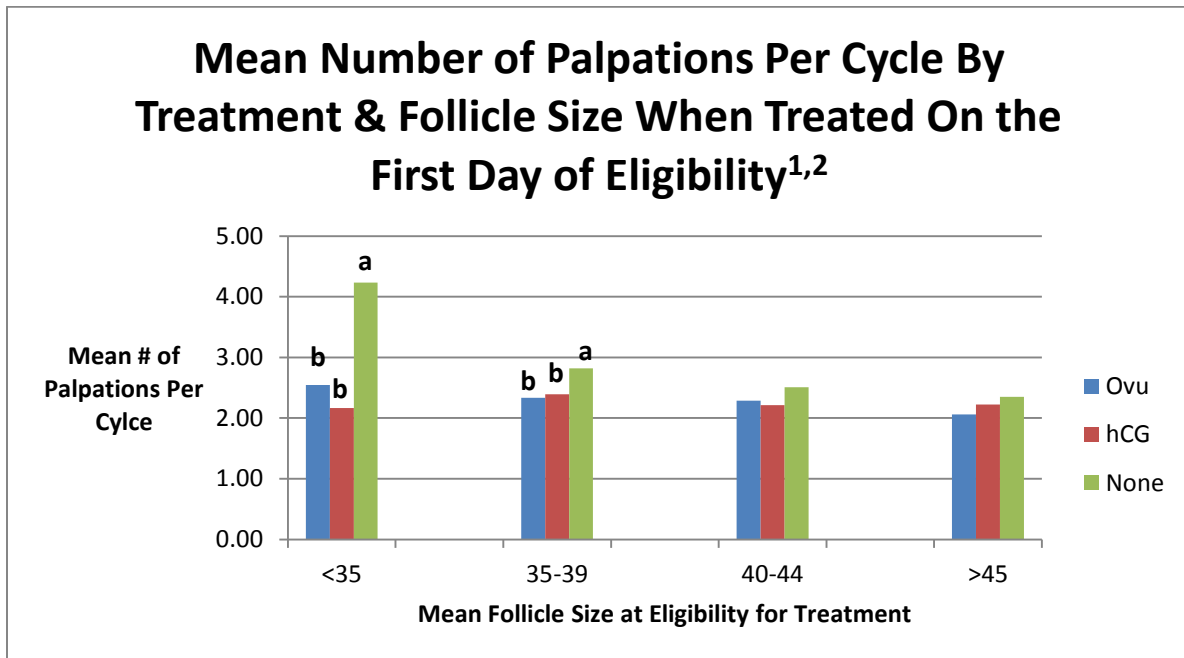
<sup>2</sup>P values: <35mm=.001; 35-39mm=.000; 40-44mm=.000

**Histogram 3:** The mean number of palpations performed in an estrus cycle at a particular follicle size when ovulation induction was performed with either hCG or deslorelin in comparison to no treatment. [Ovu= deslorelin (Ovuplant™); hCG=human chorionic gonadotropin (Chorulon™); None= untreated mares]



<sup>1</sup>P values: <35mm=.009; 35-39mm=.000; 40-44mm=.000; >45mm=.001

**Histogram 4:** The mean number of palpations performed during an estrus cycle at a particular follicle size when ovulation induction was performed with either hCG or deslorelin on the first day a mare was examined for breeding and met the requirements for ovulation induction. [Ovu= deslorelin (Ovuplant™); hCG=human chorionic gonadotropin (Chorulon™); None= untreated mares]

