Adenoviral Vectored Gonadotropin Releasing Hormone Vaccine for Estrus Suppression in Mares

by

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Abstract

The objective of this study was to evaluate an adenoviral vectored gonadotropin releasing hormone (GnRH) vaccine as a method to suppress estrus behavior and cyclicity in mares. An additional objective was to determine the effects of a heterologous vaccination strategy against gonadotropin releasing hormone on estrus behavior and cyclicity in mares using an adenoviral vectored gonadotropin releasing hormone vaccine to prime, and a protein based gonadotropin releasing hormone vaccine to boost. Twelve normal cyclic mares were included in the study which was divided into two phases. The first phase (weeks 0-46) included one ovulatory season and phase two (weeks 47-70) included the subsequent ovulatory season.

During phase one, treatment mares (n=5) were vaccinated twice, 4 weeks apart, with an adenoviral vectored gonadotropin releasing hormone vaccine. The vaccine was a replication-defective E1/E3 deleted adenovirus (Ad5) vector expressing antigens consisting of 16 multimers of GnRH, bacterial leukotoxin, T-helper epitopes, and other various hinge and linker amino acids (Ad-GnRH). Each 1 milliliter dose of the vaccine contained 4.64E10 infectious units. Five additional mares served as controls for estrus behavior, cyclicity, and seasonality. During phase two, mares that had been vaccinated during phase one (previous ovulatory season) were administered a single vaccination using a quarter of the labeled dose (sub-effective) of a protein based gonadotropin releasing hormone vaccine (100 µg protein conjugate per quarter of the labeled dose: Equity® Oestrous Control Vaccine, Zoetis, Australia). Two naïve mares (protein vaccine control mares) received an equivalent dose of the protein based gonadotropin releasing hormone vaccine to determine if the 100 µg protein conjugate dose was sub-effective for suppression of cyclicity and estrus. Anti-GnRH antibodies, estrus behavior, reproductive tract sonography, and serum progesterone concentrations were monitored over two consecutive breeding seasons.

Following homologous prime and boost using Ad-GnRH, all treatment mares developed anti-GnRH

antibodies and the antibody response remained significantly different from that of time zero for 32 weeks during phase one. There was no effect on mean interestrus interval, reproductive cyclicity, or estrus behavior. Following heterologous boost during phase two, all treatment mares experienced an anti-GnRH antibody response that was maintained for at least 17 weeks (remainder of study period). All treatment mares became anestrus based on serum progesterone concentrations and transrectal sonographic findings. Estrus behavior became erratic and unpredictable, and therefore, interestrus interval could not be calculated. Protein vaccine control mares developed anti-GnRH antibodies after vaccination and the response was maintained for 15 weeks. There was no effect on interestrus interval, reproductive cyclicity, or estrus behavior. The day following Ad-GnRH boost, three of five treatment mares developed a non-painful 3-5cm raised nodule at the injection site. Following heterologous boost, two of four treatment mares developed a small (<2cm) raised, non-painful nodule. All injection sites reactions resolved within three days without treatment.

This study demonstrates that mares are capable of developing an anti-GnRH antibody response to homologous immunization using a replication-defective E1/E3 deleted replication-defective adenovirus vector encoding GnRH peptide, bacterial leukotoxin, and T-helper epitopes. Homologues prime-boost vaccination of mares with Ad-GnRH at the dose and frequency used in this study does not result in suppression of reproductive cyclicity and estrus behavior.

This study demonstrates that heterologous prime-boost vaccination of mares using an Ad-GnRH prime and protein based GnRH vaccine boost results in an antibody response that suppresses reproductive cyclicity, and interferes with estrus behavior. Vaccine-induced suppression of reproductive cyclicity and estrus was maintained for at least 17 weeks. Vaccinated mares experience minimal side effects following vaccination with either Ad-GnRH or heterologous boost using a sub-effective dose (100µg protein conjugate) of a protein based GnRH vaccine.

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CL	Corpus luteum
ml	Milliliter
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
LH	Luteinizing hormone
FSH	Follicle stimulating hormone
HPG	Hypothalamic gonadal axis
eCG	Equine chorionic gonadotropin
GnRH	Gonadotropin Releasing Hormone
ER	Estrogen receptor
KNDy	Kisspeptin/neurokinin B/dynorphin
HCG	Human chorionic gonadotropin
IU	International units
GPCR	Rhodopsin-like G protein coupled receptor
CTL	Cytotoxic $CD8^+$ T lymphocytes
Th	T helper
Th1	T helper 1
Th2	T helper 2
Treg	Regulatory T cell
APC	Antigen presenting cell
DC	Dendritic cell
Ig	Immunoglobulin
GCs	Germinal centers

FDC	Follicular dendritic cell
MHCI	Major compatibility complex class 1
MHCII	Major compatibility complex class 2
Tfh	Follicular helper T cell
CAR	Coxsackie/adenovirus receptor
DNA	Deoxyribonucleic acid
Ifu	Infectious units
IEI	Interestrus interval

1. Literature Review

1.1 Mare Reproductive Physiology

Mares are seasonally polyestrus, long day breeders, with a natural breeding season extending from April to October in the northern hemisphere and from October to March in the southern hemisphere [1]. Day length serves as the predominant circadian pacemaker that synchronizes ovarian cyclicity with environmental conditions that are favorable for survival.

During the ovulatory season, mares exhibits an 18-22 day reproductive cycle that is divided into two phases: estrus and diestrus [2]. Estrus is defined as the period during which the mare is sexually receptive to a stallion. Behavioral estrus is stimulated by increasing amounts of estrogens secreted by one or more dominant (≥25mm) ovarian follicles in the absence of progesterone. The average length of estrus in mares is 6.5 days, but it is highly variable and can range from 4.5-8.5 days [3]. On average, estrus is of the longest duration during the spring. It decreases progressively to become stable during summer, and then increases in duration gradually during the fall [1].

Diestrus is the phase in which the mare is not receptive to the presence of a stallion (non-receptivity) and averages 14 days. Non-receptivity occurs when circulating progesterone levels secreted by the ovarian cells of the corpus luteum (CL) are greater than 1-2ng/ml [1, 4]. Towards the end of the luteal phase prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is released from the endometrium. This occurs in a pulsatile manner between days 13 and 16 after ovulation. The $PGF_{2\alpha}$ diffuses into uterine venous drainage and reaches the ovaries via systemic circulation where it induces luteolysis, and initiation of the next follicular phase of the cycle during the breeding season [1, 4].

The anovulatory season (anestrus) extends from the last ovulation of the ovulatory season to the first ovulation of the subsequent ovulatory season. The anovulatory season can be further divided into three phases: receding/recession, inactive, and resurging phase [5]. The receding phase begins after the last ovulation for the year and is characterized by failure of a large follicle to ovulate at the expected time. The

resurging phase occurs as a gradual return to the ovulatory season. During this time period follicles repeatedly grow and then regress to be replaced by another growing follicle until eventually a large dominant follicle grows and ovulates, thus beginning cyclicity [5]. The mechanism by which photoperiod regulates endogenous rhythms of seasonality in horses involves relay of photoperiodic signals from the retina to the suprachiasmatic nucleus in the hypothalamus, which acts as the central circadian pacemaker and controller of pineal melatonin secretion. Circadian clock genes that control pacemaker function are suggested to govern seasonal reproduction, and preliminary studies to identify equine homologues of core molecular clock genes such as ARNTL, PER2, CLOCK, and CYR1 have been identified and may help elucidate the exact mechanisms that regulate seasonality in mares [6].

