THESIS

AN EVALUATION OF ESTRUS SUPPRESSION IN THE MARE THROUGH THE USE OF AN ALTRENOGEST DELIVERING INTRAVAGINAL DEVICE

Submitted By

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ABSTRACT

AN EVALUATION OF ESTRUS SUPPRESSION IN THE MARE THROUGH THE USE OF AN ALTRENOGEST DELIVERING INTRAVAGINAL DEVICE

The ability to avoid adverse behavior in mares through manipulation of their reproductive cyclicity has been a desired technique for many years. The mare's sour reputation when in heat, is known by competitive professionals and hobby riders alike. As the breeding season approaches during long daylight days in the northern hemisphere, the mare will begin to transition into her regular twenty-one day interovulatory cycle. The estrus or "standing heat" phase of a mare's cycle occurs for approximately 5-7 days within a given estrous cycle. During this phase, the mare is primarily under the influence of estrogen, produced by the dominant follicle, driving signs of the sexual receptivity. Outward signs of estrus for the mare include: overt interest in other horses (especially stallions), leaning their hind end toward another horse (teasing), lifting their tail, posturing and urinating frequently. Dangerous behaviors associated with estrus such as biting, kicking, irritability, and distraction commonly persuade owners to look into estrus suppression options. Following ovulation, a corpus luteum (CL) is formed and the luteal cells begin to produce progesterone. Progesterone levels increase once again, overcoming the effects of decreasing estrogen levels for twelve to fourteen days, and often alleviating the undesirable behavior. Altrenogest, an oral and injectable synthetic progestin, is the most effective supplement for providing estrus suppression in the mare. The equine industry is in need of a reliable pharmaceutical device to suppress adverse behavior commonly associated with the estrus phase of the mare's cycle, yet allow the mare to resume normal cyclicity upon removal of the device for

pursuant of reproductive procedures. A custom intravaginal ring specifically designed for the unique anatomy of the mare will provide a novel and effective method for sustained release of altrenogest administration while being safer to handle and administer. The first experiment focuses on the unique anatomy of the mare's caudal reproductive tract compared to intravaginal ring sizes in order to obtain a pilot device for experimentation. An unmedicated toroidal silicone intravaginal ring measuring 14.2 cm in diameter was selected during experiment one. The second experiment was to evaluate the vaginal ring delivering altrenogest in a trial along with a placebo intravaginal ring and control group to evaluate several parameters associated with estrus behavior suppression. Both oil-based and solid suspension-based intravaginal rings for drug administration was evaluated during experiment two. Finally, the third experiment examined the in vivo evaluation of solid suspension altrenogest IVR as well as the marketability of this product and what the future holds for novel medical devices in equine reproduction.

Twelve total mares were used over the course of this study to determine the pharmacokinetics (PKs) and pharmacodynamics (PDs) of intravaginally administered altrenogest. Variables such as: teasing behavior when presented with a stallion, ultrasonographic examination of reproductive changes throughout several cycles, uterine and vaginal cultures, and blood collections for drug hormone bioanalysis were collected and monitored to evaluate the PK and PD of this novel drug delivery device. Throughout experiment three, each mare went through three cycles in a crossover design consisting of an oral dose treatment group, a therapeutic vaginal ring treatment group, and placebo vaginal ring control group. This study aims to provide horse owners and trainers an alternative method for delivering behavior modulating hormones, through an effective, therapeutic, steady-state release from a vaginal ring, and importantly enable normal reproductive cyclicity to resume upon removal.

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DEDICATION

To the horses in this study and to grumpy mares everywhere- thank you.

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CHAPTER 1: INTRODUCTION

Background

The equine estrous cycle 1.1

Mares are seasonal polyestrous breeders, meaning they have multiple estrous cycles during a specific time of the year. The mare's annual reproductive cycle is broken down into four phases: the spring transition period, the natural summer breeding season, the fall transition phase, and the winter anestrous period. Increasing day length will stimulate reproductive activity in mares while shorter days will cause the ovaries to deactivate.

Summer breeding 1.2

Although the physiological breeding season varies between mares and geographical locations, mares typically begin cycling regularly around April and lasts through October. In the mare, estrogen and progesterone work hand in hand to support the mare through her twenty-one day cycle and subsequently through gestation. As seasonal long day polyestrous breeders, mares will begin to cycle regularly anywhere between April and August, depending on their geographical location. During these reproductively receptive months, the mare will continue to cycle every twenty-one days until a viable pregnancy is obtained or the winter months approach and she falls into anestrus.

The twenty-one day duration of the reproductive cycle is broken down into two major phases. The estrus phase, also known as the follicular phase, is largely dominated by estrogen produced from the dominant follicle of the ovary. As the mare initiates a new cycle, the pituitary gland will release follicle stimulating hormone (FSH) to initiate the development of ovarian

follicles. FSH will signal granulosa cells within the ovarian follicles to produce high amounts of the hormone estradiol- 17 β, an estrogen. For five to seven days of the estrus cycle, estrogen drives the production and rising levels of luteinizing hormone (LH). A surge of LH occurs in a manner unique to the mare over the course of several days and initiates the follicular wall to rupture as a process known as ovulation, followed by peak levels of LH and the formation of the corpus luteum (CL) as the collapsed follicle fills with blood. The second phase of the mare's cycle is characterized by the formation of this CL. This phase is called diestrus, or the luteal phase, and is fueled by progesterone being produced by the luteal cells of the active CL. Progesterone communicates with the pituitary gland to command a negative feedback effect on LH. In the event of a pregnancy, the CL is maintained and progesterone production continues from this source to provide the early maintenance of pregnancy. Progesterone is the essential hormone responsible for maintaining a pregnancy. Mares without adequate progesterone levels may experience early embryonic loss if an exogenous source of progesterone is not supplemented. In the event that maternal recognition of pregnancy is not established by the mare, the luteolytic effect of prostaglandins released from the endometrium will lyse the CL after thirteen to fifteen days. Estrogen will elevate in the absence of progesterone and new follicular growth cycle will resume in the estrus phase (McCue, 2019).

If a viable embryo is detected by the mare through maternal recognition of pregnancy within fourteen days following ovulation, prostaglandin release will be inhibited and several endogenous sources of progesterone will work to maintain the pregnancy for the entirety of gestation. Progesterone production is categorized by Ginther into two phases. "Output D" is the progesterone production during diestrus whereas "Output 1" is the first luteal response of early pregnancy (Ginther, 1992. The primary CL from the ovulated follicle will provide adequate levels of progesterone for pregnancy maintenance throughout the first 30 days. Serum progesterone

levels will slightly decrease from the primary CL and progesterone production is assumed by secondary CL's between day 40 and 45, stemming from the release of equine chorionic gonadotropin (eCG) from the endometrial cups; a unique process in the mare. As a result of the resurgence efforts of the primary CL, termed the "secondary luteal phase" or "output 2", and secondary CLs, termed the "third luteal phase" or "output 3", progesterone concentrations reach peak levels at approximately two to three months of gestation, likely identified as 5-*a*-pregnanes, a metabolite of cholesterol and subsequently pregnenolone (P5). Progesterone production by the ovaries begins to steadily decline as the placenta becomes solely responsible for the production of progestogens, a progesterone-like substance or metabolite, by day 90 to 100. The progestogen productions from the feto-placental unit are essential in the maintenance of pregnancy during the second half of gestation, with all luteal structures regressing by day 200. It is hypothesized that the metabolism of progestogens are highly contributed to by the fetal adrenals.

Estrogen and Progesterone 1.3

Two-Cell Two-Gonadotropin Theory 1.31

Steroid hormones have a profound influence on the secretion of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), thus greatly affecting ovulation, cyclicity, early pregnancy maintenance, and behaviors throughout the cycle. In the mare, estrogens and progesterones are primarily synthesized in the theca and granulosa cells of the ovary. This theory is known as the two cell two gonadotropin model. According to this theory, ovarian steroids are synthesized from cholesterol through the interactions between theca cells and granulosa cells, ultimately producing progesterone and estradiol, respectively. Specifically, LH binds to luteinizing/chorionic gonadotropin receptor (LH/CGR) on the cell surface of the theca cells to stimulate the expression of the steroidogenic enzymes needed for androgen production.

Steroidogenic acute regulatory protein (STAR) drives cholesterol to be converted into pregnenolone, which subsequently diffuses into the smooth endoplasmic reticulum to be converted into progesterone followed by androstenedione. Alternatively, follicle-stimulating hormone (FSH) will act on follicle-stimulating hormone receptor (FSHR) on the surface of granulosa cells to stimulate the expression of enzymes necessary for estrogen synthesis. Androstenedione produced by theca cells diffuses into granulosa cells and is converted to testosterone or to estrone, ultimately leading to the synthesis of 17β-estradiol.

Sources of Steroid Hormones 1.32

Although the ovary provides as the main source for estradiol and progesterone in the mare, there are several other sources these hormones can be traced to. For example, mare owners may opt to ovariectomize their mares in an effort to cease estrogen production for behavioral reasons. In these ovariectomized mares, estrogen levels may still be detected from the adrenal glands and some mares may continue to display signs of estrus. Estrogens or progesterones may also be administered exogenously through oral or short-acting and long-acting injectable medications for breeding management purposes. In some cases, the mare's diet may cause endocrine dysregulation and possibly effect reproductive cycles due to consumption of estrogen-like compounds found in legumes such as clover, alfalfa and soybeans. Several studies have identified disruptions in reproductive efficacy in species such as sheep, cattle, and horses when fed legume-based diets (Shemesh, 2012), however specific recommendations for feeding safe levels of plant phytoestrogens at certain times within the breeding cycle have not been determined for horses and further research is needed to identify specific possible threats to mare reproduction (K.F.M. Reed, 2016).