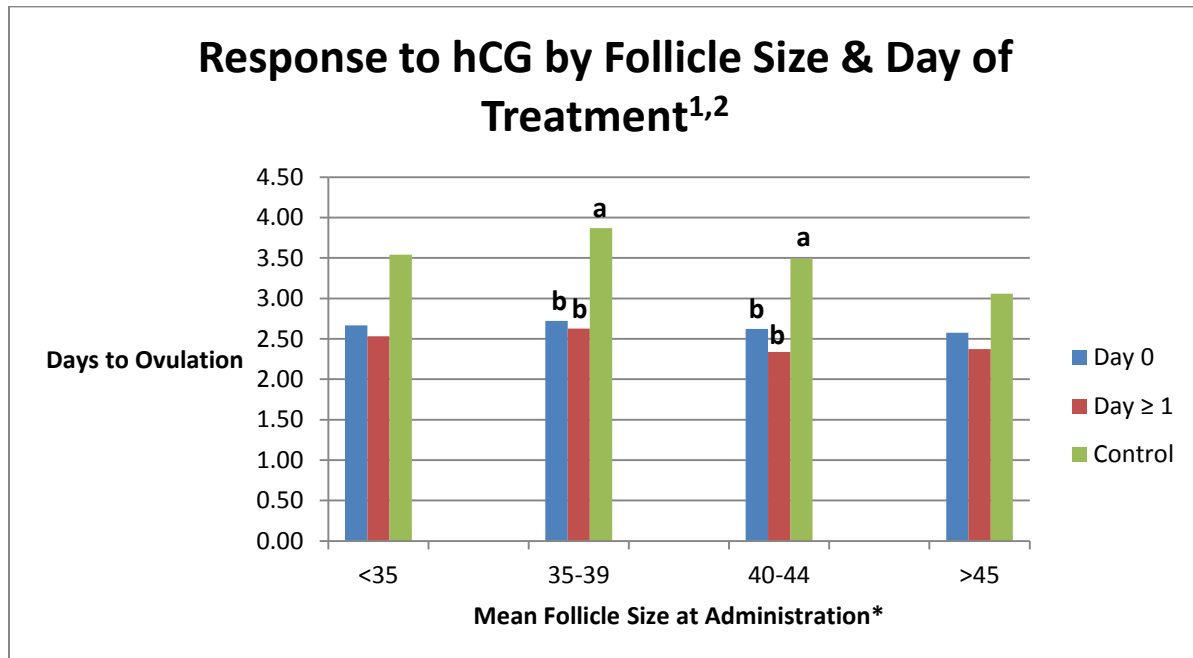


<sup>1</sup>First day of eligibility for ovulation induction for treated mares=day of ovulation induction; untreated mares first day of eligibility=first breeding date.

<sup>2</sup>P values: <35mm=.003; 35-39mm=.000



**Histogram 5:** A comparison of the days to ovulation for hCG based on time of administration and follicle size. [Day 0=first day examined and meeting criteria for ovulation induction; Day $\geq$ 1=day of estrus other than first day examined (Day 0); Control=Mares not treated with deslorelin or hCG]

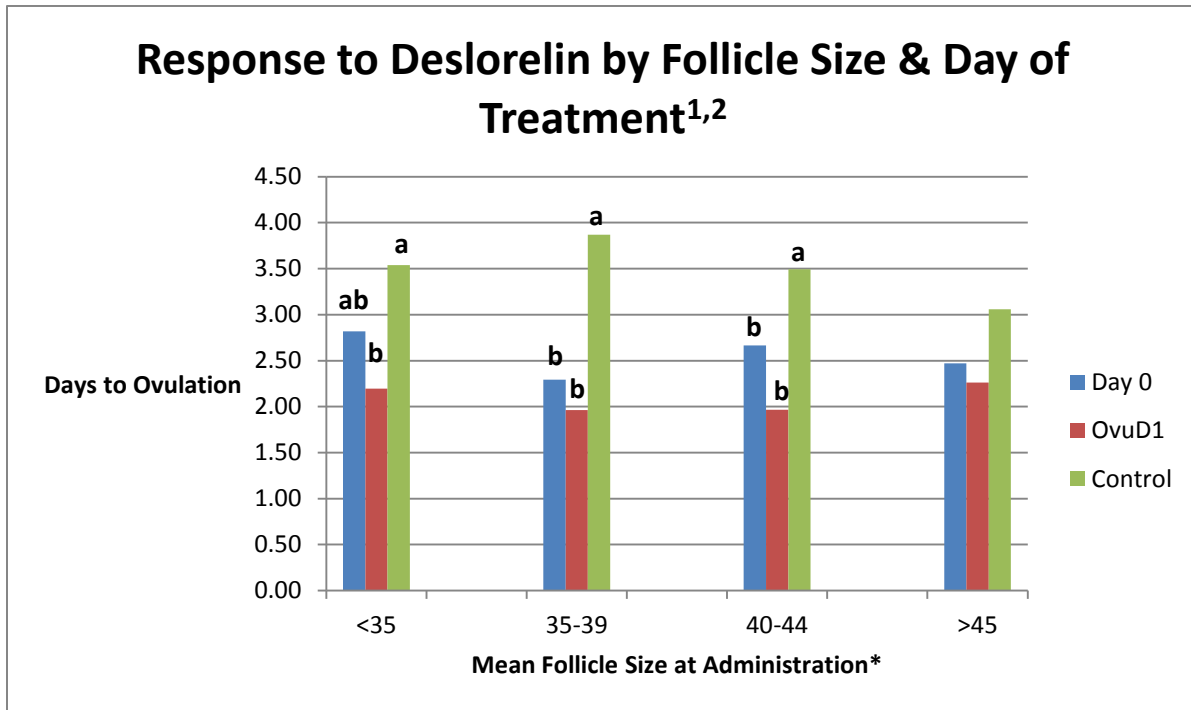


<sup>1</sup> Day of treatment=first day examined for breeding

<sup>2</sup> P values: 35-39mm=.000; 40-45mm=.000; >45mm=.033

\* Control mares=first breeding day

**Histogram 6:** A comparison of the number of days to ovulation assessing time of administration for deslorelin and follicle size at administration. [Day 0=first day examined and meeting criteria for ovulation induction; Day $\geq$ 1=day of estrus other than first day examined (Day 0); Control=Mares not treated with deslorelin or hCG]