Follicle dynamics

Small tertiary follicle (up to 10 or 15mm in diameter) populations are believed to continuously grow and regress throughout the year, thus providing a reservoir for larger follicles. This basal activity continues throughout all reproductive states (estrus, diestrus, anestrus, pregnancy) [1, 2]. A major wave of follicles initially grow in synchrony. The dominant follicle of each wave takes one of three pathways. The dominant follicle can either regress (anovulatory wave), ovulate (ovulatory wave), or become hemorrhagic (hemorrhagic anovulatory follicle). A major follicular wave beginning during mid to late diestrus and giving origin to a dominant follicle that ovulates during estrus is defined as the *primary wave* [2]. A major follicular wave that occurs during early diestrus, and is the origin of a dominant diestral follicle, is defined as a *secondary wave* [2].

Hypothalamic pituitary gonadal axis

Gonadotropin releasing hormone (GnRH) is a decapeptide that has a central role in the control of reproduction, cyclicity and sexual behavior. It was first isolated from the mammalian hypothalamus in the

1970's and has the amino acid sequence (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH_2) [7]. Twenty three forms of decapeptide have been identified in vertebrate and protochordate species. Of vertebrates, up to three forms can exist which vary in their tissue distribution, amino acid sequences, and gene location [8]. Of the forms described, NH-2 and COOH- terminal sequences are conserved and are essential for receptor binding and activation [8].

GnRH is produced by neurosecretory cells in the hypothalamus. It is processed and packaged in storage granules that are transported down axons to the median eminence where it is released in synchronized pulses from nerves endings into the hypophyseal portal system every 30-120 minutes [9]. Each GnRH pulse stimulates the release of luteinizing hormone (LH), and to a lesser extent follicle stimulating hormone (FSH), from gonadotropes in the anterior pituitary gland [8]. These gonadotropins are released into systemic circulation, and they act at the level of the gonad. FSH is essential for follicular recruitment whereas LH facilitates follicular maturation, production of estrogens, ovulation, and luteinization [1]. Estrogens and inhibin are produced by granulosa cells of one or more dominant follicles and these ovarian hormones have negative feedback effects on FSH. During the estrus period, estradiol has a positive feedback effect of GnRH secretion. Hypothalamic nuclei that are sensitive to the positive feedback effects of estradiol secrete high amplitude, high frequency pulses of GnRH once estradiol reaches a threshold concentration. GnRH then stimulates a surge of LH from the anterior pituitary. This surge in LH induces ovulation, after which, a corpus luteum (CL) forms. Luteal cells secrete progesterone which has a negative feedback effect on the release GnRH from the hypothalamus and LH for the anterior pituitary. Progesterone secretion from the corpus luteum characterizes the luteal phase.

A notable discovery in the early 1980's was made using combined autoradiography and immunocytochemistry. It was suggested that there must be a second population of neurons that act as arbitrators to pass on feedback from sex steroids to GnRH neurons because GnRH neurons do not contain estrogen receptors [10, 11]. Further research confirmed that a collection of estrogen receptor (ER)

containing neurons (predominantly ERα) named Kisspeptin/neurokinin B/dynorphin (KNDy) neurons are responsible for transmitting both positive and negative signals from sex steroids to GnRH neurons [12]. Pharmacological studies utilizing kisspeptin have demonstrated its ability induce a sustained LH response in monkeys, rats, ewes, cattle, and horses [13-17]. In female rats, the blockage of local kisspeptin action in the preoptic area of the brain with a specific monoclonal antibody to rat kisspeptin completely abolished the proestrus LH surge and inhibited estrous cyclicity [18]. Sex steroids mediate their negative feedback to GnRH neurons by stimulating dynorphin secretion and suppressing neurokinin-B and kisspeptin secretion [11]. Study into this area for the purpose of suppression of the hypothalamic gonadal (HPG) axis is limited, but reveals another potential target for control of reproduction in these species.

Estrus behavior

During estrus, mares exhibit behaviors to indicate receptivity to a stallion. These behaviors include tail raising, eversion of the clitoris, urinating, and lowering of the pelvis. These behaviors are exacerbated by the presence of a stallion, but in some cases, they are exhibited in the presence of other mares and geldings [3]. The intensity of estrus (heat) varies between mares, and ranges from undetectable to very intense.

During the luteal phase, mares will elicit behaviors signaling non-receptivity to the presence of a stallion. These signs include kicking, biting, holding back the ears, switching the tail, moving away from the stallion, head shaking, pawing, or other nervous and repelling behaviors [1]. Comparison has been made between the intensity of non-receptive signs (observed during diestrus) and the intensity of non-receptive signs displayed during the anovulatory season. The intensity of non-receptive signs was greater for diestrus mares than for anestrus mares, providing evidence that progesterone has a stimulatory effect on the intensity of signs of non-receptivity in mares [19].

The expression of estrus by mares is arguably an important factor in reduced athletic performance. Many

mares and fillies fail to perform at their best due to strong estrus behavior [20]. They may become hard to manage, are aggressive, perform irregularly, or even appear lame during the time of estrus. Additionally, aggressive or unpredictable behavior can result in serious injury for handlers and riders. This behavior is intermittent, and usually corresponds to the follicular phase of the estrous cycle when the female is receptive to the stallion. In addition to the direct hormonal effects, poor behavior may also be related to pain associated with growing follicles, stretching the ovarian tunic, or from rupture of the follicle at the time of ovulation. This theory is supported by the fact that many mares exhibit pain during ovarian palpation immediately before or after ovulation [21]. In a survey of American Association of Equine Practitioner members in 1996, 90% of the veterinarians that responded believed that the estrous cycle altered mare athletic performance [22]. The impedance of athletic performance, risk of injury to handlers and riders, and potential ovarian pain experienced around the time of ovulation substantiate the need to develop methods that temporarily suppress estrus, especially in those mares that need to maintain value as a breeding animal.

1.2 Non Pharmacological Treatment for Estrus Suppression

Ovariectomy

Ovariectomy if often performed as a 'last resort' for suppression of estrus behavior in mares. In addition to elimination of any future reproductive potential, it is a common misconception that an ovariectomized mare ceases to display estrus behavior. In a survey by Jorgensen and coworkers, 44% of respondents elected to have their mare ovariectomized in order to prevent unwanted estrus behavior. Only 60% of the respondents that had their mare's ovariectomized reported an improvement in behavior [22]. Other reports show that at least 30% of ovariectomized mares continue to display estrus behavior following ovariectomy [23].

Induced pseudopregnancy

A remarkable feature of the equine placenta is the formation of endometrial cups at approximately day 38 of gestation. These cups are derived from trophoblastic cells of the chorionic girdle and secrete a glycoprotein hormone known as equine chorionic gonadotropin (eCG). This hormone assists the formation of supplementary corpora lutea and subsequent sustained serum progesterone concentration for at least 120 days, even in the event of embryo/fetal loss [3]. Elimination of pregnancy after the formation of endometrial cups has been utilized as a way to sustain increased plasma progesterone concentration and therefore eliminate estrus behavior [3]. However, terminating a pregnancy for the purpose of estrus suppression is ethically and logistically unacceptable for most owners and trainers. Instead, methods of induced pseudopregnancy that do not require establishment of an initial pregnancy have been studied as a preferred alternative.