Outward Behavior Associated with Steroid Hormones 1.33

Steroid hormones, derived from numerous sources both endogenous and exogenous, have a profound effect on outward behavior. 17β-estradiol produced by the granulosa cells of the growing follicles will stimulate the expression of behavioral estrus, typically lasting 5-7 days. Specifically in the mare, behaviors associated with estrus often include receptiveness to the stallion, tail raised, frequent posturing to urinate, vocalization (squealing), and becoming easily distracted by other horses. Progesterone produced from the luteinized granulosa and thecal cells within the CL will bring the mare out of behavioral estrus and into diestrus for a period of 13-15 days, often characterized by complete disinterest in the stallion.

Hormonal manipulation

Endogenous Hormone Manipulation 1.4

Artificial Photoperiod 1.41

Endogenous manipulation of hormones in the mare can be achieved using light therapy techniques to advance the first ovulation of the breeding season and possibly cycle and ovulate more times throughout the breeding season than mares allowed to come into their natural breeding season. Equine owners who desire a foal closer to the start of the year (between January and March) may choose to expose their mares to an artificial photoperiod. Through light therapy, the natural secretion of melatonin during the hours of darkness from the pineal gland in the mare is disrupted and ultimately reduced, leading to the production of gonadotropin releasing hormone (GnRH) from the hypothalamus. Typically, allowing mares to remain under light therapies beginning in

December will on average advance the first ovulation of the season by approximately two or three months (McCue, 2019).

Exogenous Hormone Manipulation 1.5

In the reproductive maintenance of mares, it is common practice to supplement the mare with exogenously administered hormones in an effort to manipulate their reproductive cycle. Hormonal manipulation is often utilized by breeding management facilities throughout the mare's cycle for a number of desired outcomes including but not limited to stimulating follicular development, inducing ovulation, luteolysis, pregnancy termination, stimulation of uterine contractions, suppression of estrus, stimulation of behavioral estrus, induction of labor, induction of lactation, and treatment of retained placentas.

Gonadotropin releasing hormone (GnRH) 1.51

Hormone therapy for mares intended to be bred earlier in the season can be a valuable tool for owners who opted out of or did not have access to light therapy. As Dr. McCue evaluates in his book, GnRH and GnRH analogs can be administered effectively by twice daily intramuscular injections in order to increase follicular development and ovulations. Once mares are identified as in their spring transition period, administration of GnRH analog over the course of approximately 5-10 days, such as deslorelin acetate which is available as both an intramuscular injection as well as an implant (available in Canada, Australia, and Europe), will greatly increase follicular diameter and may produce fertile ovulations throughout the duration of treatment. Buserelin, another well-known GnRH agonist, binds with high affinity to the GnRH receptor on anterior pituitary cells to

stimulate the secretion of LH and FSH, ultimately resulting in increased follicular growth (McCue, 2019).

Follicle Stimulating Hormone 1.52

The use of FSH in the mare for stimulation of follicular growth in transitional mares has been widely studied and found to be highly successful by equine theriogenologists. FSH originating from porcine or ovine species is shown to be ineffective in stimulating follicular development in mares, while twice daily administration of equine follicle stimulating hormone and recombinant FSH results in the advancement of the first ovulation of the season, as well as shorter intervals between ovulations throughout treatment (Checura, 2010).

Luteinizing Hormone 1.53

In the mare, LH is critical for final follicular and oocyte maturation. When recombinant FSH is coupled with genetically cloned recombinant LH, anovulatory follicles decreased while the number of follicles and subsequent ovulations increased (Meyers-Brown, 2017). Unfortunately, recombinant FSH and LH are still being studied and are not yet available for commercial purchase for use in equine breeding. Human Chorionic Gonadotropin (hCG) is a glycoprotein hormone that consists of an alpha subunit identical to LH and FSH, and is often used to induce ovulation in mares in estrus.

Prostaglandins 1.54

Once a mare is brought out of her spring transition phase and into the summer breeding phase, prostaglandins are commonly used in the diestrus phase to “short cycle” her by lysing the

existing CL. Endogenous progesterone produced by the endometrial lining of the mare's uterus are produced and secreted in pulses from day 13 to 15 of the reproductive cycle. Uterine prostaglandins enter the blood stream and are transported to the ovaries to signal the destruction of the CL. Common exogenous forms of progesterone such as Lutalyse® and Estrumate® may be administered to mares as early as five days after ovulation to act effectively on the CL. Typically, one to two doses of prostaglandins are effective in lysing the CL, causing a rapid decline in endogenous progesterone levels and allowing subsequent follicular development and estrogen production to occur, bringing the mare back into heat (McCue, 2019). When administered in large doses to a potential nurse mare, prostaglandins may stimulate maternal behavior and increase the success of grafting an orphan foal by stimulating the release of oxytocin from the brain (Podico, 2022).

Estrogens 1.55

Estrogens are utilized in the equine breeding industry for a variety of reproductive therapies including non-cycling recipient mare preparation, nurse mare preparation, and tease mares. Ovariectomized mares may receive exogenous 17β-estradiol to stimulate behavioral estrus and allow the mare to be used as a tease mare or jump mare for semen collection. While endogenous progesterone levels are elevated, 17β-estradiol injections will not cause behavioral estrus to occur in intact mares. However, 17β-estradiol injections may be given to a mare without an active CL, followed by administration of a short-acting progesterone to manually "ovulate" a recipient mare in preparation to receive an embryo from a donor mare. Estrogen supplementation may also be used to stimulate milk production in a nurse mare matching with an orphaned foal. Research shows that pretreating mares with estrogen prior to domperidone therapy, a medication used to induce

lactation in nurse mares and follicular development in transitional mares (McCue, 2019), resulted in higher prolactin levels. Following four to six days of domperidone therapy, mammary gland development is sufficient enough to begin hand milking, thus promoting greater milk production.

Progestins 1.56

Throughout the regular breeding season, it is common practice for facilities to utilize natural and synthetic progesterone to aid in the processes of advanced reproductive procedures such as (artificial insemination) AI, embryo transfer (ET), and oocyte recovery. Examples of native progesterone as a therapeutic agent are commercially available through compounding pharmacies in short-acting and long-acting dosages. Short-acting progesterone (SA-P4) is available in oil to achieve rapid, short-term elevated levels of blood progesterone concentrations. Administered as an intramuscular injectable formula, SA-P4 is able to elevate progesterone levels for a maximum of 24 hours, and therefore is administered intramuscularly once daily. Additionally, progesterone administration is widely used as a method of estrus suppression to overcome the effects of native estrogen in the mare. The following sections will evaluate the effects of native and synthetic progesterones when administered to the mare.

Synthetic Progesterone Administration 1.57

There are several brands of synthetic progestins available to equine owners and veterinarians that fail to adequately suppress estrus in mares. Medroxyprogesterone, more commonly recognized as Depo-Provera®, is an example of a synthetic progestin hormone disproved to manipulate the estrus cycle. By monitoring mares through transrectal ultrasonography and teasing behavior using stallions, it was concluded that Depo-Provera® was ineffective even at

higher doses for keeping mares out of heat (Gee, 2009). Hydroxyprogesterone caproate (brand name Hyproval®), a 27-carbon synthetic progestin that has been attempted for use in mares, was ultimately proven ineffective for both pregnancy maintenance and estrus suppression when McKinnon et al demonstrated that a 500 mg intramuscular injection every other day failed to maintain progesterone levels at or above 1.0 ng/ml (McKinnon, 1993). Melengestrol acetate (MGA) is a synthetic progestin in a feed additive commonly used to suppress estrus in cattle. MGA has been investigated for its ability to bring mares out of seasonal transition, as well as for its efficacy in pregnancy maintenance, displaying inconsistent results across the board (Loy and Swan, 1966). Canine estrus suppression is largely achieved by the oral product Ovaban® or Megace®, also referred to as megestrol acetate (Burke, 1975). Past studies have also examined the use of megestrol acetate as well as hydroxyprogesterone caproate, hydroxyprogesterone hexanoate, and norgestomet in an effort to suppress equine estrus or maintain pregnancy, and have remained ineffective (McKinnon, 2000). A vast array of unsuccessful synthetic progesterone treatments for estrus suppression in the mare have been tried and failed, most likely due to the inability of the synthetic progestin to bind to the equine progesterone receptor. For those equine owners not intending to breed their mares, hormonal manipulation may still interest them by means of estrus suppression, a crucial aspect of the behavioral modification in mares exhibiting untoward behaviors during the estrus phase. Exogenous manipulation of progesterone and estrogen is the saving grace for most mare owners looking for a short term or long term effective attitude adjustment for their mare.

Behavioral estrus suppression in the mare

History 1.6

The estrus phase of the mare's reproductive cycle is dominated by estrogen produced by the dominant ovarian follicle and often associated with common behavioral adversities within individual mares. During the five to seven day period of the follicular phase, the mare is receptive to the stallion's advances to breed, and adverse behavior such as biting, kicking, distraction, posturing to urinate, and generally undesirable behavior is likely to occur. Conversely, progesterone during the luteal phase of the estrus cycle will overcome the effects of estrogen for twelve to fourteen days, thus alleviating many of these untoward behaviors. Subsequently, the search for the unrivaled estrus suppressant has led to numerous discoveries thus far.

Current Methods of Estrus Suppression 1.7

Intrauterine Marble 1.71

One of the most historical methods of estrus suppression is the use of a marble placed within the uterus of the mare. Intended to mimic an early pregnancy, placing a marble inside the mare's uterus is effective for estrus suppression in approximately 39% of mares (Nie, 2003). Unfortunately, these methods commonly cause prolonged diestrus even after removal, as well as incidences of uterine infection and vaginitis. One case describes the shattering of intrauterine marbles, suggesting that utilizing a softer material such as a plastic may be more beneficial to the health and safety of the mare and veterinarian (Diel de Amorim, 2016).