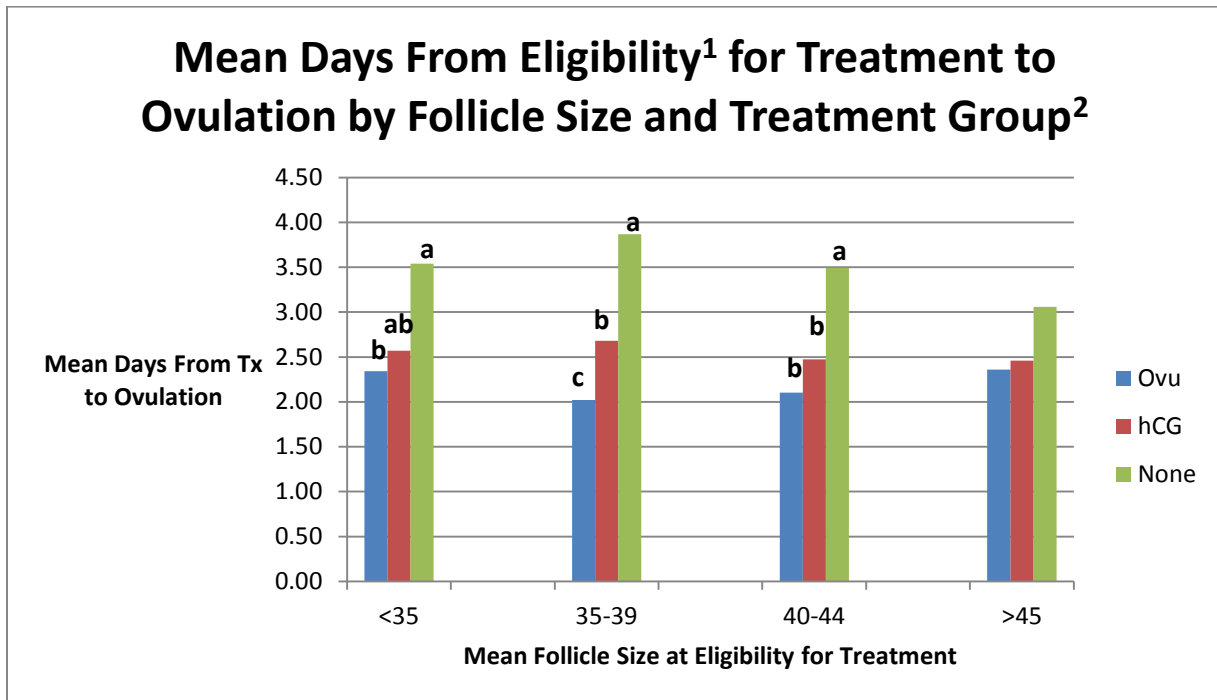


<sup>1</sup> Day of treatment=first day examined for breeding

<sup>2</sup>P values: <35mm=.002; 35-39mm=.000; 40-44mm=.000.

\* Control mares=first breeding day

**Histogram 7:** The number of days to ovulation for varying follicle sizes comparing mares not induced to ovulate (but meeting the minimum requirements for ovulation induction=eligibility) to mares induced to ovulate with hCG or deslorelin. [Ovu= deslorelin (Ovuplant™); hCG=human chorionic gonadotropin (Chorulon™); None= untreated mares]