Intrauterine devices

Intrauterine devices such as glass marbles have been shown to prolong the luteal phase in mares [24-26]. It is proposed that the device might mimic a conceptus and prevent the endometrial release of $PGF_{2\alpha}$ via the movement and physical contact of the device with the endometrium [24]. Placement of a 25mm-35mm glass marble into the uterine lumen immediately after ovulation results in prolonged CL function in approximately 40% of mares for an average of 3 months [26]. However, there are reports of mares becoming pregnant despite the presence of a glass marble in the uterine lumen with subsequent compromise or loss of pregnancy [27]. In addition, there are reports of the glass marble fragmenting in the uterine lumen leaving glass shards that induce severe endometrial damage and reduced fertility [28, 29]. To prevent these complications, a water filled 20mm plastic ball inserted into the uterus 2-4 days post ovulation successfully extended luteal function in 75% of treated mares for an average of 57 days (range 44-75 days) [24]. While apparently simple, the insertion of intrauterine devices has an efficacy that is not

reliable, and the duration of effect too short for practical application.

Intrauterine oil infusion

A one milliliter dose of either coconut oil or peanut oil administered into the uterus on day 10 after ovulation prolonged luteal function for up to 30 days with equal efficacy in 11/12 treated mares [30]. Plant and vegetable oils are rich with fatty acids which are known to modulate eicosanoid synthesis and may mimic the mechanisms underlying luteostasis achieved by the maternal recognition of pregnancy signal in the mare [30]. A later study was performed with the objective to compare and understand the utero-ovarian response to two different amounts of coconut oil administered into the uterus of mares on day 10 after ovulation. Mares received either 1ml or 0.5ml of coconut oil, however the investigators were unable to replicate the results of previous studies. In fact, the mares that received the 1ml dose of coconut oil experienced an early return to estrus (mean 12.2 days) [31].

Herbal supplements

Many herbal supplements have a label claim for calming or modifying undesirable behavior in horses, but in most instances, the efficacy of these products has not been tested or proven [32]. In addition to a lack of evidence regarding efficacy, there is some research that suggests that herbal supplements may have detrimental effects on future fertility. For example, a study by Abdulhakeem *et. al,* demonstrated that oral administration of valerian root to mice resulted in abnormalities in spermatozoal production [33]. Extracts of the fruits of the chase tree (*Vitex agnus*) are widely used to treat premenstrual symptoms in women [34]. However, there is no research evaluating the effects these herbal supplements on follicular activity in mares. Additionally, many herbal supplements are prohibited in performance horses due to drug testing regulations.

1.3 Hormonal Treatments for Estrus Suppression

Induced diestral ovulation

Secondary follicular waves can yield diestrus ovulations. The frequency of naturally occurring diestrus ovulations can vary according to breed. In thoroughbreds and quarter horses, as many as 20% of ovulations occur during the luteal phase [35]. The frequency is much less in ponies [1]. Administration of human chorionic gonadotropin (hCG) (3000IU) injected intramuscularly during the luteal phase when a diestrus follicle ≥30mm is detected can result in diestrus ovulation from a secondary wave and a subsequent prolonged luteal phase of up to 82 days [36]. A major challenge posed by this method of estrus suppression is that not all mares respond to hCG treatment during diestrus, despite the presence of a large dominant follicle, and some mares never develop a large enough follicle (>30mm) during the luteal phase of the estrous cycle [36]. Mares may require multiple transrectal sonographic examinations before a follicle of suitable size is detected and hCG can therefore be administered. This significantly influences the practicality of this method of estrus suppression.

The use of oxytocin to disrupt normal luteolysis was first described by Stout and Allen [37]. Oxytocin and its endometrial receptor are important for stimulating the pulsatile release of $PGF_{2\alpha}$ from the endometrium required for luteolysis. In this study, oxytocin was administered continuously via a subcutaneous minipump and, when administered before day 8, luteolysis was delayed in four of five mares with a diestrus period that continued for more than 30 days [37].

Further work has demonstrated that once to twice daily injections of oxytocin starting during the mid-luteal phase disrupts luteolysis thereby prolonging luteal function in 60-100% of treated mares [38, 39]. An oxytocin analogue, carbetocin binds endometrial receptors similarly to oxytocin [40]. When compared with oxytocin, it has an increased half-life, and therefore allows for decreased administration frequency. In the horse, the half-life of carbetocin is 17.2 minutes [41], whereas the half-life of oxytocin is only 6.8 minutes [42]. Despite the apparent advantage of carbetocin over oxytocin for prolongation of diestrus, a

recent study demonstrated that administration of carbetocin at a dose of 1.19mg once daily by intramuscular injection from days 7 to 14 post ovulation failed to increase the interovulatory interval [43]. The mechanism by which oxytocin, but not carbetocin, disrupts normal luteolysis after mid-diestrus administration remains unknown at this time.

Progesterone and progestin therapy

Progesterone and other progestins have been used widely for the suppression of estrus [32, 44, 45]. For women, progestins are available for oral, injectable, implantable, and intrauterine use [46]. The number of available progestin delivery modalities are more limited in horses. While an intrauterine product is not available for mares, intravaginal devices offer a relatively inexpensive and convenient method of progesterone delivery. Devices designed for cattle have not been widely accepted for use in mares because they commonly induce vaginitis [47]. An intravaginal device designed specifically for mares has been developed which contains 1.72g of progesterone (Cue-Mare^{T-M}, Bioniche Animal Health Australasia, Victoria, Australia) [48]. This device maintains serum progesterone concentrations above 1ng/ml for at least 10 days and induces only mild vaginal irritation that resolves within 48 hours of removal [48]. While the use of an intravaginal device obviates the need for daily oral administration, the product is not widely used for the purpose of estrus suppression and is not commercially available within the United States. The only oral product approved for use in mares in the United States is a synthetic progestin called altrenogest (Regu-mate, Intervet, Inc, Millsboro, Del; Altresyn, CEVA Sante Animale, Libourne, France) [32]. Altrenogest is a 17α-allyl derivative of the potent anabolic agent trenbolone which is structurally similar to testosterone [23]. Altrenogest does not adequately inhibit follicular activity and ovulation is still possible. Mares that usually exhibit signs of pain as a result of a large dominant follicle will continue to experience discomfort despite the absence of estrus [3]. Furthermore, altrenogest administration to horses is prohibited by many equine performance regulatory authorities. These regulations exist despite

evidence that, when administered at the recommended dose, altrenogest has no obvious anabolic effects that may affect competition and performance in the mare [23]. In addition, altrenogest requires daily oral administration which is inconvenient, time consuming, and in some cases, may make mares reluctant to be handled.

The injectable administration route has been used widely for estrus suppression. Natural progesterone is compounded with an oil base and is administered intramuscularly [32]. Local swelling and pain often occurs following each injection and this can interfere with training and performance due to muscle soreness [32, 49]. Other synthetic progestins, such as medroxyprogesterone acetate, norgestomet, megesterol acetate, and hydroxyprogesterone hexanoate have been administered to mares in an attempt to prevent estrus behavior without success. The reason for lack of efficacy of these compounds is presumably failure to bind adequately to equine progesterone receptors [50].