Equine Intra-uterine Devices (IUD) 1.72

Several devices have mimicked the concept of inserting a device into the mare's uterus to simulate pregnancy in hopes of suppression estrus. As one step up from the average marble technique, several equine intrauterine devices (IUD) have made their way onto the mare estrus suppression market such as self-assembling magnets and silicone devices. It is hypothesized that an intrauterine device in continuous contact with the endometrium of the mare's uterus will prevent the release of prostaglandins, thus prolonging the function of the CL (Alamo, 2008). A self-assembling, 3-part polymer-coated magnetic IUD marketed under the name "Upod" is an effective, hormone-free contraceptive option when studied under pasture breeding conditions (Hoopes, 2021). Although highly successful as a contraceptive, this device showed high evidence of uterine fluid accumulation throughout treatment, as well as severe vaginal discharge and presence of Streptococcus equi subspecies zooepidemicus, a common uterine pathogen.

O-shaped equine IUDs were examined for contraceptive efficacy as well as estrus suppression. When 24 mares were studied in 2021, O-ring retention rates never exceeded 50% regardless of ring size or material (Lyman, 2021). When the O-ring IUDs were compared to the alternatively shaped Y-design IUDs (Holyoak, 2021), the Y-design IUDs had a retention rate of greater than 75% and did not show success in estrus suppression, however was highly successful in pregnancy prevention, possibly due to evidence of a hostile uterine environment resulting from the IUD. The use of equine IUDs has been met with some controversy surrounding evidence of uterine pathology, however some researchers report no complications and high efficiency of these devices (Gradil, 2019).

Plant Oil Administration 1.73

It has been hypothesized that intrauterine administration of plant oils on day 10 post ovulation will delay luteolysis and prolong the function of the CL due to the estradiol or fatty acid content. In one study, 48 mares received an intrauterine infusion of either 1ml of fractionated coconut oil, peanut oil, mineral oil or a slow-release preparation of estradiol (10 mg/ml) in mineral oil on Day 10 post ovulation. Results showed that luteolysis was delayed in 92% of mares, providing an effective and practical method of prolonging luteal function likely due to a disruption in the uterine environment of the mare (Wilsher, 2011).

Oxytocin Administration 1.74

In contrast to the use of uterine or vaginal devices, oxytocin has proven to offer an alternative method of estrus suppression. When administered around day 11 or 12 after ovulation, oxytocin will stimulate the secretion of PGF2α. When oxytocin is administered mid-luteal phase however, it does not induce a PGF2α release. Instead, an oxytocin administration before day 10 after ovulation will disrupt luteolysis and prolong CL function. Experimentally, oxytocin blocked luteolysis in four out of five mares when administered a continuous infusion from day 8 to 20 after ovulation (Stout, 1999). When administered twice daily for days 7 to 14 after ovulation, CL function was prolonged for 30 days in all treated mares (Vanderwall, 2007). Similar results were found in several other subsequent studies, resulting in prolonged CL activity in 71% of mares (Vanderwall, 2012), 67% of mares (Vanderwall, 2012), and 83% of mares (Gee, 2012).

Ovariectomy Procedures 1.75

Surgical ovariectomy procedures in mares are often misunderstood as the "fix-it" solution to behavioral estrus. In 1980, ten ovariectomized mares and ten intact mares in seasonal anestrus were observed for estrus behavior while being introduced to a stallion. Mare behavior in the absence of steroid hormone production from the ovaries was analyzed in this study. No statistical differences were found between the two groups, and all mares showed perceptive behaviors throughout the study (Asa, 1980). In a 2020 study, 28 mares underwent surgical ovarian removal, and behavioral improvements were only observed in 40% of mares with normal ovaries and were not statistically significant within the scope of this study (Melgaard, 2020).

Intravaginal Devices 1.76

High incidences of cumbersome behavior or injection site irritation has led researchers to explore an alternative to administering injections. Equine intravaginal devices offer several advantageous aspects over intrauterine devices (IUD's), which in most equine models do not contain hormones. It is hypothesized that incidences of uterine infection are far fewer by avoiding passing a device all the way through the cervix into the uterus. In one influential study, Hanlon *et al*, treated two hundred twenty seven transitional thoroughbreds with a synthetic progestin via an intravaginal device in a Y-shape, mimicking the controlled intravaginal device (CIDR) commonly utilized in cattle. This study demonstrated high device retention rates and reported a significantly earlier breeding season initiation than the control groups, on average coming into heat approximately 13 days earlier than control mares and offering advantageous breeding opportunities earlier in the season (Hanlon, 2012). This proved to be one example of an

economical, convenient, and effective method of progestin delivery in the mare, however this device is not yet approved for use in the United States.

A variety of progesterone releasing intra-vaginal devices (PRIDs) have been utilized in clinical broodmare practice. One intravaginal device in particular called the PRID® Delta, is a triangular shape with a long external string, similar to the CIDR model, and contains 1.55g of progesterone. Ten total mares were used to evaluate progesterone delivery through this device for a total of 10 days. Safety of the device was determined using a scale of 0 to 3 using the following system: 0, no discharge; 1, mild discharge; 2, moderate discharge; 3, severe discharge with scalding of the skin. Visual assessments were made using a speculum examination, culture swab and cytology brush. Progesterone delivery was evaluated by blood samples taken on days 0 through 14. All intravaginal devices in the PRID® Delta study demonstrated successful delivery of progestins defined by elevated mean progesterone levels for the 10 days of treatment (3.79 ±0.34 ng/ml), and no mares ovulated during this time. Unfortunately, researchers also identified an increasing presence of vaginal discharge associated with this device (Crabtree, 2018). Although often harmless to the mare, vaginal discharge in association with this method of drug delivery is a concern among horse owners. Other studies have looked into the use of an antibiotic and corticosteroid administered alongside the intravaginal device to prevent vaginitis or at least reduce its severity. Polasek et al used intravaginal devices with or without sprayed oxytetracycline and hydrocortisone and found that the intensity of the vaginal irritation was greater in the group that did not receive the oxytetracycline/hydrocortisone spray than in the group that did receive the spray (Polasek, 2016). Regardless of vaginitis presence in most treatment groups, the mechanism for progesterone delivery was not effected and can therefore be concluded that progesterone releasing intravaginal devices are a reliable method for progesterone delivery.

Intravaginal Rings 1.77

Intravaginal rings have been used in human and animal medicine for over 50 years. The first patent for human and animal IVR was filed in 1968 by Gordon Duncan. Recent studies have evaluated the pharmacokinetics and tolerability of intravaginal rings in sheep, macaques, and several other species. In 2019, Weiss et al. demonstrated the effectiveness of an intravaginal ring in sheep to retain placement for a 28 day period as well as deliver steady states of 17b-estradiol and progesterone. Intravaginal rings have also been studied in pre-clinical trials, such as looking at an intravaginal ring for the real-time evaluation of vaginal delivery (Moss and Baum, et al. 2017). Several preclinical research studies such as Moss et al. 2014 have examined the properties of an intravaginal ring in species such as the macaque. Previously conducted research was used to develop the experimental design and objectives of the equine intravaginal ring.

Altrenogest 1.8

Several commercially available progestins are marketed for human and animal use, but only altrenogest (Regu-mate®; Merck, USA) has been found to be consistently progestogenic in mares, making it the gold standard for estrus suppression (Mckinnon et al. 2000b; McCue 2003; Storer et al. 2009). Altrenogest, known more commonly by the brand names of Regu-Mate®, OvaMedTM, or Altren®, provides the industry gold standard for estrus suppression as well as pregnancy maintenance. Available in oral and injectable forms equine veterinarians often use altrenogest for: management during spring transition periods, pregnancy maintenance, estrus synchronization, but is only marketed for estrus suppression.

Chemical Structure 1.81

Altrenogest, also known as allyl-trenbolone, was originally approved for use to control estrus in mares in the 1980's under the brand name Regu-Mate®. Soon thereafter, altrenogest was approved for over-the-counter use in pig operations under the name MATRIX™. In addition to its use for estrus suppression in pigs and horses, off-label uses in horses, dogs and other mammals to maintain pregnancy is common. The synthetic progesterone altrenogest (17-allyl-17β-hydroxyestra-4,9,11-trien-3-one) is a derivative of the C19-nortestosterone series (figure 1). Altrenogest has strong progestomimetic and anti-gonadotropic effects, acting on the hypothalamus and pituitary progesterone receptors in order to achieve a negative feedback effect on the follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The principal effects of altrenogest are the progestomimetic and anti-gonadotrophic effects, however altrenogest also has weak oestrogenic, anabolic and androgenic effects with no corticoid or anti-inflammatory effects.

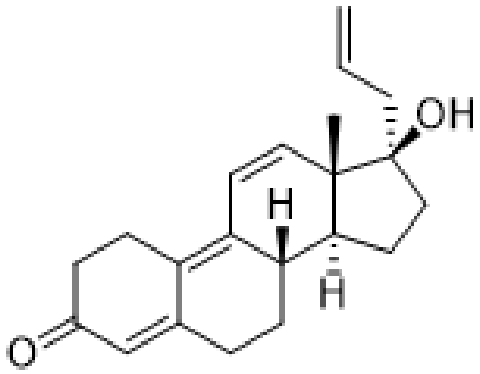

Figure 1: The chemical structure of altrenogest

Pharmacokinetic Properties 1.82

Altrenogest concentrations can be detected in the blood as little as 15 minutes after oral administration, and can still be detected up to 72 hours after the last oral treatment. Oral administration is the most common altrenogest dosage form, where it is distributed mainly to be

metabolized by the liver through Phase II conjugation with glucuronic acid, as the principal route into glucuronide, and sulfonation as a minor route, into sulfate, followed by the kidney, muscle and fat. No Phase I metabolites have been observed. The terminal half-life following oral administration of altrenogest is 10.7 hours ± 4.3 hours. Phase II conjugation is the rate limiting step of the metabolic process and biotransformation is independent of the dose level. Metabolites of altrenogest show little or no hormonal activity. Altrenogest is excreted mainly through the urine and fecal matter within 24 hours, with peak concentrations in the urine within three to six hours after the first dose. Altrenogest can be traced by looking for the phase II metabolites within the blood through the use of ultra-performance liquid chromatography coupled to tandem mass spectrometry.