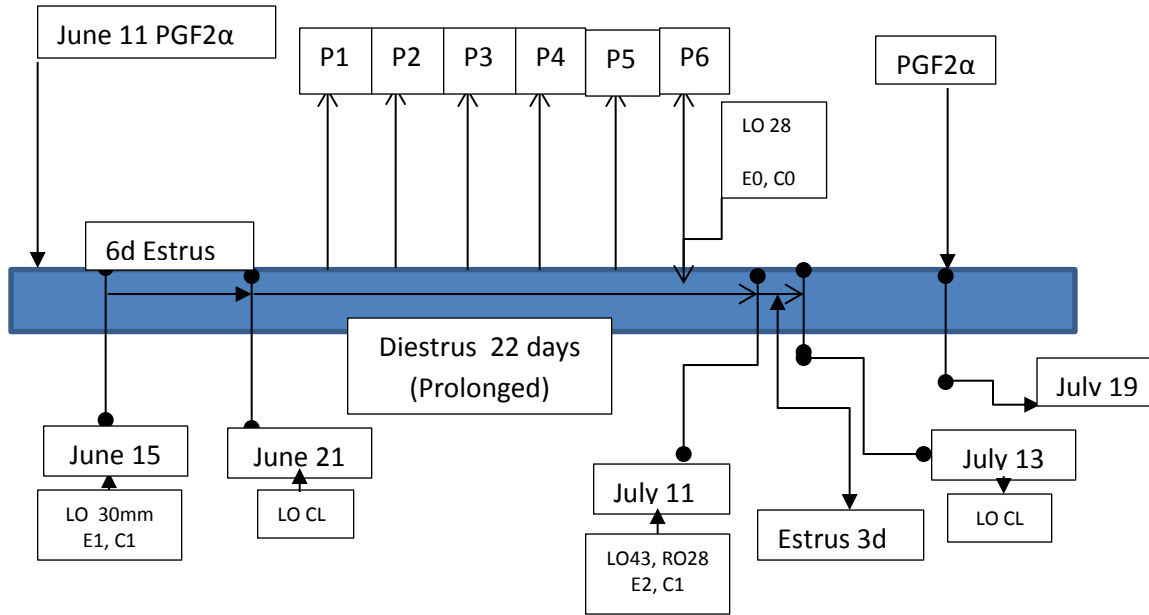


<sup>1</sup>First day of eligibility for ovulation induction for treated mares=day of ovulation induction; untreated mares first day of eligibility=first breeding date.

<sup>2</sup>P values: P value: <35mm=.001; 35-39mm=.000; 40-44mm=.000; >45mm=.027



**Figure 2:** Timeline displaying treatment protocol and progesterone sampling for Mare 1 during the **Control cycle** of Study 1. Control cycle was the second treatment cycle for this mare.



**P**=Progesterone sampling (every 3 days in diestrus)

**OV**=Ovulation

**RO**=Right Ovary

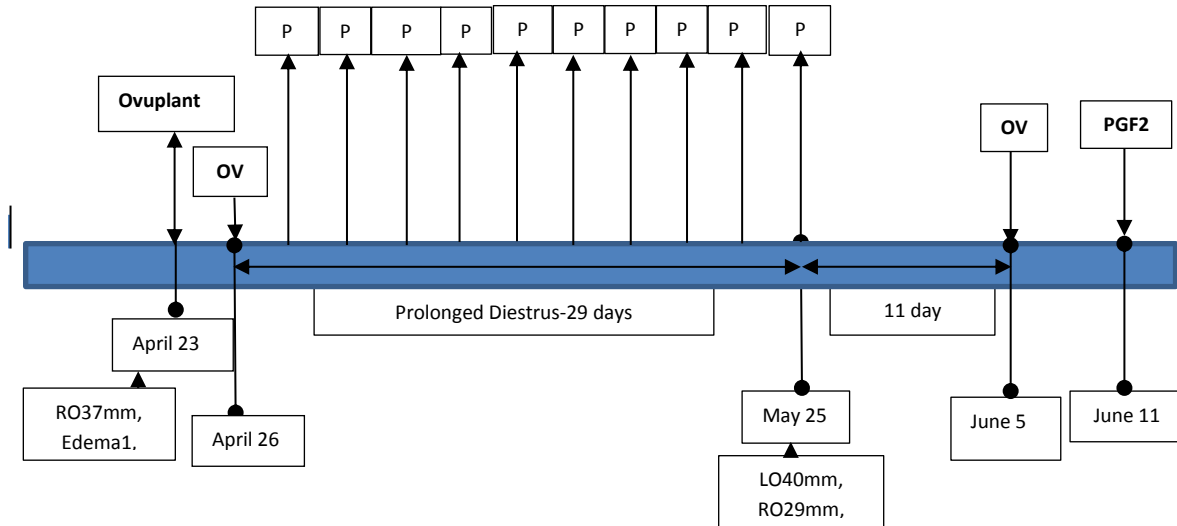
**LO**=Left Ovary

**Edema**=Endometrial edema scored from 0-3 with 0 equal to no edema present to 3 indicating maximal edema visible on ultrasound

**Cervix**= Assessment of cervical relaxation determine by rectal palpation on a scale of 0-3 with 0 tightly closed and 3 fully relaxed

**PGF2α**=Prostaglandin injection given to return mare to estrus

**Figure 3:** Timeline displaying treatment protocol and progesterone sampling for Mare 1 during the Ovuplant™ treatment cycle of Study 1.