GnRH agonists

GnRH is an attractive target for reproductive control in many species due to it homology among mammals [51]. It acts via GnRH receptors on gonadotropes in the anterior pituitary gland. GnRH receptors belong to the rhodopsin-like G protein coupled receptor (GPCR) family [52]. GnRH receptors of humans, rats, mice, pigs, sheep, and horses have more than 80% sequence homology [53]. Binding of GnRH, or its agonists, with pituitary GnRH receptors leads to activation of phospholipase C, thus elevating cytoplasmic calcium and activating protein kinase C isozymes, both of which are important for gonadotropin synthesis and secretion [52]. GnRH agonists bind pituitary GNRH receptors, inducing an initial rise in gonadotropin secretion. This rise is followed by a marked reduction in gonadotropin secretion and suppression of the pituitary gonadal axis as a result of GNRH receptor internalization [54]. Deslorelin acetate is a potent GnRH agonist and has been used successfully as an implant for reproductive control in several species including ferrets, rats, rabbits, dogs, and cats [55-59]. Deslorelin

differs from natural GnRH in two amino acid substitutions: position 6 where L-glycine is replaced with the amino acid D-tryptophan, and in position 10 where glycine and its amino acid terminal are replaced with N-ethylamide [7, 60]. Deslorelin's affinity for equine pituitary GnRH receptor is 144 times greater than natural GnRH [61]. Ovuplant™ is a GnRH agonist implant consisting of deslorelin acetate, which was approved for use in mares in the United States in 1998 but is no longer available, although is still commonly used in other nations. Studies concluded that when used at the recommended dose, Ovuplant™ extends the interovulatory interval (time from one ovulation to the next) in mares by 4.4-6.2 days [62, 63]. The increase in interovulatory interval occurs due to hyposecretion of LH and FSH from the anterior pituitary, consistent with down regulation of GnRH receptor expression in the anterior pituitary [62] .

Fitzgerald *et al*. evaluated the effects of another GnRH agonist, goserelin acetate, on reproductive activity in mares. LH secretion and ovulation was suppressed for 30-90 days in 75% of treated mares using biodegradable depots that released 360-1,200 µg of the GnRH agonist per day [64]. Despite these results obtained 1993, little further research has been done to evaluate the use of GnRH agonists for the purpose of estrus suppression in mares. A major limitation to their use is the need for repeated or continuous administration and variable responses between individual mares.

GnRH antagonists

GnRH antagonists suppress the release of pituitary gonadotropins via inhibition of GnRH activation of pituitary gonadotrope receptors. There is limited research on the use of GnRH antagonists in mares. In one study, mares were administered a subcutaneous injection of a potent GnRH against, Antarelix™ (Europeptides, Argenteuil, France) [65]. The antagonist was administered during mid diestrus and treated mares experienced a prolonged interestrus interval (35 ± 3.8 days). The estrous cycle was lengthened because the follicular phase was extended from 6 days to 17.5 days [65]. A later study shared similar

findings. Antarelix™ was administered at a rate of 0.01mg/kg intravenously, twice daily for three days during the preovulatory period and ovulation was postponed for approximately 13 days [66]. The follicular phase of the cycle was extended under both conditions. While estrus behavior was not evaluated in either of these studies, it is likely that prolongation of the follicular phase of the cycle would in fact increase the duration of estrus. In order for a treatment to be considered successful for the suppression of estrus, it must maintain either diestrus, or anestrus.

1.4 Immunization against Gonadotropin Releasing Hormone

Immunology of vaccination

Vaccine induced immune effectors are essentially antibodies, produced by B lymphocytes [67]. Other potential effectors are cytotoxic $CD8^+$ T lymphocytes (CTL). The generation and maintenance of B lymphocyte and $CD8^+$ T cell responses is augmented by growth factors and signals from helper CD_4^+ T lymphocytes (Th) [67]. Th lymphocytes can be further divided into T helper 1 (Th1) and T helper 2 (Th2) subtypes. These effectors are controlled by regulatory T cells (Treg) and these cells are involved in maintenance of immune tolerance. Most vaccine antigens trigger both a B and T cell response, and $CD4^+$ T cells are required for most antibody responses [67].

The construct of a vaccine influences the type of immune effectors that are predominantly elicited and mediate efficacy. The induction of antigen-specific B and T cell responses requires their activation by specific antigen presenting cells (APCs), such as dendritic cells (DC), monocytes, and neutrophils [67]. Upon antigen exposure, DCs undergo rapid maturation and migrate towards secondary lymph nodes, where they induce B and T cell responses [67]. DCs possess a unique capacity to induce vaccine responses via co-stimulation and activation of naïve T cells, and thus the initial inflammatory response to vaccination is mediated by cells of the innate immune system. In the absence of DC activation, T cells do not differentiate into immune effectors but into regulatory Treg cells which maintain immune tolerance [68].

Initial antigen exposure initiates a B cell differentiation pathway that occurs outside of spleen/node germinal centers (extrafollicular response). In an extrafollicular response, naïve B cells generated in bone marrow circulate until they encounter a protein antigen that their specific surface immunoglobulin M receptors can bind. Antigen-receptor binding activates B Cells and upregulates a chemokine receptor named CCR7, that drives antigen-specific B cells towards recently activated DCs and T cells that provide B cell activating signals [69, 70] T cell activation of B cells drives differentiation into immunoglobulin (Ig) secreting plasma cells that produce low affinity (germ line) antibodies [70]. The extrafollicular reaction is rapid, and IgM and low levels of IgG antibodies appear in the blood a few days following primary immunization. The extrafollicular reaction is short lived, as most cells are eliminated via apoptosis within just days.

Antigen-specific B cells that receive adequate signals from T cells proliferate in specialized structures called germinal centers (GCs). Germinal centers exist within the spleen and lymph nodes, in which antigen-specific B cells proliferate and differentiate into antibody secreting B cells, or memory B cells [67]. Follicular dendritic cells (FDCs) play an essential role in B cell responses. B cells receive additional activation from FDCs and follicular T cells, after which they undergo massive clonal proliferation. This intense proliferation is associated with a switch from IgM towards IgG, IgA or IgE, and maturation of the affinity of B cells for their specific antigen [67]. This results in an increased production and higher binding affinity of antibodies [67]. B cells process vaccine antigens into small peptides that are displayed on their surface through major histocompatibility complex class II (MHCII) proteins. These complexes become available for binding with follicular helper T cells (Tfh).

The interactions that occur between antigen-specific B cells, FDCs and antigen specific Tfh cells results in proliferation and selection of B cells that have the highest possible antigen specific affinity. Additionally, they provide the signals for differentiation of B cells into either antibody-secreting B cells, or towards memory B cells [67]. Under most conditions, peak IgG antibody response is reached within 4-6 weeks after

primary immunization. Plasma cells elicited in the spleen and lymph nodes have a short life span (weeks to months). A subset of plasma cells migrate towards the bone marrow where they can continue to produce antigen-specific antibodies for years. Vaccine mediated protection requires not only an antigen specific increase in antibody titer, but production of antibodies with high avidity and persistence of vaccine antibodies and/or generation of immune memory cells capable of rapid and effective expansion upon subsequent exposure [67].

The reactivation, proliferation and differentiation of memory B cells occurs independent of GC responses. The antibody affinity maturation process is initiated in the GCs but continues for several months after the end of the GC reaction. As a consequence, high avidity antibodies are only induced after a sufficient time period following primary immunization. Secondary vaccine antigen exposure results in the production of higher avidity antibodies than the primary response [67].