Injectable Altrenogest 1.83

Estrus suppression therapies through the use of altrenogest are available to be administered in several different ways. There are injectable options for administering altrenogest to mares. Strengths from this injectable altrenogest range from 60 mg/ml to 225 mg/ml. Altrenogest injection costs can be a factor for some horse owners when used for estrus suppression. Injectable altrenogest can be advantageous to oral altrenogest if a mare's gastrointestinal diseases do not allow her to absorb altrenogest adequately or she resists oral medication administration. Injection sites are, however, prone to inflammation and irritation after repeated administration. One study examined the effectiveness of three sustained release injectable formulations of altrenogest, as well as one injectable formulation of medroxyprogesterone acetate (MPA) for long term suppression of estrus. All altrenogest mares increased their number of days between ovulation and returning to heat, while MPA mares did not show any differences in their estrus cycles.

Intra-rectal Altrenogest 1.84

Another study investigating a novel method of the use of altrenogest chose an intrarectal administration method to aid in the treatment of pregnant mares who are systemically compromised for any number of reasons including: neurologic diseases, refluxing anterior enteritis, postoperative ileus cases, and horses with esophageal disease. Although this study opened up an additional route of administration for altrenogest, it is a less practical delivery route for routine scenarios due to the decreased bioavailability and increased absorption rate, leading to an increase in dosing.

Oral Altrenogest 1.85

Oral altrenogest is the industry standard for the administration of altrenogest. Regumate for the equine species is a 2.2 mg/ml oral solution. Each ml contains 2.20 mg of altrenogest suspended in oil. This product is intended for use in the suppression and prevention of estrus during the prolonged estrus periods. Oral altrenogest can also be used to control of the time of initiation of estrus, with approximately 90% of mares showing signs of estrus within 5 days following the end of treatment.

Adverse effects of Altrenogest 1.9

Studies have not demonstrated any adverse or toxicological effects following administration on horses. This product has been shown to be administered for periods up to 305 days and five times the recommended dose at a given time leading to no negative effects. Owners themselves should take precaution when administering the commercially available oral product. If

administering over grain, any left-over feed should be properly disposed of without risk of other farm animals accessing the mixture. Any spilled drug should be adequately cleaned up to reduce risk of accidental exposure at a later time. In a one-and-two-generation reproduction study in rats using oral altrenogest, reproductive effects were demonstrated as depression of spermatogenesis in males, decreased litter size and weight in females, and decreased weight of hormone dependent organs.

The global concern with widespread altrenogest usage is the adverse effects the drug has on public health. Traditionally, altrenogest administration is through an oral solution marketed as "Regu-Mate®" at 10 mL orally once daily often with a large syringe. This method is frequently wasteful if the horse moves suddenly and the handler is not successful in administration. Often this oily solution comes in contact with skin or clothing, which can lead to negative reproductive effects on pregnant or cycling women. The Food and Drug Association (FDA) has received numerous reports of adverse effects involving altrenogest (FDA.gov, 2021). Reports have included alterations with women's reproductive cycles, decreased libido, abdominal pain, and nausea. Although precautions such as wearing gloves are a requirement when handling altrenogest, the FDA furthers their restrictions by specifying who is not permitted to handle the drug: women who are or suspect they are pregnant, those diagnosed with thrombophlebitis or thromboembolic disorders or a history of these problems, those with cerebral-vascular or coronary-artery disease, women with known or suspected carcinoma of the breast, people with known or suspected estrogen-dependent neoplasia, women with unexplained vaginal bleeding, people with benign or malignant tumors that developed during the use of oral contraceptives or other estrogen-containing products, and anyone with liver dysfunction or disease. Longer term exposure to altrenogest will cause an increase in these affects as seen in teenage girls specifically. When an enormous facet of

the equine owning and riding population are minors, this concept must be considered at the forefront of altrenogest handling and safety.

Banned and controlled substances 1.10

Founded in 1921, the Fédération Equestre Internationale (FEI) is the world governing body for most equine related sports events including Jumping, Dressage & Para Dressage, Eventing, Driving & Para Driving, Endurance and Vaulting. Every year, the FEI publishes an 'Equine Prohibited Substances List (EPSL) to ensure horses are not being treated or fed any substances that are not permitted during competition or use in the horse at any time. A banned substance is one that is considered to have no legitimate use in the competition horse and/or have a high potential for abuse. On the other hand, controlled medications are those that are deemed by the FEI to have therapeutic value and/or be commonly used in equine medicine. Controlled Medications do have the potential to affect performance and/or be a welfare risk to the horse, and therefore are still highly regulated within the equine community. The official list for 2022 banned and controlled substances list altrenogest as a controlled substance for use in males and geldings only. At this time altrenogest is not a banned or controlled substance for the use of estrus suppression in mares. One previously discussed method of estrus suppression, medroxyprogesterone, is banned according to the FEI, while others such as 17-alpha-hydroxy progesterone are controlled within the competition environment. Clomiphene, an estrogen receptor modulator, along with several substances that have anti-estrogenic properties are also banned according to the FEI. Although altrenogest was moved from the banned list to the controlled list for male horses and geldings in 2011, it is unlikely that it will be moved to the banned or controlled list for the use of estrus suppression in mares. It is likely that with the currently available

information, it is permissible to continue to use the altrenogest product “Regu-Mate” solution 2.2 mg/mL for mares as per the manufacturer's instructions. A complete list of up to date banned substances in accordance with the FEI can be found on their website at “https://inside.fei.org/fei/cleansport/ad-h/prohibited-list#”.

A proposed method of estrus suppression in the mare 1.11

Horse owners are in need of a safe, reversible, and reliable method of estrus suppression in the mare. Altrenogest administration is by large the most common and most effective method of estrus suppression on the market today. A novel intravaginal ring designed for sustained altrenogest delivery to the mare may provide steady-state systemic drug exposure for a prolonged period of time and allow the mare to resume normal cyclicity upon removal of the device while suppression estrus throughout the duration of treatment. The design, development, and beginning stages of the evaluation of an altrenogest releasing equine intravaginal ring is examined in this thesis.

CHAPTER 2: EXPERIMENTAL DESIGN

Research hypothesis 2.1

This study hypothesizes that a custom intravaginal ring specifically designed for the unique anatomy of the mare will provide a novel and effective method for sustained release altrenogest delivery. Uniquely, this device will suppress adverse behavior commonly associated with the estrus phase of the mare's cycle, yet allow the mare to resume normal cyclicity upon removal of the device for pursuant of reproductive procedures.

Facilities 2.2

All experimentations were performed at the Equine Reproduction Lab (ERL) at Colorado State University (CSU). The ERL is a world-renowned equine breeding facility that houses over 300 horses on over 70 acres of land during the busy breeding season. The faculty and staff are devoted to a busy clinical practice; robust research programs; and education opportunities for veterinary residents, graduate students, veterinary students, interns and undergraduate students. There are more than 100 stalls and 40 acres of land including many dry lot paddocks. All personnel have years of experience with handling both mares and stallions within a reproductive environment.

Experiment one: design and development of an equine intravaginal ring 2.3

Purpose 2.31

Two grade mares of average ages were used in this experiment. An intravaginal ring device in the mare is a novel concept that required significant design and development efforts. Experiment

one was dedicated to the invention of this equine intravaginal ring by utilizing multiple ring diameters and designs to evaluate retention rates and overall safety of the ring.

Hypothesis 2.32

A silicone ring designed to rest in the vaginal vault of the mare will remain comfortably within the vagina for the duration of treatment.

Objective 2.33

In experiment one, two objective were explored. The first objective was to determine the design and size of an IVR for ultimate retention in the mare. The second objective was to determine the safety of an IVR in the mare.

Results 2,34

As a result of these trials, two vaginal ring prototypes (14.2cm and 13 cm in diameter) proved to remain within the vaginal vault of the mare for 7 days. Ultimately, the 14.2 cm ring retained its position the most effectively over time without posing health issues to the mare. Therefore, this IVR represented the size and material utilized for the subsequent experiments.

Experiment two: optimal form and concentration of altrenogest within the ring 2.4

Purpose 2.41

Nine grade mares of average ages were used to evaluate the pharmacokinetic values of altrenogest delivery through an intravaginal device in the mare. The three rings tested in this

experiment were: Ring type “ALTA-xx”, “ALTB-xx”, and ALTC-xx” containing .22% oil in altrenogest, 1% powder altrenogest, and 5% powder altrenogest, respectively.

Hypothesis 2.42

A 14.2 cm silicone intravaginal ring will deliver altrenogest to the mare through a specific and ideal form and concentration of altrenogest.

Objective 2.43

In experiment two, the objectives were to determine the optimal levels of altrenogest in this experimental ring, as well as determine the ideal form of altrenogest within the experimental ring.

Results 2.44

55% of mares retained a 14.2 cm silicone vaginal ring containing 1% of altrenogest suspended as a matrix within the ring. After the analysis of three types of altrenogest releasing intravaginal rings, the suspended altrenogest within a silicone ring design allowed for longer-term studies to take place in experiment three.

Experiment three: Efficacy of altrenogest intravaginal rings for estrus suppression 2.5

Purpose 2.51

The final experiment of this study was to evaluate pharmacodynamic values of altrenogest delivery through an intravaginal device in the mare. This aim focused on the use of altrenogest releasing intravaginal rings for the purpose of estrus suppression and whether it will effectively

hinder the untoward behavior of the mare associated with the estrus phase of the reproductive cycle. IVRs developed in the previous objectives were used to move forward in a longer experiment with a unified and specific ring diameter and altrenogest concentration for each mare.

Hypothesis 2.52

A 14.2 cm silicone intravaginal ring containing 1% altrenogest suspension will deliver adequate steady-state levels of altrenogest for estrus suppression in the mare over a period of time.