**P**=Progesterone sampling (every 3 days in diestrus)

**OV**=Ovulation

**RO**=Right Ovary

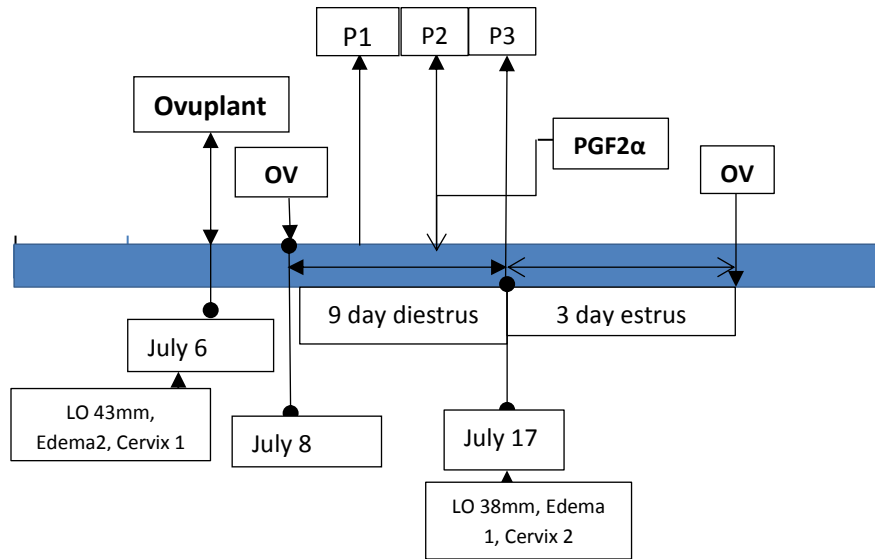
**LO**=Left Ovary

**Edema**=Endometrial edema scored from 0-3 with 0 equal to no edema present to 3 indicating maximal edema visible on ultrasound

**Cervix**= Assessment of cervical relaxation determine by rectal palpation on a scale of 0-3 with 0 tightly closed and 3 fully relaxed

**PGF2 $\alpha$** =Prostaglandin injection given to return mare to estrus

**Figure 4:** Timeline displaying treatment protocol and progesterone sampling for Mare 3 during the Ovuplant™ with PGF2α administered 6 days after ovulation treatment cycle of Study 1.



**P**=Progesterone sampling (every 3 days in diestrus)

**OV**=Ovulation

**RO**=Right Ovary

**LO**=Left Ovary

**Edema**=Endometrial edema scored from 0-3 with 0 equal to no edema present to 3 indicating maximal edema visible on ultrasound

**Cervix**= Assessment of cervical relaxation determine by rectal palpation on a scale of 0-3 with 0 tightly closed and 3 fully relaxed

**PGF2α**=Prostaglandin injection given to return mare to estrus

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## APPENDIX A:

### Association of ovulation induction and the number of palpations performed per cycle for all mares in the study and the subset of mares becoming pregnant:

Results for all mares in the study:

The results for the inclusive group of mares in the study showed that mean number of palpations for all mares, pregnant or non-pregnant, across the 3 treatment groups had a statistically significant association with treatment ( $F=47.033$ ,  $p=0.000$ ,  $r^2=.231$ ) (table follows). Potential confounders were introduced into the model: mare, number of AIs, size of follicle at treatment, day of ovulation and pregnancy ( $r^2=.634$ ,  $F=286.326$ ,  $p=0.000$ ). Treatment group and all of these covariates except mare were significantly associated with the mean number of palpations ( $p<0.001$ ). After adjusting for the set of covariates, at least one of the mean numbers of palpations was determined to be significantly different from the other treatment groups. Post hoc pairwise comparison (Table 7ii) indicated that the mean number of palpations for mares receiving deslorelin (3.5) was significantly higher than for those receiving hCG (2.9) or those in the Control group (3.2)( $p=.000$ ). There was no significant difference between those receiving hCG and those in the Control group ( $p=.000$ ). The median number of palpations for all the agents was 3.00.

Results for the subset of mares which become pregnant on the induced cycle:

A significant association between treatment group and the number of palpations received per cycle for mares becoming pregnant was determined ( $F=45.845$ ,  $p=0.000$ ,  $r^2=.087$ ). Controlling

for mare, number of AI, size at treatment, day of ovulation and pregnancy proved statistically significant ( $p < 0.001$ ,  $r^2 = 0.063$ ). Treatment group and all of the covariates except mare were significantly associated with the mean number of palpations ( $p < 0.001$ ) for pregnant mares. Adjusting for the set of covariates supported the conclusion that at least one of the mean numbers of palpations for mares becoming pregnant was significantly different. ( $p = 0.000$ ).

Reducing the number of palpations per cycle would have labor and financial advantages for the owner, farm manager, and veterinarian. In this study we did not note a significant decrease in the overall number of palpations performed on the mares after inducing ovulation. The use of deslorelin was actually associated with a significant increase in the number of palpations when compared to hCG and untreated mares. The increase in palpations is most likely related to its high rate of use on Farm 2, which managed a higher number of shipped chilled semen, embryo donor, and subfertile mares. Such mares require closer monitoring of the uterine environment during breeding, fewer inseminations, and insemination closer to ovulation to improve pregnancy rates. (Crowe, et al, 2008; Sieme, et al, 2003; Pycock, 2006) No studies were found that looked at the effect of ovulation induction on the number of palpations performed.