T cell responses to vaccination parallel B cell responses. Vaccine induced activation of Th cells supports differentiation of B cells (Th2) or $CD8^+$ T cells (Th1). Vaccine antigens that are processed by APCs are displayed at their cell surface on MHC molecules. As a rule, MHC class I (MHC1) molecules present peptides from antigens that are produced within infected cells, whereas phagocytosed antigens are displayed on MHC II molecules [67]. At the T cell zone of lymph nodes, $CD4^+$ recognize antigens displayed by MHC II molecules, and $CD8^+$ cells recognize antigens displayed by MHC I molecules. Activated $CD4^+$ T cells provide support to DCs which provides signals that result in activation of B cells and $CD8^+$ cytotoxic T cells. $CD4^+$ activation leads to differentiation along two mutually exclusive pathways. Th1 cells participate in the elimination of intracellular antigens via activation of macrophages and $CD8^+$ T cell proliferation. Th2 cells produce interleukins that mediate defense against extracellular antigens. Effector T cell responses are short lived, and immune memory is essential for T cell mediated vaccine efficacy. The memory T cell response is dependent on the extent of initial T cell expansion, and subsequent contraction during which a small number of T cells become memory T cells [67].

Peptide based GnRH vaccines

Inhibiting GnRH binding of pituitary receptors via the induction of neutralizing anti-GnRH antibodies induces infertility and prevents reproductive behavior in many species [71]. Effective immunization against GnRH is posed with two major challenges: 1) GnRH is a small decapeptide and therefore is poorly immunogenic, and 2) GnRH is naturally present in the body and, therefore, is recognized by the immune system as "self", with minimal or no immunostimulatory effects [72]. Various strategies have been used to overcome these challenges. In the case of the mare, coupling GnRH to a highly antigenic carrier protein and combining an adjuvant has proven successful with the induction of anti-GnRH antibodies that suppress the HPG axis [73-76].

Peptide-adjuvant combinations used in many of the commercial protein based GnRH vaccines are largely proprietary. Regardless of the unique peptide-adjuvant combination used, it has been shown these GnRH vaccines can effectively suppress ovarian function and cyclicity in mares [3, 32, 73, 75-79]. These studies demonstrate a rapid increase in GnRH antibody titer following immunization, and cessation of reproductive activity based on reduced circulating progesterone concentrations and suppressed follicular activity [73, 75, 78]. The duration of estrus suppression can vary greatly from 3 months to over 2 years [74, 76]. This variable duration of suppression has been attributed to vaccine formulation and variability in the immune response between mares. An equine labeled protein based GnRH vaccine is licensed for use in Australia (Equity® Oestrus Control Vaccine, Zoetis, Australia). The vaccine comprises GnRH-diphtheria toxoid conjugate (Personal communication, Zoetis, Australia). In addition there are immunostimulating complexes formed from Saponin Quil A, cholesterol, and dipalmitoylphosphatidycholine. Each milliliter dose contains 200µg peptide conjugate, 300µg complexes, and 0.01% thimerosal as a preservative [74].

GnRH vaccines have been shown to be reversible, but effects can also be extended by re-immunization and therefore boosting the antibody response [80]. Tshewang, *et al*., confirmed reversibility using a

protein based GnRH vaccine, with all mares returning to normal cyclicity and producing normal foals within two breeding seasons [78]. In addition to the variability in interestrus interval following GnRH immunization, a major deterrent to the use of protein based GnRH vaccines in mares is the high incidence of side effects such as local tissue irritation at the injection site and transient fever. These effects result from the use of highly immunogenic adjuvants necessary to induce an immune response to GnRH. These side effects limit field use of protein based GnRH vaccines in high performance mares [73, 74].

Adenovirus as a vaccine vector

Adenoviruses have been well characterized since their initial description in the 1950's [81, 82]. They consist of an icosahedron made of three major proteins, hexon (II), penton base (III) and a knobbed fiber (IV), along with a number of minor proteins, VI, VIII, IX, IIIa and Iva2 [83]. The viral genome is linear and contains double stranded DNA. The delivery of viral genome to the nucleus of infected cells, and high efficiency of replication, make them ideal candidates for the expression of therapeutic genes [83]. Fifty five different serotypes of human adenovirus have been identified and classified into seven subgroups (A-G) [84].

Adenoviral infection of cells begins with entry of the virus into the host cell and the passage of virus genome into the nucleus, followed by transcription and translation of early genes. These early genes facilitate transcription and translation of late genes that lead to the assembly the viral structural proteins. The process from host cell entry to structural protein assembly and maturation of infectious virus requires approximately 10-14 hours [83]. Host cell entry requires binding via the knob portion of the virion fiber to target cell receptors termed coxsackie/adenovirus receptor (CAR). CAR is a plasma membrane protein belonging to the immunoglobulin superfamily [85]. It has been found that all human adenovirus species bind to CAR with the exception of members of the subgroup B [86, 87]. Other receptors that have been shown to be available for adenovirus fiber knob binding include MHCI, and sialoglycoprotein receptors

[83]. Following receptor binding, endocytosis of the virus occurs followed by release into the cell cytoplasm. Thereafter, the virus capsid undergoes proteolysis and the partially disrupted virus is transported to the nuclear membrane. The genome is passaged through the nuclear pore into the nucleus. Transcription occurs with a series of slicing events with cassettes of gene transcription termed E1, E2, E3, and E4. E1 gene products modulate cellular metabolism and are essential for viral gene transcription, shutting off of cellular proteins and cellular transformations, and is essential for replication [88]. The E2 gene products provide machinery for replication of virus deoxyribonucleic acid (DNA), including DNA-binding protein and DNA polymerase [83, 88]. E2 also ensures transcription of late genes, mediated by complex pathways. The E3 genes are dispensable for replication in tissue culture. E3 gene products facilitate late cytolysis of infected cells and assist release of progeny virus. Additionally, E3 delays expression of MHCI molecules and inhibits proapoptotic pathways [83]. E4 gene products predominantly facilitate virus messenger RNA metabolism and provide functions to promote DNA replication, and suppress host cell protein synthesis [89]. Following late transcription and translation of virus structural components, and encapsidation, the viral particles mature in the nucleus. The encapsidation process is accompanied by major changes in the host nuclear membrane that facilitates egress of the virus into the cytoplasm and is followed by disintegration of the host plasma cell membrane and the release of the virus from the cell [83].

Adenoviral vectored vaccines: development and applications

Replication deficient adenoviruses entail deletions of one or multiple viral genes. Usually, transgenes are inserted in place of the deleted genes. For first generation vectors, the deletion and transgene insertion involves E1 and possibly E3 regions. Sequences up to 5.1 to 8.2 kilo-base pairs can be accommodated [90]. These vectors are able to infect cells and express the transgene, but are not able to replicate efficiently due to the lack of E1. Second generation adenoviral vectors are also non-replicating but have further

deletions in the E2A, E2B, or E4 regions. These were developed to overcome the problem with first generation adenoviral vectors, in which expression of other early genes allows for a low degree of replication. The challenge with these second generation adenoviral vectors is in the engineering of stable cell lines that complement the vector, resulting in poor cell growth and viral titers [91]. Helper-dependent adenovirus vectors, also called “gutless” vectors, represent a third generation of adenoviral vectors. They are devoid of all viral coding sequences and possess only the 5’ and 3’inverted terminal repeats. They are able to accommodate large (~35 kb) insertions of DNA. The elimination of adenoviral antigen expression permits long term gene expression by avoiding problems with the cellular immune response against viral gene products [90]. Replication competent vectors are developed with the expression cassette for foreign antigen in place of the E3 gene [92].

Adenoviral vectored vaccines, particularly those based on human adenoviral serotype 5 (Ad5), have been used extensively for cancer gene therapy and for protective immunity against many infectious diseases [93-98]. Vectored vaccine technology offers the ability to elicit an intense humoral and cell-mediated immune response and an effective memory response leading to increased antibody formation following a booster vaccine [95]. Ad5-vectored vaccines have been shown to be adaptable for induction of protective immunity in a large variety of animal species [94, 99-101].