Objective 2.53

The objective of experiment three was to examine the pharmacokinetics and pharmacodynamics of altrenogest releasing intravaginal rings over a prolonged period of time.

Results 2.54

The 14.2 cm intravaginal ring containing 1% powder altrenogest concentration demonstrated in this study shows a promising future for effective, safe, and reversible estrus suppression method in the mare. Additional studies are required to determine the efficacy of its long-term use through ring retention and drug delivery rates.

Materials and methods 2.6

Uterine and vaginal cultures 2.61

To evaluate bacterial growth or contamination within the vaginal vault and uterine environment, culture sampling of the uterus and vagina in contact with the device were obtained at the beginning and end of every trial to evaluate her normal parameters before placing the IVR

and once the IVR is removed. Culture plates were examined at 24 and 48, and 72 hours while being kept in an incubator at 37 degrees Celsius. Culture swabs were kept at 4 degrees Celsius for three days. If no growth was identified on the plate, both the culture swab and plate were discarded. If growth was identified during the three day period, saved culture swabs were used to isolate individual colonies on a quad plate with multiple agars to select for both gram-negative and gram-positive bacteria.

Speculum exams 2.62

The safety of the intravaginal vaginal ring was monitored through visual inspection utilizing an endoscopic exam of the mare's vaginal vault containing the silicone ring at the beginning of her trial as well as daily speculum exams. The mare's normal parameters were evaluated prior to inserting the IVR, and any redness, irritation or abnormalities were recorded during exams.

Transrectal ultrasonography 2.63

Mares were monitored through transrectal ultrasonography every five days upon identifying an ovulation. Prominent structures such as CL's, varying sized follicles, uterine edema, and uterine fluid were noted during these examinations. In each mare, the dominant follicle was identified and followed through ovulation. Upon ovulation, each mare was monitored every five days and the two largest follicles were identified and recorded. At the end of each treatment cycle, mares continued to be transrectally ultrasounded and guided into their next treatment cycle following an additional ovulation.

Blood sample collection 2.64

Blood samples were obtained in purple top collection tubes and centrifuged to generate serum that was frozen at -80◦C until hormone analysis was performed. Samples for altrenogest levels in the serum were collected prior to, throughout, and following the cessation of treatment for experiments two and three.

Placement of the intravaginal ring 2.65

The mare's tail was wrapped and secured to the side. The vulva and perineal area was cleaned at least three times with a gloved hand and antibacterial soap and her caudal vestibule and clitoral region and checked for excessive debris. The clean intravaginal rings were then wiped with 70% ethanol wipes prior to insertion and folded within the palm of the person inserting. Donning a sterile palpation sleeve and sterile gloves, a small amount of sterile lubricant was applied to the back of the hand, and the ring effectively passed through the caudal reproductive tract. Ultimately the ring was placed within the anterior vaginal vault, ventral to the cervix and allowed to expand and rest naturally on the floor of the vaginal vault. Correct placement was verified by a speculum exam immediately following placement.

Removal of the intravaginal ring 2.66

In the event that the ring was expelled prior to the end of the trial, the ring was easily located in the dry lot paddock where the mares were housed. Rings that remained in place within the vaginal vault were manually removed at the end of each trial. The vaginal area was prepped in the same manor when inserting the ring. Using a sterile sleeve and sterile lubricant, the ring is

gently removed from the vaginal vault, and a final speculum exam and culture/cytology was performed.

Intravaginal Ring Materials 2.67

Silicone tubing used for IVR fabrication was USP Class VI, platinum-cured Sani-Tech® ULTRA-C (Saint-Gobain, Taunton, MA). Springs for IVR cores were obtained from McMaster-Carr (Santa Fe Springs, CA). Liquid silicone resin (LSR) used to fabricate fit-test IVRs was MED-4940 (Nusil, Carpenteria, CA), and LSR used to fabricate drug containing IVRs and associated placebos was Elkem Silbione RTV-4420 (Factor II, Inc., Lakeside, AZ). Altrenogest powder was obtained from Toronto Research Chemicals, Inc (Toronto , ON). Altrenogest (Regu-Mate®) in oil was obtained as a 0.22% (w/v) solution from Merck (Kenilworth, NJ).

Intravaginal Ring Fabrication 2.68

The IVRs used for fit testing were fabricated from silicone tubing with dimensions ranging from 13-18.5 cm in diameter. For spring-supported IVRs, the spring was threaded inside tubing of appropriate length, leaving a ~25 mm gap at each end of the tubing. A tubing segment of OD matching the IVR tubing ID was used to form a joining "plug" of ~50 mm length. The plug was placed in one end of the IVR tube, and the IVR tube formed into a circular shape and attached to the opposite end of the plug. The plug was bonded to the IVR tubing using LSR and cured 10 min at 160°C. Any gaps remaining in the IVR joint were filled with LSR and cured to form a smooth surface. For silicone supported IVRs, the IVR tubing was filled with LSR, leaving ~25 mm at each end empty. The LSR was cured 10 min at 160°C. The IVR was formed into a circular shape and bonded as described for the spring-supported IVRs.

Intravaginal Ring Fabrication for "ALTA-xx" 2.69

Experiment two evaluated various concentrations and forms of altrenogest within the silicone ring. Rings labeled "ALTA-xx" were prepared in the following manner: The unmedicated inner core was prepared by filling a silicone tube with RTV 4420 LSR and allowing the LSR to cure, forming a solid silicone core (9.5 mm OD, 376 mm length). The core was placed inside a silicone tubing sheath (15.9 mm OD, 12.7 mm ID, 406 mm length) such that a 15 mm gap remained at each end of the sheath. A silicone tubing support (12.7 mm OD, 9.5 mm ID, 10 mm length) was placed around one end of the core and bonded to both core and sheath with LSR to close off one end of the IVR assembly. A second 10 mm length silicone support tube was placed around the other end of the core, but not bonded, so that a reservoir was formed in the gap between the inner wall of the sheath and the core. This reservoir was filled with altrenogest solution using a syringe and Teflon needle inserted between the core and 10 mm support. A LSR layer of ~ 3 mm thickness was placed in the end of the sheath tube and allowed to cure overnight to seal the oil filled reservoir. The filled IVR assembly was formed into a ring and bonded as described for Type A IVRs.

Intravaginal Ring Fabrication for ring types "ALTB-xx" and "ALTC-xx" 2.6a

Rings labeled "ALTB-xx" or "ALTC-xx" were prepared in the following manner: Liquid silicone resin (LSR; Silbione RTV 4420, Elkem) parts A and B were mixed 1:1 in an asymmetrical centrifugal mixer (SpeedMixer DAC 150.1 FVZ-K, FlackTek, Inc.). Altrenogest powder was added to the LSR mixture at 1, 2, or 5% (w/w) and blended in the SpeedMixer to form a smooth, yellowish, opaque mixture. Entrapped air was removed from the LSR blend by placing in a vacuum chamber at ~ 25" H2O vacuum for 5 minutes. Outer sheaths of IVRs were fabricated from commercially available silicone tubing (15.9 mm OD, 12.7 mm ID). A silicone plug (12.7 mm

diameter, 30 mm length) fabricated by filling a silicone tube with LSR and allowing to cure was inserted 15 mm into one end of the sheath and bonded with LSR. The sheath was secured vertically with the open end up and filled with altrenogest-LSR mixture, leaving ~ 25 mm empty sheath at the top. The LSR was allowed to cure overnight at 60°C. The sheath was then formed into a circular shape, inserting the protruding end of the plug in the open space at the opposite end of the sheath. Any remaining unfilled space around the plug was filled with LSR, using LSR as an adhesive to seal the plug inside the sheath. Teflon tape was wrapped around the IVR joint to hold the ring together during curing and to form a smooth surface of LSR at the joint. Following curing overnight at 60°C, any remaining gap in the joint was filled with additional LSR to obtain a smooth surface.

Pharmacokinetic Analysis of Altrenogest 2.6b

Plasma samples, stored at -80°C, were thawed on ice, and 200-µL aliquots were mixed with high purity water (> 18 MOhm-cm, 200-µL) and dispensed into 96-well plates, along with a minimum of six standards and a minimum of three quality controls prepared in the appropriate matrix in accordance with FDA guidelines (FDA, 2018). Samples were spiked with internal standard (IS) solution (10 µg mL-1 levonorgestrel, LNG, solution for a final concentration of 1 µg mL-1). Sample purification was carried out in a 96-well format using a supported liquid extraction plate (ISOLUTE® SLE+, Biotage, Charlotte, NC) according to the manufacturer's instructions. The sample was eluted with ethyl acetate (2×900 µL) into a collection plate. The purified samples were dried in vacuo using a SpeedVac concentrator system (Savant SC210A Plus; Thermo Fisher Scientific, Inc.) and were reconstituted in 0.1% (vol/vol) formic acid in water-acetonitrile (70:30, vol/vol, 200 µL) prior to analysis. The samples were analyzed by LC-MS/MS on the same day as

reconstitution. The concentration of altrenogest (ALT) was measured by LC-MS/MS using a 5-µL injection volume and an HPLC system consisting of a model G1367C well plate autosampler and a model G1312B binary pump (1200 Series; Agilent Technologies, Santa Clara, CA) operating at 0.6 mL min-1 interfaced to an API 4000 triple-quadrupole tandem mass spectrometer (AB Sciex, Framingham, MA) with a Turbo VTM electrospray ionization (ESI) source. An Agilent Zorbax Eclipse XDB-C18 Rapid Resolution column (2.1 by 50 mm; 3.5 µm pore size) controlled at 40°C was used for the stationary phase. The following gradient program was used (solution A, 0.1% [vol/vol] formic acid–water; solution B, 0.1% [vol/vol] formic acid–acetonitrile): 0.5 min ramp from 70:30 A:B to 30:70 A:B; 0.5 min hold at 30:70 A:B; 0.2 min ramp from 30:70 A:B to 70:30 A:B; 3.8 min hold at 70:30 A:B (total run time, 4.0 min; ALT retention time, 2.19 min; LNG retention time, 2.21 min). The measured transition ions, m/z, in positive ESI mode were as follows: for ALT, 311.5 atomic mass units (amu) (parent) and 227.5 amu (product); for LNG (IS), 313.2 amu (parent) and 245.2 amu (product). The analytical measuring range was 0.1-10 ng/ml ALT. The lower limit of quantitation (LLOQ) was 0.1 ng/ml, and the lower limit of detection (LLOD) was 0.03 ng/ml. Data sets were analyzed using GraphPad Prism (version 9.3.1; GraphPad Software, Inc., La Jolla, CA). Samples that were below the LLOQ, but above the LLOD were set to 0.05 ng/ml in the analysis. (US FDA). All analyses were conducted under FDA Guidance for Industry- Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), and Center for Veterinary Medicine (CVM) (2001. p. 22) in Rockville, MD.