The result of a higher number of palpations is not unreasonable. Mares being managed for chilled semen or frozen semen inseminations will require more frequent palpations to ensure timely arrival of the semen and an appropriately timed insemination to improve the odds of conception. Mares which are of poor fertility secondary to age or uterine pathology may require frequent palpation to not only ensure optimal timing of insemination but to also reduce the amount of semen placed in the tract (to reduced post mating inflammation of the endometrium

and improve the post mating uterine environment). Poor stallion fertility may also create a situation in which mares must be managed to reduce the number of breeding's they receive.

Hence it may be concluded that ovulation induction may not reduce the number of palpations in certain circumstances where timing of insemination and ovulation are essential.

**Tables:**

**One-Way ANOVA comparing the mean number of palpations for all mares across the three treatment groups ( hCG, deslorelin, and no treatment )**

ANOVA<sup>a</sup>

	Sum of Squares	Mean Square	F value	P Value
<b>Regression</b>	78.286	78.286	47.033	.000 <sup>b</sup>
<b>Residual</b>	1468.129	1.664		
<b>Total</b>	210.266			

a. Dependent variable: Palpations

b. Predictor: Treatment (p=.000)

c.  $r^2=.231$

**Multivariable Regression Model comparing the mean number of palpations for all mares across the three treatment groups, controlling for: Mare, Number of AIs, Size at Treatment, Day of Ovulation and Pregnancy**

ANOVA<sup>a</sup>

	Sum of Squares	Mean Square	F value	P Value
<b>Regression</b>	930.116	186.023	287.326	.000 <sup>b</sup>
<b>Residual</b>	538.013	.647		
<b>Total</b>	1468.129			

a. Dependent variable: Palpations

b. Predictor: Days to Ovulation, Mare, Size at Treatment, Treatment, AI

c.  $r^2=.634$

**Coefficients<sup>a</sup>:**

	T Value	P value
<b>Treatment</b>	9.033	.000
<b>AI</b>	3.542	.000
<b>Size at treatment</b>	-4.249	.000
<b>Mare</b>	-.858	.391
<b>Days to Ovulation</b>	27.832	.000

(Dependent Variable: Palpations)

**Post Hoc Comparisons (Wilcoxon Signed Ranks Test) for Mean Number of Palpations for Mares Receiving Ovuplant™(PO), Mares Receiving hCG(PH), and Mares Receiving No Treatment(PN).**

	PO-PH	PN-PH	PO-PN
<b>Z Value</b>	-6.037 <sup>a</sup>	-0.795	-6.710 <sup>a</sup>
<b>Asymp. Sig. (2 tailed)</b>	0.000	0.427	0.000

Based on negative ranks.

**One-Way ANOVA comparing the mean number of palpations for pregnant mares across the three treatment groups (hCG, deslorelin, and no treatment)**

**ANOVA<sup>a</sup>**

	Sum of Squares	Mean Square	F value	P Value
<b>Regression</b>	69.759	69.759	45.845	.000 <sup>b</sup>
<b>Residual</b>	712.122	1.552		
<b>Total</b>	781.881			

a. Dependent variable: Palpations

b. Predictor: Treatment

**Coefficients<sup>a</sup>:**

	T Value	P value
<b>Treatment</b>	-6.771	.000

a. Dependent Variable: Palpations

**Multivariable Regression Model comparing the mean number of palpations for pregnant mares across the treatment groups controlling for: Mare, Number of AIs, Follicle Size at Treatment, Day of Ovulation and Pregnancy.**

ANOVA<sup>a</sup>

	Sum of Squares	Mean Square	F value	P Value
<b>Regression</b>	495.962	99.192		<b>.000<sup>b</sup></b>
<b>Residual</b>	285.919	.616		
<b>Total</b>	781.881			

a. Dependent variable: Palpations

b. Predictor: Days to Ovulation, Size at Treatment, Mare, Treatment, AI

**Coefficients<sup>a</sup>:**

	T Value	P value
<b>Treatment</b>	-7.165	<b>.000</b>
<b>Mare</b>	-.102	.919
<b>AI</b>	4.557	<b>.000</b>
<b>Size at treatment</b>	-2.609	<b>.009</b>
<b>Days to ovulation</b>	20.345	<b>.000</b>

a. Dependent Variable: Palpations

## APPENDIX B:

Association of ovulation induction and the number of artificial inseminations performed per cycle for all mares in the study and the subset of mares becoming pregnant:

ANOVA did not show a significance of treatment with the number of artificial inseminations. A comparison between treatments using multivariable regression introducing the potential effects of mare, number of palpations, size of follicle at treatment, day of ovulation and pregnancy was attempted and found significance of treatment ( $p=.000$ ;  $r^2=.303$ ). A Wilcoxon-Signed Ranks test for post hoc pairwise comparison found that the mean number of AI for mares receiving deslorelin ( $1.19\pm.024$ ) and those not treated was significantly lower than for those receiving hCG ( $1.59\pm.045$ ). There was no difference between mares treated with deslorelin and those not induced to ovulate ( $1.21\pm.044$ ) ( $p=.000$ ). The individual mare was found to not be significantly associated with the mean number of AI. Although this model showed significance, other variables could be introduced into the model. Since ANOVA did not prove a significance of treatment, model fit could be improved before drawing conclusions on this association.

For mares that became pregnant, treatment was shown to be significantly associated with the number of AIs by multivariable regression analysis controlling for mare, number of palpations, size at treatment, and day of ovulation ( $p=0.000$ ). Results however were similar to the inclusive set of mares and did not show a difference between mares induced to ovulate and untreated mares. Only a difference between mares treated with deslorelin and hCG treated mares was determined. Treatment, days to ovulation (DaysO) and number of palpations, were significantly associated with the mean number of AI ( $p<0.001$ ) in this model. Unlike for all

mares, pregnant or not pregnant, the size of the follicle at treatment was not significantly associated with the number of AI for mares becoming pregnant.

Multivariable regression analysis performed on the inclusive group of mares found an effect of treatment ( $p=.000$ ) along with several other variables. Pairwise comparison testing determined there was a decrease in the number of artificial inseminations on mares induced to ovulate with deslorelin but not hCG. This same relationship was detected in the pregnant mares also. Finding that deslorelin performed better than hCG at reducing the number of artificial inseminations agrees with both a large retrospective study (Allen, 2007) and a controlled study (Jochle, et al, 1994). This outcome may be driven by the fact that deslorelin has been shown to have a more consistent and reliable rate of ovulation induction than hCG. (Samper et al, 2002)

**Multivariable Regression Model comparing the mean number of AIs across the three treatment groups for all mares, controlling for: Mare, Number of Palpations, Size at Treatment, Day of Ovulation and Pregnancy. (r2=.303)**

**ANOVA:**

	Sum of Squares	Mean Square	F Value	P Value
<b>Regression</b>	330.623	55.104	99.367	0.000
<b>Residual</b>	761.392	0.555		
<b>Total</b>	1092.014			

**Coefficients:**

	T Value	P value
<b>Treatment</b>	-2.640	.008
<b>Mare</b>	0.500	0.617
<b>Palpations</b>	2.741	0.006
<b>Size at Treatment</b>	10.925	0.000
<b>Days to Ovulation</b>	11.203	0.000
<b>Pregnancy</b>	5.263	0.000

**Wilcoxon Signed Ranks Test comparing the mean number of AI for mares receiving treatment with deslorelin (Ovuplant™, PO), hCG (PH), and no treatment (PN)**

	PO-PH	PN-PH	PO-PN
<b>Z Value</b>	-12.410 <sup>a</sup>	-0.129 <sup>a</sup>	-2.163 <sup>a</sup>
<b>Asymp. Sig. (2 tailed)</b>	0.000	0.897	0.031

<sup>a</sup> Sign Test; based on negative ranks.



**Multivariable Regression Model comparing the mean number of AIs across the three treatment groups for pregnant mares only, controlling for: Mare, Number of Palpations, Size at Treatment, Day of Ovulation and Pregnancy.**

ANOVA<sup>a</sup>

	Sum of Squares	Mean Square	F value	P Value
<b>Regression</b>	66.069	13.214	42.520	.000 <sup>b</sup>
<b>Residual</b>	144.197	.311		
<b>Total</b>	210.266			

a. Dependent variable: AI

b. Predictors: Palpations, Mare, Size at Treatment, Treatment, Days to Ovulation

c.  $r^2 = .314$

**Coefficients<sup>a</sup>:**

	T Value	P value
<b>Treatment</b>	7.630	.000
<b>Mare</b>	.259	.796
<b>Palpations</b>	4.557	.000
<b>Size at Treatment</b>	1.536	.125
<b>Days to Ovulation</b>	4.473	.000