Following vaccination with a replication-competent human recombinant Ad5, both chickens and weanling piglets developed a robust antibody response to avian influenza hemagglutinins [101]. In the rabbit, protective hem agglutination inhibition antibody titers against Rabbit Hemorrhagic Disease Virus were detected shortly after a single vaccination with a replication-defective human Ad5 encoding the Rabbit Hemorrhagic Disease Virus VP60 capsid protein. IgG antibodies corresponding with inhibition percentages over 85% persisted up to one year [102]. A replication defective human Ad5 expressing the rabies glycoprotein has been developed and can induce immunity in rodents, canines, and foxes when given intramuscularly [103]. Protection of cattle and pigs against Foot and Mouth Disease Virus by inoculation

of a human Ad5 expressing capsid proteins is also now well established [99, 100, 104]. In spite of these important characteristics and their successful use in animals, adenoviral vectored vaccines have not yet been used in the horse. The only published report for development of an adenoviral vectored vaccine in the horse utilized a human Ad5 vaccine containing the *Rodococcus equi* VapA gene. The vaccine was developed and tested in mice for safety, immunogenicity and efficacy. The vaccine generated a strong antibody and cytokine response in mice and clearance of *R.equi* was demonstrated following challenge [105]. This vaccine is yet to be evaluated in the horse. The validation of an adenoviral vectored GnRH vaccine would carry significant scientific advancement for both reproductive control in horses, and application of adenoviral vectored vaccine technology in equids.

Heterologous boost and enhanced immunogenicity

Homologous prime-boost vaccination utilizes the same vaccine formulation in both the prime and boost components of the regimen. Alternatively, heterologous prime-boost approaches use different vaccine formulations for the prime and boost injections. Heterologous prime-boost can therefore utilize different antigen delivery systems for induction of both humoral and cellular immunity. Recently, studies have shown that heterologous prime-boost regimens with a chimpanzee adenoviru1s type 7 viral vector expressing Human Immunodeficiency Virus (HIV) F4 fusion protein induced a polyfunctional HIV-1 specific $CD4^+$ T-cell response in macaques [106]. Additionally, vector-prime protein-boost immunization induced broad hepatitis C virus-specific CD8+ and CD4+ T cell responses and functional Th1 type IgG responses in mice and guinea pigs [107]. In that study, heterologous prime-boost induced an immune response that surpassed homologous vaccination alone. The study utilized a human adenovirus vector 6 expressing E1E2 glycoprotein as the priming vaccine, followed by recombinant protein vaccine (HVC genotype 1a E1E2p7) and MF59 adjuvant [106]. Due to the small size and poor immunogenicity of GnRH, heterologous prime-boost vaccination utilizing a viral vector prime coupled

with a protein based boost, offers the advantage of two separate antigen delivery systems to elicit an immune response.

2. Immunization of Mice with an Experimental Adenoviral Vectored GnRH Vaccine

An experiment was performed in male mice to evaluate the immunogenicity and effects of several adenoviral vectored anti-GnRH vaccines using five different antigen carriers on gonadal size, histoarchitecture, and systemic testosterone concentration. It was hypothesized that GnRH immunization would suppress the HPG axis resulting in a reduction of gonadal size and systemic testosterone concentration, ultimately suspending reproductive capacity. The vaccines were shown to be antigenic and effective in blocking reproductive function (A vectored GnRH contraceptive vaccine to control animal populations; manuscript in preparation, permission granted by Baker *et al*., 2017). The vaccine used in this study consisted of a thoroughly characterized, safe, adenoviral (Ad5, E1/E3 deleted) infectious, but non-replicating and non-shedding vector. The vector was engineered to expresses a GnRH antigen consisting of 16 multimers of native GnRH linked to one of five highly antigenic carriers, the most immunogenic being a bacterial leukotoxin (leukotoxin A1 gene of *Pasteurella haemolytic*a). Vaccinated mice produced anti-GnRH antibodies with suppression of circulating testosterone concentration and profound testicular dysplasia (disruption) and complete loss of spermiogenesis, as shown in Figures 1 and 2a-2c. (Unpublished, permission granted by Baker *et al*., 2017).

3. Hypotheses and Objectives

Hypotheses

The primary hypothesis was that homologous prime-boost vaccination of mares using an adenoviral vectored GnRH vaccine (Ad-GnRH) would result in temporary suppression of reproductive cyclicity and estrus behavior, with a return to normal cyclicity and estrus behavior at the beginning of the subsequent ovulatory season. A secondary hypothesis was that following a return to cyclicity, heterologous boost using a quarter of the labeled dose (sub-effective) of a protein based GnRH vaccine would reactivate immune memory and suppress reproductive cyclicity and estrus behavior. It was also hypothesized that vaccination using both Ad-GnRH, and the sub-effective dose of a protein based GnRH vaccine would induce minimal or no adverse local or systemic side effects.

Objectives

The initial objective of the study was to determine the production of anti-GnRH antibodies following a standard prime and boost vaccination protocol in normal cycling mares. The vaccine chosen to be tested was that observed to be the most immunogenic in mice. It was a replication-defective E1/E3 deleted adenovirus (Ad5) vector expressing antigens consisting of 16 multimers of GnRH decapeptide, bacterial leukotoxin as an immunogenic “carrier” antigen, T-helper epitopes, and other various hinge and linker amino acids (Ad-GnRH). Reproductive physiological response of mares would be evaluated utilizing score values assigned for sonographic findings of the reproductive tract, serum progesterone concentration, and behavioral indices of estrus/diestrus/anestrus following exposure to a stallion (anestrus index). The anestrus index could then be used to define the expected time period from vaccination to suspension of cyclicity and suppression of estrus behavior, and to determine the duration of effect.

Another objective of this study was to determine the antibody and physiological response of Ad-GnRH primed mares to a single vaccination using a 'sub-effective' (one quarter of the labeled dose) dose of a protein based GnRH vaccine (Equity® Oestrous Control Vaccine, Zoetis, Australia). The vaccine label states that two one milliter doses should be administered four weeks apart. Each one milliter dose contains 200 µg of the GnRH peptide conjugate. Mares is this project were to be vaccinated only once using 100 µg of the peptide conjugate. Naive cyclic mares would receive the same dose of the protein based GnRH vaccine to differentiate immune responses that occurred as a result of Ad-GnRH priming from immune responses that occurred as a result of protein based GnRH vaccination alone. Finally, evidence of local or systemic side effects of vaccination would to be identified by visual inspection of the injection site and daily physical examinations.

4. Materials and Methods

4.1 Mare Inclusion Criteria

Twelve healthy, non-pregnant mares between 14-23 years of age with normal estrous cycles were included in the study. The mares were part of the Auburn University Equine Reproduction Center teaching herd. All mares were of average size (400-600kg) and of various light horse breeds. Mares were housed by groups in large pens and were fed free choice coastal bermuda hay and supplemented with grain. The study took place from June 2015 to October 2016. Mares were examined via transrectal palpation and ultrasound to establish normal reproductive organ anatomy and ovarian activity (confirmed by the presence of a CL and an average interovulatory interval of 21 days). Additionally, all mares underwent a breeding soundness examination to establish normal reproductive health before inclusion in the study. Diagnostic procedures such as uterine culture and cytology where performed to ensure mares were free of endometritis and an endometrial biopsy was performed to ensure normal histoarchitecture. Finally, a

complete blood count and serum biochemical analysis was performed for each mare to establish normal physiological health. Normal estrus behavior was confirmed via exposure to a stallion and characterized using the following scale:

One: Mare completely rejects the stallion, presenting one or more of the following refusal manifestations: squealing, pawing, kicking, switching tail, holding ears back.