Statistical Analysis 2.6c

Paired t-tests and were performed to analyze the variables associated with comparing placebo intravaginal rings, altrenogest intravaginal rings, and oral altrenogest. P-values for teasing scores, follicular growth, and luteal phase length were used to examine the null hypothesis that there would be no difference between the estrus behaviors demonstrated between study groups. Analyses were performed using R Studio® software, and significance was established at $P \leq 0.05$.

CHAPTER 3: RESULTS

Uterine and vaginal cultures 3.1

Uterine and vaginal cultures were obtained prior to and following 16 day treatment cycles for the altrenogest releasing intravaginal ring and the placebo intravaginal ring. Overall, there were 6 incidences of bacterial growth evident throughout all three experiments. Six total incidences of bacterial growth were noted across all trials. One positive vaginal culture was noted prior to a 21 day treatment cycle. Five positive uterine cultures were observed before and after a treatment and placebo cycles. Positive cultures resolved over time as was indicated by repeat cultures. Streptococcus zooepidemicus, E. coli, and staphylococcus aureus, were the three main pathogens identified through the positive cultures.

Follicular growth and luteal function 3.2

Mares participating in placebo ring, altrenogest ring, and oral altrenogest groups preserved their corpus luteum for an average of 14.4 ± 1.2, 20.8 ± 1.3, and 21.4 ± 0.75 days, respectively. Mares receiving an altrenogest ring had a significantly longer luteal phase as compared to mares receiving a placebo ring ($P = 0.002$). A statistical difference ($P = 0.0001$) was also found between the number of days a CL remained present in placebo ring groups and the number of days a CL remained present in oral altrenogest groups (known as the luteal phase). There were no significant differences found amongst the altrenogest ring group and the oral altrenogest group (figure 2). On average, mares ovulated approximately a 44 ± 1.89 mm follicle. Average follicle sizes across all groups measured on days 5, 10, 15, 20, and 25 were 19.2 ± 2.29, 21.25 ± 3.69, 27.8 ± 3.77, 30.25 ± 1.95, and 31.9 ± 2.5 millimeters, respectively. Follicular waves occurred normally in all three

groups, and no statistical differences were found between the placebo ring, altrenogest ring, and oral altrenogest groups, as illustrated in figure 3.

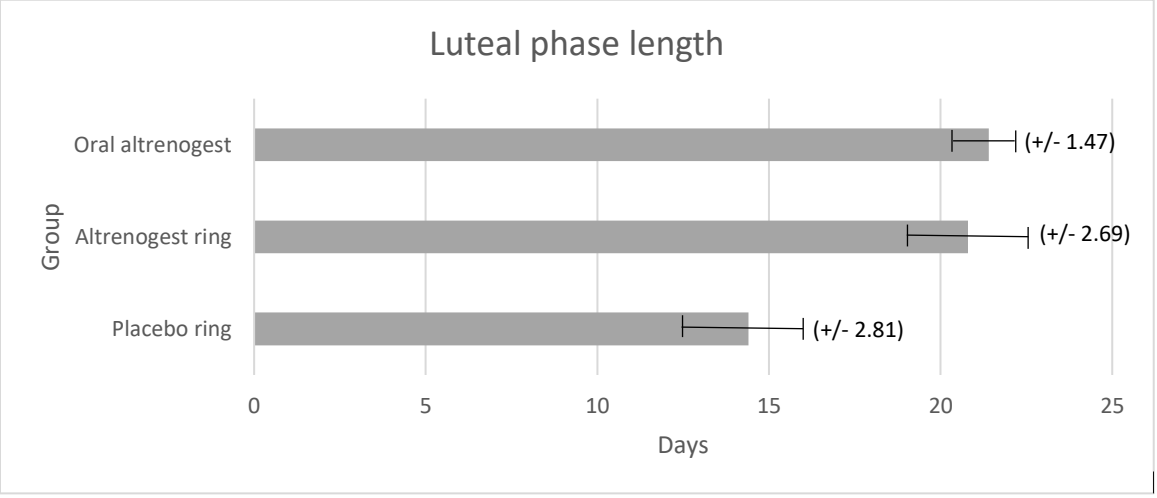

Figure 2: The average length (in days) of the luteal phases of mares in altrenogest ring (P = 0.002) and oral altrenogest groups (P = 0.0001) as compared to placebo ring groups. The luteal phase is defined as the period of time in which a functioning luteal structure is present.

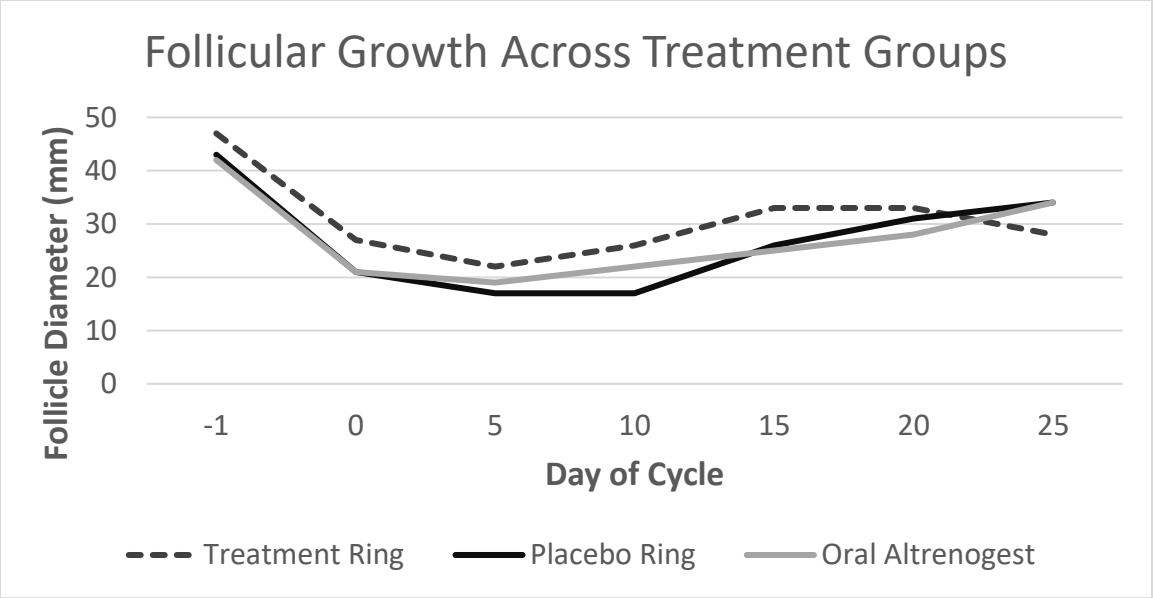

Figure 3: The average size follicle (in millimeters) for placebo ring, altrenogest ring, and oral altrenogest groups the day before ovulation and on days 5, 10, 15, 20, and 25 following ovulation. ($P > 0.05$)

Pharmacokinetic data 3.3

Maximum serum concentrations of altrenogest (C_{man}) were reached within two hours of administration for both oral altrenogest and altrenogest IVR groups. The average altrenogest serum concentration found in mares receiving oral altrenogest was approximately 0.55 ng/ml throughout the treatment period (Figure 4). Peak plasma concentrations from oral altrenogest were approximately 1.8 ng/ml upon administration. Plasma concentrations begin to drop as the dosing interval approaches, averaging at approximately 0.15 ng/ml prior to re-medicating. Approximately 72 hours after the final oral administration of altrenogest, blood concentrations were below the limit of quantification (Figure 4). The average altrenogest concentration found in mares receiving altrenogest rings was approximately 0.3 ng/ml throughout the treatment period (figure 5). Peak plasma concentrations from altrenogest rings were approximately 1.45 ng/ml upon administration.

Immediately after the altrenogest releasing ring is removed, altrenogest plasma concentrations begin to drop. Within two hours of ring removal, concentrations were below the limit of quantification (figure 5).