Dependent Variable: AI

## APPENDIX C:

### Effect of ovulation induction on the length of estrus:

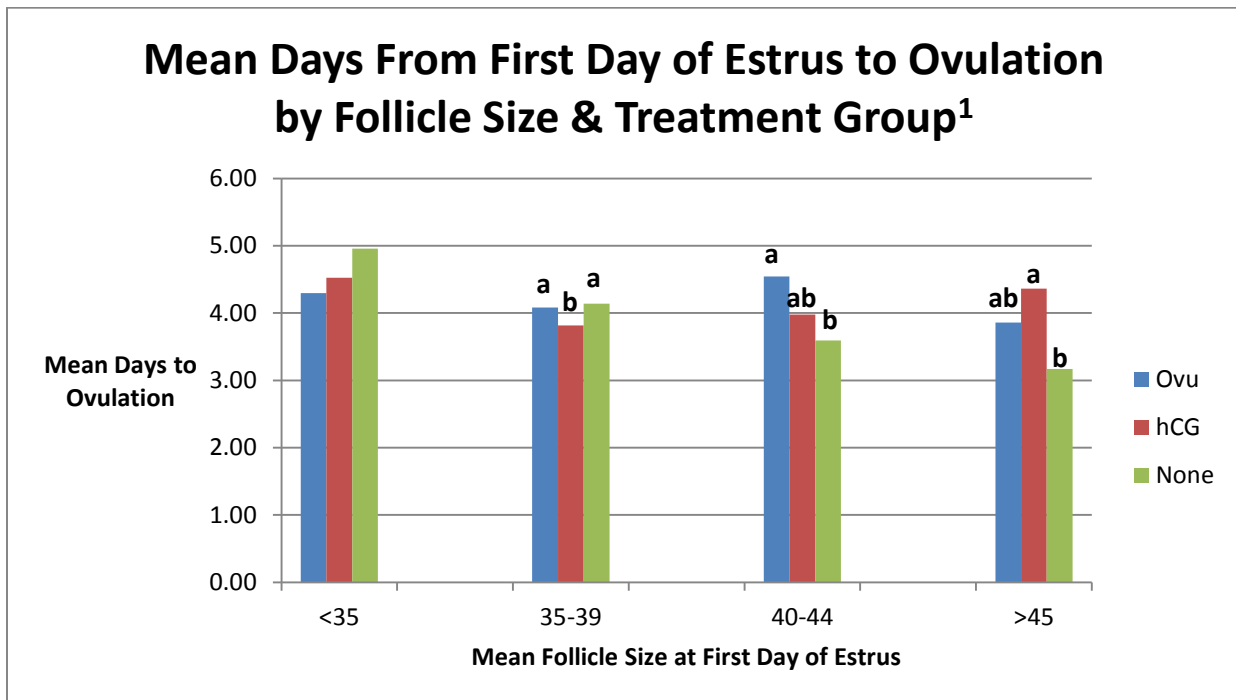
When the overall length of estrus was examined for mares treated and compared to mares not treated, ovulation induction did not consistently shorten the estrus interval. At smaller follicle sizes there is a trend toward the ovulation inductions creating a shorter estrus period. At larger follicle sizes, over 40mm in diameter, the mares not induced to ovulate proved to have a shorter period to ovulation than the mares treated. To determine if this effect is real, several factors would need to be examined and controlled for such as time of year the mares were examined, the individual cycles of the mares, and reproductive management of the mares. There can be much individual variation in the length of estrus among mares. (Stabenfeldt et al, 1975; Adams, et al, 1988; Witherspoon, 1971; Ginther, et al, 1972; Hughes, 1980) Season of the year affects estrus length do to photoperiod variation. (Adams, 1988; Ginther, et al, 1974; Hughes, et a, 1977) One farm used teasing as a staging method and may not have observed mares until teasing was noted. Hence, a more precise way to determine the first day of estrus and more frequent palpations would improve accuracy. More frequent palpations would benefit to know the exact beginning of estrus also.

At follicles 35-39 mm in diameter, the deslorelin treated mares and mares not induced to ovulate had comparable average days to estrus while hCG treated mares appeared to have a slightly shorter period of days to ovulation as expected with ovulation induction. Management of the mares would most likely account for these varying results as Farm 2 managed more chilled semen and infertile mares. Farm 2 also used more of the slow release implants, Ovuplant™. Sensitive mares treated with Ovuplant™, which can effect return to estrus and the ability to

evaluate a mare for the return to estrus. Mares treated with Ovuplant on the previous estrus have decreased levels of FSH and hence produce a smaller dominant follicle (i.e. a smaller follicle at ovulation) and will display less endometrial edema. This leads to a later average ovulation date in comparison to non-treated, non-sensitive mares. (Vanderwall, et al, 2001; Morehead, et al, 2000; Johnson, et al, 2000) In this study, interovulatory period was not specifically examined so the effect of sensitive mares is unknown.

In order to determine the effect ovulation had on the overall length of estrus, a controlled study will need performed and more frequent palpations required.

**Histogram: An analysis of the mean number of days from the first day of estrus to ovulation for mares treated with hCG, for mares treated with deslorelin, and for mares not treated.**



<sup>1</sup>P value: 35-39mm=.010; 40-44mm=.001; >45mm=.003