Two: Mare is indifferent to the presence of the stallion; she does not move away, but does not lift the tail or display rhythmic eversion of the labia to expose the clitoris (clitoral eversion).

Three: Mare is interested in the stallion and approaches him, raising the tail, and everting the clitoris.

Four: Mare presents similar behavioral signs as score three: clitoral eversion, elevation of the tail, plus urination, change in posture to one that facilitates copulation (arched tail, flexed stifles and hock, abducted rear limbs and tipped pelvis with associated lowering of the perineal area).

Only healthy mares demonstrating regular estrous cycles, with normal uterine health, and normal estrus behavior in response to a teaser stallion were included in the study.

4.2 Construction of Replication Incompetent Adenovirus 5 Encoding anti-GnRH Antigen

An adenovirus 5 (Ad5) vector was constructed to encode a gene sequence that included human tissue plasminogen activator (tPA) leader sequence followed with 8 copies of GnRH (EHWSYGLRPG) linked to LKT (leukotoxin A1 gene of *Pasteurella haemolytic*a) followed by another 8 copies of GnRH. The amino acid trimers GSS or SGS were used as spacers between each of the GnRH monomers. The peptide TCPPCPAP was used as hinge sequence between each of the GnRH 8mers and the LKT sequence. Finally, the peptide MATVIDLS was added between the hinge peptide and the N-terminus of the LKT. The infusion cassette was codon-optimized for dog cell expression, synthesized by GenScript (Piscataway, NJ), and cloned into HindIII and XbaI sites of the pAdHigh vector (Altimmune) to generate pAdLKTGnRH16dog

which was used for generation of AdLKTGnRH16dog vaccine virus as described before [108]. AdLKTGnRH16dog vaccine virus was propagated on HEK293 cells and purified by ultracentrifugation over a cesium chloride gradient as described before [109]. The purified AdLKTGnRH16dog vector was sterilized by a 0.22-µm filtration then stored at -80°C in a formulation buffer. [110]. AdLKTGnRH16dog viral titer was determined by Adeno-XTM rapid titer kit (BD Biosciences, Palo Alto, CA) on HEK293 cells. The correct structure of the AdLKTGnRH16dog antigen within the vector was verified by DNA sequencing (Genewiz, Germantown MD). Each one ml dose of the vaccine contained 4.64E10 infectious units (ifu) of the vector. This vaccine will now be identified subsequently as Ad-GnRH.

4.3 Heterologous Boost Protein Based Vaccine Construct (Equity®)

The protein based GnRH vaccine was comprised of GnRH peptide conjugated to a diphtheria toxoid and admixed with an adjuvant immune-stimulating complex formed from Saponin Quil A, cholesterol, and dipalmitoylphosphatidycholine. Each 1.0ml dose of this vaccine contained 200 µg peptide conjugate, 300 µg immunostimulating complexes, and 0.01% thimerosal, as a preservative, and isotonic buffered solution to volume (Equity® Oestrus Control Vaccine, Zoetis, Australia).

4.4 Experimental Treatment Phases

Phase 1 (week 0-46)

Treatment group: Five healthy, normally cyclic mares were vaccinated against GnRH by injection of one ml (4.64E10 ifu) of Ad-GnRH into the left cervical musculature twice, four weeks apart.

Control group: Five healthy, normally cyclic mares did not receive any treatments, and served as controls for reproductive cyclicity, estrus behavior, and seasonal changes in cyclicity.

Phase 2 (week 47-70)

Treatment group: Mares that were immunologically primed with the Ad-GnRH vaccine during phase one received a single vaccination with 0.5ml of a protein based GnRH vaccine (100 µg peptide conjugate: one quarter of the recommended dose: Equity®) into the left cervical musculature 49 weeks after initial Ad-GnRH vaccination. One treatment mare that had been primed with Ad-GnRH mare was removed from phase two of the study and was euthanized for reasons unrelated to the study.

Protein vaccine control group: Two healthy, normally cyclic mares that were not included in phase one of the study (naïve mares) were vaccinated once with 0.5ml of the protein based vaccine (100 µg peptide conjugate: Equity®) into the left cervical musculature. These control mares served to determine if vaccination using a single injection at an equivalent dose of that administered to treatment mares (100 µg peptide conjugate: Equity®) had an effect on reproductive cyclicity and estrus behavior.

4.5 Vaccination procedure

Vaccinations were performed using an aseptic technique. At the time of vaccination, mares were restrained in palpation stocks and a 3cm x 3cm area of hair was clipped from the vaccination site, over the left cervical region. This site was scrubbed using alternating chlorhexidine scrub and ethanol. Vaccines were administered to all mares (treatment and control mares) during the same day, within a three hour time period.

4.6 Data Collection

Anti-GnRH antibodies, estrus behavior, reproductive tract sonography, and serum progesterone concentrations were monitored over two consecutive breeding seasons. A venous blood sample was

drawn from each mare immediately prior to initial injection with Ad-GnRH, and repeated weekly for the remainder of the study. Blood samples were collected by jugular venipuncture. Serum was centrifuged upon clotting at a rate 3000 x g for 10 minutes. Sera was immediately separated, aliquoted, and frozen (-80^{o}C) until analysis. Data collection for this project ended 17 months after initial injection of treatment mares with Ad-GnRH.

4.6.1 Anti-GnRH antibody assay

Anti-GnRH antibody was detected from blood drawn every other week via binding of ^{125}I-labeled GnRH (L8008, Sigma, St Louis, MO, USA) in serum using a radioimmuno-precipitation technique previously validated in mice and cats (Baker, personal communication). Samples were assayed in duplicate and were performed as follows; 100µl of ^{125}I-labeled GnRH was added to 100 µl of test serum diluted 1:100, and 200µl PBS/BSA (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, 1% (w/v) bovine serum albumin; pH 7.4 (Sigma-Aldrich). After overnight incubation at 4^{o}C, 100µl of bovine IgG PBS/BSA solution (total 250µg bovine IgG) was added to each sample (19640, Sigma-Aldrich, St. Louis, MO, USA). Bound ^{125}I-labeled GnRH was precipitated from unbound hormone by adding 500µl of a 24% solution of polyethylene glycol (PEG; Carbowax™ PEG 8000, P156-500, Thermo Fisher Scientific, Waltham, MA, USA). Tubes were then vortexed and incubated at 4^{o}C for 10 minutes. Tubes were centrifuged at 1400 x g for 15 minutes and the supernatant was aspirated. Radioactivity in the pellet was measured in a gamma counter. Nonspecific binding (NSB) of ^{125}I-labeled GnRH was determined from the mean of duplicate tubes in which the diluted serum was replaced by PBS/BSA buffer. Mean NSB was subtracted from individual sample measurements. The serum anti-GnRH antibody that was bound to ^{125}I-labeled GnRH was expressed as a proportion of an internal standard control.

4.6.2 Progesterone

Serum progesterone concentration was assayed weekly and was analyzed using a chemiluminescence immunoassay, Immulite® Progesterone (Diagnostic Products Corporation, Los Angeles, California). This assay was previously validated in the horse [73].

4.6.3 Transrectal palpation and ultrasound of the reproductive tract

The reproductive tract of all mares was examined via transrectal palpation and ultrasound with a 5 MHz linear array transducer (Sonosite, Sonosite Inc., Bothell, Washington). During phase one (week 0-46), sonographic examination was performed twice weekly during the summer and early fall (week 0-25). During the subsequent winter (from week 35), sonographic examinations were performed once to twice weekly.