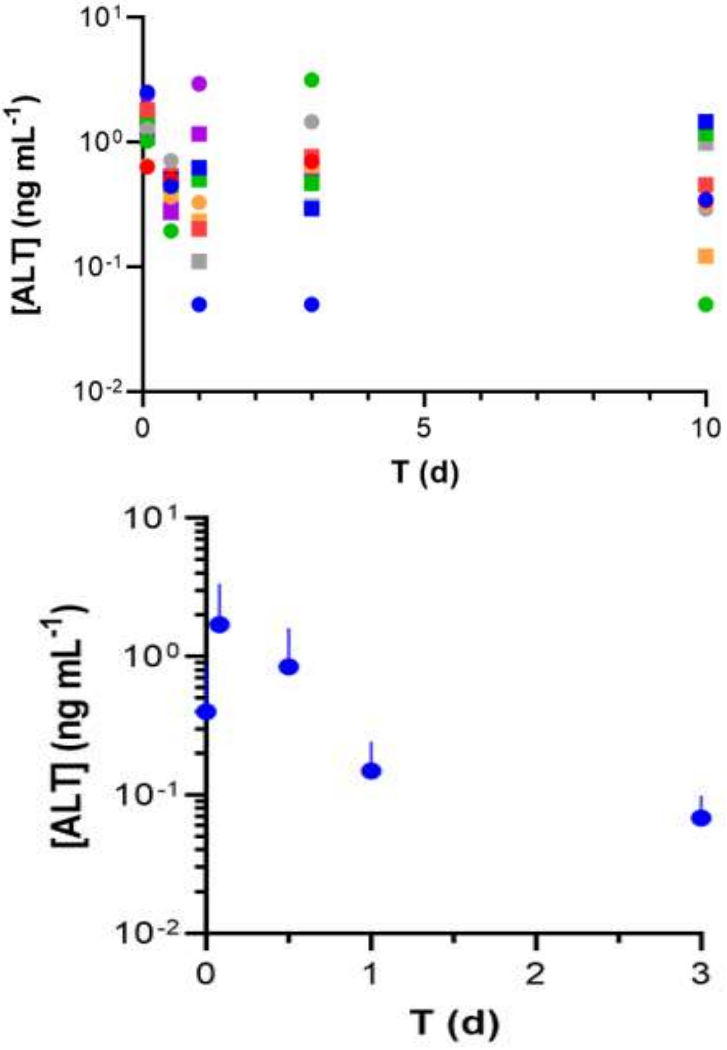

Figure 4: Top- plasma concentrations (individual) of altrenogest over 10 days while being administered orally (PO). Bottom- plasma concentrations of altrenogest for 72 hours following the last administration of oral altrenogest.

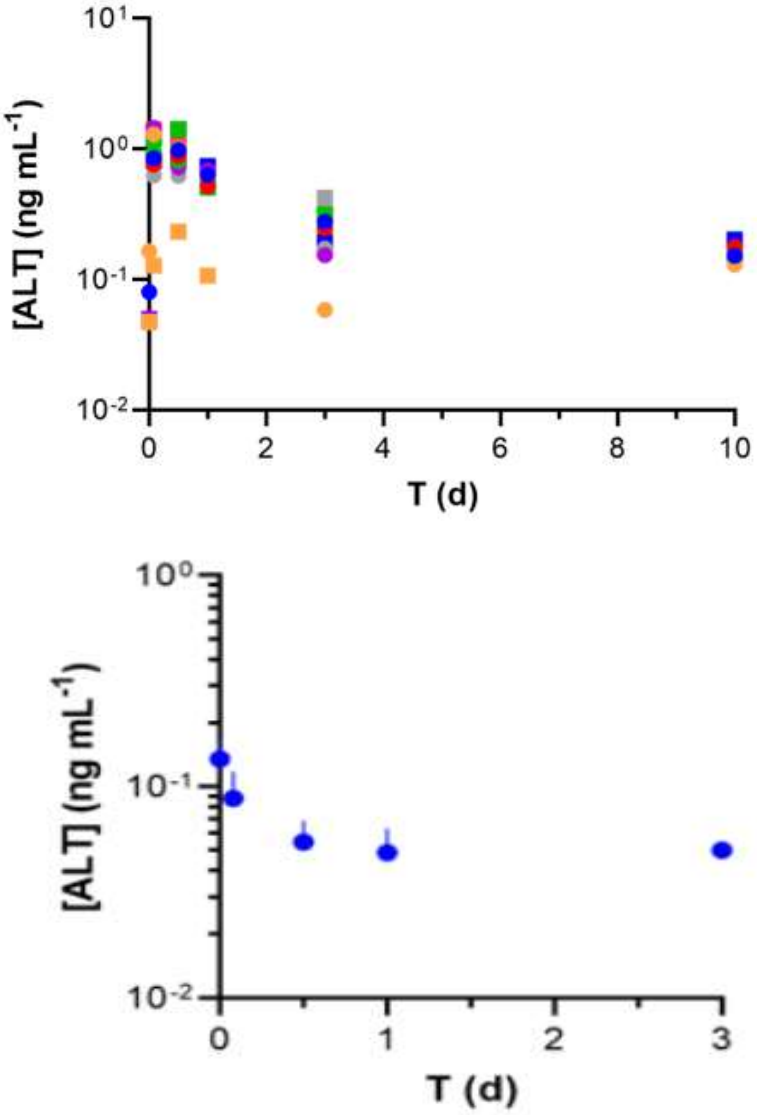

Figure 5: Top- plasma concentrations (individual) of altrenogest over 10 days while being administered vaginally using an intravaginal ring. Bottom- plasma concentrations of altrenogest for 72 hours following the last administration of vaginal altrenogest using an

Behavioral Estrus 3.4

Mares were teased two to three times a week throughout the 3 month period of this experiment. Average teasing scores for treatment rings, placebo rings, and oral control cycles are shown in figure 6. 0% of mares demonstrated teasing scores above 0 or 1 while they underwent their treatment ring cycles. Similarly, 0% of mares demonstrated teasing scores above 0 or 1 while they underwent their oral altrenogest control cycles. Conversely, 45% of mares showed teasing scores above 1 during their placebo ring cycle.

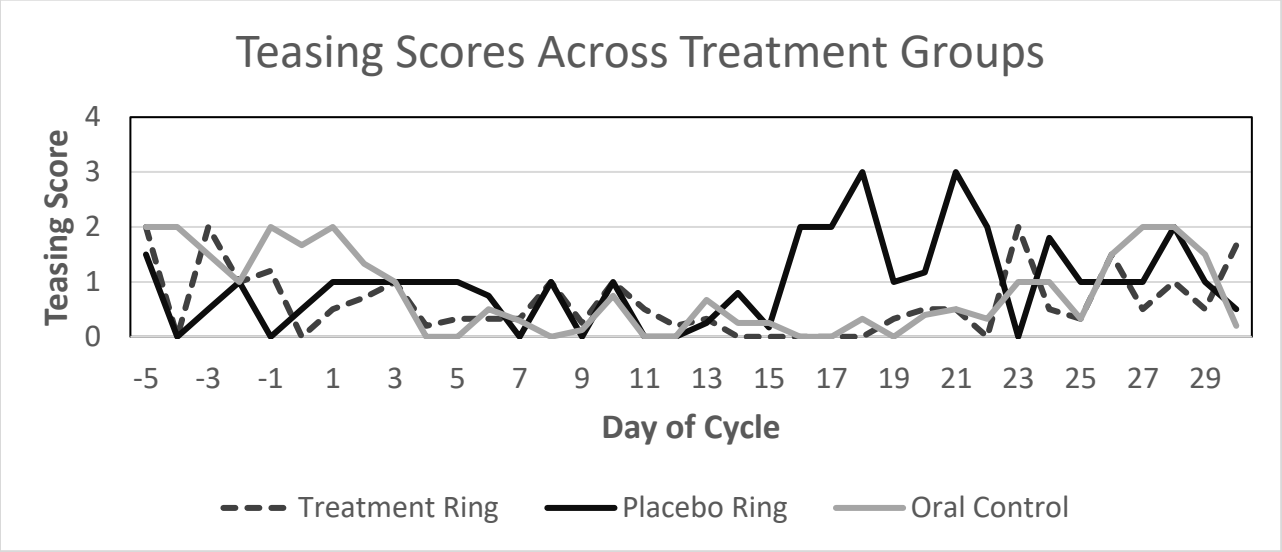

Figure 6: Average teasing scores across altrenogest ring (P = 0.018) and oral altrenogest groups (P = 0.005) as compared to placebo ring groups are shown in this graph. A teasing score of a 0 or 1 is indicative of a mare in diestrus. A teasing score of a 2 or 3 is indicative of a mare in estrus.

CHAPTER 4: CONCLUSIONS

Conclusion 4.1

Estrus suppression in the mare is widely desired amongst the equine community for a variety of reasons. Mares in training programs for competition are required to be focused and well-behaved throughout the year, despite cyclic changes in the spring and summer months. Many other mare owners desire pleasant, calm, and relaxed behaviors of their mare associated with the diestrus phase as opposed to the irritable, distracted, and likely aggressive behaviors displayed during the mare's estrus phase of her cycle. Altrenogest has proven to be a safe and effective method of estrus suppression in mares over the last five decades. Oral administration of altrenogest is associated with public health concerns when handling the drug. Concerns with mare compliance and practicality of daily oral altrenogest have also been expressed. A long-term, effective method of estrus suppression that does not require daily administration is needed in the equine industry.

Summary 4.2

The 14.2 cm intravaginal ring containing 1% altrenogest concentration suspended in silicone demonstrated in this study shows a promising future for an effective, safe, and reversible estrus suppression method in the mare. Though this study provides the much needed groundwork for a marketable altrenogest delivering equine intravaginal device, additional studies are required to determine the efficacy of its long-term use through ring retention and drug delivery rates.

CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

Discussion 5.1

Study limitations 5.11

A sample size of 12 mares was used for the final experiment of this study. Although larger sample sizes can be seen in traditional PK studies, it is not uncommon for equine pharmacokinetic studies to be limited to a sample size of fewer than 20 animals. The duration of this study was sufficient for achieving the objectives within each experiment, however it is important to note that future studies will likely examine the long-term efficacy of this device. Treatment time should extend through at least two full 21 day treatment cycles in the next steps. As seasonal long day polyestrous breeders, mares tend to cycle an average of 10 times throughout the year, all concentrated within the months of April and October. Although the number of cycles depends on many variables such as geography and hormone therapies, this is a relatively short window of time to conduct pharmacokinetic studies relating to estrus behavior. The reproductive performance of the mare during this period of time influences how effectively researchers were able to collect data.

Uterine and vaginal cultures 5.12

The vaginal ring method offers several advantageous aspects to administration. As a vaginal mode of delivery, the ring will never pass through the cervix of the mare. The cervix offers the mare's reproductive tract a barrier to the harmful pathogens it may be exposed to. Some intrauterine estrus suppression devices (IUD) were passed through the cervix into the uterus of the mare, potentially dragging external pathogens with it. The vaginal ring is also unique from other vaginal devices in that it is a completely internal device within the vaginal vault. A great deal of

vaginal device designs being researched or on the market today contain an external string for the purpose of removing the device. An intravaginal ring aims to keep the uterine environment as sterile as possible by preserving the vaginal vault as a closed environment. The four most common equine uterine pathogens in Colorado are streptococcus zooepidemicus, escherichia coli, klebsiella pneumoniae, and pseudomonas aeruginosa. Mares are routinely treated for these pathogens in breeding programs, and it is not unusual for these infections to clear without the interference of treatments.

Follicular growth and luteal function 5.13

Follicular waves were still expected to occur normally throughout all three groups. Altrenogest does not appear suppress or enhance follicular growth throughout the treatment groups, however further studies should be conducted to evaluate the long term effects of vaginally delivered altrenogest. Several studies looking at altrenogest administration have shown varying results. Studies such as Lofstedt, et al. examined the effects of oral altrenogest delivered over 15 to 20 days. This study showed that altrenogest was unable to suppress the growth of follicles and subsequent ovulation, however it did show a prolonged luteal phase in several of their mares. Conversely, James et al. (1998) found short-term administration of 0.044 mg/kg altrenogest upon the detection of a larger follicle will successfully delay ovulation when administered. When attempting to replicate these results, Bruemmer et al. (2000) achieved conflicting results, showing that administration of 0.088 mg.kg altrenogest did not delay ovulation in a larger field setting. Many variables play a role in the effect of altrenogest on follicular growth. In this current study, it was shown that a slightly prolonged luteal phase may be present amongst treatment groups, however longer-term studies are needed.

Pharmacokinetic evaluation of altrenogest 5.14

Past pharmacokinetic evaluations of altrenogest have shown a much higher LLOQ (approximately 2.0 ng/mL in Machnik et al. 2007) and LLOD than this current study, which has a LLOQ of 0.1 ng/mL. Injectable altrenogest administered intramuscularly by McConaghy et al. (2016) at 0.3 mg/kg showed the mean circulating concentration of altrenogest was determined to be 33.52 ng/mL, and plasma altrenogest concentrations remained above 0.5 ng/mL for 6.2 days. The pharmacokinetics of intra-rectal altrenogest has also been assessed. Ellis et al. (2019) administered 0.088 mg/kg of altrenogest both per rectum and per oral for 5 days and found that the C_{max} of rectal altrenogest was considerably lower than that of oral altrenogest, and clearance was much more rapid through intrarectal administration than oral administration. Intrarectal administration of altrenogest suggested a lower bioavailability than oral altrenogest, and was suggested that use in hospitalized mares may be beneficial.

The blood analysis of altrenogest administered through an intravaginal ring suggests more consistent bioavailability compared to oral altrenogest, as evidenced by the distribution of serum drug concentrations at days 3 and 10 in figures 4 and 5. The initial spike of altrenogest in the blood from the IVRs can be attributed to the loading dose built into the design of the ring, meant to deliver a burst release upon administration and reach steady-state thereafter.

One study looked at the effects of a vaginally delivered detomidine (Seddigh et al. 2019), and determined that it was well-absorbed when administered intravaginally and maintained a higher mean plasma concentration compared to intravenous administration. Although vaginal administration is less commonly studied in mares, rectal administration of other drugs has been widely studies in horses. Many physiological factors affect drug absorption across the vaginal

mucosa such as the thickness of the vaginal epithelium and the composition, pH and volume of vaginal fluid (Hussain & Ahsan et al. 2005). Therefore, it is possible that drug absorption could be affected by the reproductive stage of the mare, which may change the thickness of the epithelium, volume of cervical mucus and pH of vaginal fluid. Outliers in this study can be attributed to mares who have expelled their rings prior to the completion of their trial, and therefore blood altrenogest levels were not an accurate representation of altrenogest delivery through the IVR.

Establishing therapeutic levels of altrenogest 5.15

Past studies have determined therapeutic concentrations of altrenogest to be at or above 0.5 ng/ml. The serum altrenogest data from experiment three coupled with teasing scores from altrenogest ring, placebo ring, and oral altrenogest groups suggest the therapeutic level of altrenogest may be much than previously thought. Past PK studies analyzing altrenogest serum concentrations have established a LLOQ of 0.5 ng/ml. The altrenogest analysis method developed for this IVR study established a LLOQ of 0.1 ng/ml and a LLOD of 0.03 ng/ml, allowing for altrenogest concentrations to be identified at a much lower level. The teasing scores of altrenogest IVR groups and oral altrenogest groups were consistent with previous studies examining the efficacy of altrenogest for estrus suppression, while maintaining serum altrenogest concentrations between 0.08 ng/ml and 0.5 ng/ml in altrenogest IVR groups (figure 5) and between 0..8 ng/ml and 4.0 ng/ml in oral altrenogest groups (figure 4).

Behavioral Estrus 5.16

Teasing scores were collected multiple times a week for the duration of this study. Teasing scores zero through three were used to evaluate and assign mares to a category relating to the phase

of their cycle. Mares exhibiting a teasing score of zero or one were considered to be in diestrus while mares exhibiting a teasing score of two or three were considered to be in diestrus. Mares on oral or intravaginal altrenogest showed a prolonged diestrus period (demonstrated scores zero or one) compared to mares receiving a placebo ring. Teasing scores were significantly reduced in the altrenogest ring groups compared to the placebo ring groups throughout treatment, while treatment ring groups and oral altrenogest control groups demonstrated similar teasing score results. The intravaginal ring offers a steady state delivery of altrenogest, eliminating the fluctuation in therapeutic levels often observed with oral altrenogest administration.

Future directions 5.2

Altrenogest patent Information 5.21

The development of altrenogest for the control of estrus suppression in the mare has been a coveted area of research since its initial development and patent information nearly 5 decades ago. A complete list of the 557 patent results found for altrenogest development or use can be found at https://ppubs.uspto.gov/. Some more common brand names and patents are shown in table 1. Altrenogest can be found most commonly as oral and injectable medications, however recent research has evaluated altrenogest delivery through a variety of other mechanisms.

Altrenogest use over Time 5.22

Altrenogest is mainly utilized on mares and sows by industry professionals. Some of the highest altrenogest use growth rates have been shown from 2015 in North America (United States, Canada and Mexico), Europe (Germany, UK, France, Italy, etc.), South America (Brazil, Argentina, Columbia etc.) and Asian-Pacific countries. As demonstrated by a survey conducted

by Equus Magazine, equine ownership is expected to increase in the future, increasing the desire for estrus suppression. When current equine owners are asked about future expectations of ownership, 17.3% of owners expect to own/manage more horses in 2022. When this study is compared to a 2018 survey, there is an increase in expected stability regarding the number of horses owned/managed. According to the American Horse Council, a study conducted in 2017 accounts for the most current comprehensive equine population number in the United States, which counts 1,013,746 horse owners owning or leasing farms housing 7,246,835 recreational horses in the US, not including a total of 459,526 horse farms in the US, with an agricultural population of 2,847,289 working horses. Current populations and expected growth in equine ownership in the future support promising ideas that altrenogest administration and desire to suppress estrus will continue to greatly increase.

Table 1: Common FDA approved products containing altrenogest

Brand name	**Patent information**	**Uses**	**Manufacturer**	**Average Cost (per dose)**
Regu-mate®	Approved 1983. NADA 131-310, Merck Animal Health, 800-211-3573	Estrus suppression in mares at a dose of 0.044 mg/kg	Merck Animal Health	$1.50
Ovamed™	Previously Altresyn, approved 2012. ANADA 200-481, Bimeda, Inc., 888-524-6332	OvaMed is a progestin used to suppress estrus cycles in mares to maximize breeding efficiency	Bimeda	$1.30
Altren®	Approved 2017. ANADA 200-620, Aurora Pharmaceuticals, LLC, 888-215-1256	Estrus suppression in mares containing 2.2 mg/mL	Merck Animal Health	$1.65
Matrix® and SWINEMATE®	U.S. patent Registration Nos. 5,468,485 and 5,378,744	For synchronization of estrus in sexually mature gilts that have had at least one estrous cycle.	Merck Animal Health	$1.90

Future Directions of Altrenogest Use 5.23

Altrenogest has been trusted and used in an oral and injectable form for decades. The expiration of the patent on altrenogest has led to the research development of several alternative methods for altrenogest delivery such as oral tablets, sustained release injections, soft capsulation, and subcutaneous implants. The continued use of altrenogest and the desire for estrus suppression in the mare will ensure the upward trend of altrenogest usage other than oral administration in the upcoming years.

Future Directions of Equine Intravaginal Rings 5.24

Longer-term studies are indicated for the use of altrenogest within these intravaginal rings. Retention rates and alternative designs for altrenogest releasing intravaginal devices should be examined in the future. Retention rates increased throughout each experiment, however the ultimate goal for retention rates of this IVR is greater than 80%. Longer-term studies will likely be required to achieve FDA approval and build a true market for IVRs.

The development of an equine intravaginal ring has the possibility of leading to numerous other drug delivery devices in the mare. Novel drug delivery devices in the horse such as this are an unexplored area and represent opportunities for future pharmacologic application. An intravaginal ring device may become a vehicle for a variety of other drug delivery possibilities. The long term administration of non-steroidal anti-inflammatory drugs (NSAIDs) may have a future with intravaginal rings. There is also a possibility of looking into the effects of long-term and short-term antibiotic administration through intravaginal rings, as well as examining the long-term treatment of mares with chronic diseases such as equine metabolic syndrome (EMS) or

Cushing's disease, to name a few. Greater ring retention over a long period of time while continuing to suppress estrus behavior is the ultimate goal of this study as the development of altrenogest releasing equine intravaginal rings continues to be evaluated. The preliminary results of this study lay the groundwork necessary to build on this design concept in order to create a marketable product available to the equine industry.

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