During phase 2 (week 47-70), sonographic examinations were performed twice weekly for the remainder of the study. At each examination, the presence or absence of a CL was noted. Uterine edema was classified as follows: No uterine edema (score 0), slight uterine edema (score 1), moderate uterine edema (score 2), and heavy uterine edema (score 3). The diameter of the largest follicle on each ovary was measured and these values were used to calculate the anestrus index (see *4.5.5*).

4.6.4 Estrus behavior and interestrus interval

Estrus behavior was assessed on the same schedule as sonographic examinations. A teaser stallion was led to the paddock and the mares allowed to approach and make contact through a fence. If a mare did not approach the fence, she was haltered and led to the fence line to ensure contact with the stallion. Estrus behavior was scored using the same scale described in the mare inclusion criteria. Mares with an assigned teasing score of one or two were considered in either anestrus or diestrus, and those that were assigned a teasing score of three or four were considered to be in estrus. Interestrus interval (IEI) was

calculated as the time period from when a mare first displays an estrus score of 3 or more followed by scores consistent with diestrus/anestrus for two or more days, to when the mare next first displayed an estrus score of 3 or more when presented with a stallion.

4.6.5 Anestrus index

Anestrus index was assigned to identify behavior consistent with anestrus. Anestrus index was based on scores assigned for parameters of sonographic findings of the reproductive tract, serum progesterone concentration, and estrus behavior. A score of 3 was assigned for parameters consistent with anestrus, while a score of 0 was assigned for a parameter that was not consistent with anestrus, and included parameters that represent both diestrus and estrus. Measured parameters included tease score (>2 not anestrus), largest follicle diameter (≥25mm not anestrus), uterine edema score (≥1 not anestrus), the presence of a CL, and serum progesterone concentration (≥2ng/ml not anestrus). The table below shows the allocation of score for anestrus index parameters.

	Score 0	Score 3
Tease Score	2 or less	>2
Largest follicle diameter	≥ 25mm	<25 mm
Corpus Luteum	Present	Absent
Uterine edema score	<1	≥1
Serum progesterone	≥2ng/ml	<2ng/ml

For example, a mare with a tease score of 2 or less, largest follicle size less than 25mm, no CL or uterine edema noted during sonographic examination, and base line serum progesterone concentration would receive a score of 3 for each parameter, and therefore a cumulative score of 3+3+3+3=12. Alternatively,

a mare with a tease score of 3 or more, largest follicle greater than 25mm, no CL noted on sonographic examination, and a uterine edema score of 3 would receive a score of zero for each parameter, and therefore a cumulative score of 0+0+0+0=0. A total score greater than 10 was considered consistent with anestrus (acyclic), while a score less than 10 was considered consistent estrus or diestrus (cyclic).

4.6.6 Vaccine associated side effects

Immediately prior to and following vaccination mares were monitored by complete physical examination and inspection of the injection site. Examinations were performed twice daily for 3 days or until resolution of vaccination induced side effects, which ever was the longest time period. The injection site was observed both visually and digitally for evidence of pain (aversion to palpation of the injection site), warmth, or swelling.

5. Data Analysis

Statistical analysis

Data was analyzed using the statistical software Statistical Analysis System (JMP®, Version 13. SAS Institute Inc., Cary, NC). The presence of anti-GnRH antibody at approximate two week intervals were compared to that of time zero, and IEI was examined using Wilcoxon Rank Sum Test. Anestrus index scores were compared using Fischer's exact test. Significance was set at 0.05.

6. Animal Welfare

This study was approved by the Institutional Animal Care and Use Committee (IACUC), Office of Animal Resources, Auburn University.

7. Results

7.1 Anti-GnRH Antibody

Phase One (Week 0-46): Ad-GnRH prime day 0, Ad-GnRH boost week 4

All mares were seronegative for GnRH antibodies prior to first vaccination. All mares were considered seronegative when antibody radioligand binding as a proportion of the internal standard (PBIS) was less than 0.0066 (based on a day 0 PBIS value treatment group mean of - 0.0174 ± standard error of the mean). All Ad-GnRH vaccinated mares responded with the production of anti-GnRH antibodies. Antibodies were detectable in all vaccinated mares at the time of the second boost (week 4). At the first occurrence that anti-GnRH antibodies could be detected, mean antibody production for treatment mares, as measured by PBIS, was 0.0825 ± 0.0498. This was significantly different from that of time zero (P=0.01). Peak mean anti-GnRH antibody response in treatment mares occurred 6 weeks after homologous Ad-GnRH prime (range 6-25 weeks). The PBIS value at this time was 0.2087 ± 0.0263, which is 12.5 times that of the upper end of the confidence limit from what was considered negative at time zero. Antibody response gradually waned until it decreased to a value that was considered negative for anti-GnRH antibody at week 36 weeks after Ad-GnRH prime (Figure 3a).

Phase Two (Week 47-70): Heterologous boost with protein antigen at week 49

At the beginning of phase two, which was 47 weeks after Ad-GnRH prime, treatment mare mean anti-GnRH antibody PBIS value was 0.01058 ± 0.0220. This was not significantly different from the value of 0.0174 ± 0.0086 that was considered negative for anti-GnRH antibody at time zero (P =0.25). Three weeks following heterologous boost (week 49 after Ad-GnRH prime), a significant increase in antibody response of treatment mares was detected (P=0.01). At this time, the mean PBIS value for treatment mares was 0.5945 ± 0.0582. This value is 90 times greater than the upper limit of the confidence interval for what was considered negative for Anti-GnRH antibodies. Maximum mean antibody response from treatment

mares occurred five weeks after heterologous boost (54 weeks after Ad-GnRH prime), with a PBIS value of 0.8234 ± 0.1798. This peak was 124.75 times higher than the upper limit for the confidence interval for what was considered negative at time zero. Mean anti-GnRH antibody response for treatment mares remained significantly higher than that of time zero for the remainder of the study period (Figure 3b).

Anti-GnRH antibodies were detectable for protein vaccine control mares three weeks after vaccination (Week 52). The PBIS value representing the mean anti-GnRH antibody response for these two mares was 0.11048 ± 0.0659, which is 16.749 times greater than what was considered negative for treatment mares at the beginning of phase one. Peak anti-GnRH response for these protein vaccine control mares occurred five (mare 210) and seven (mare 211) weeks post vaccination. These PBIS values were 0.15135 and 0.0191 respectively (Figures 4a, 4b). By 18 weeks post vaccination (week 67), anti-GnRH antibody response for protein vaccine control mares decreased to -0.01616 and was no longer different from what was considered negative for treatment mares.

There was a wide frequency distribution of antibody responses for treatment mares, especially following heterologous boost with the protein based GnRH vaccine (Figure 5). The highest antibody response during both phase one and phase two occurred in Mare 214. During phase one this mare developed anti-GnRH antibodies that peaked 21 weeks after Ad-GnRH prime vaccination with a PBIS value of 0.36977 (Figure 6a). During phase two, this mare's peak anti-GnRH antibody response occurred 5 weeks after heterologous boost (54 weeks after Ad-GnRH prime), with a PBIS value of 1.23665 which is 3.3 times greater than peak production during phase one (Figure 6b).

The second highest antibody response during both phase one and phase two occurred in mare 16. During phase one the mare reached peak anti-GnRH antibody response, with a PBIS value of 0.36063 10 weeks after homologous Ad-GnRH priming (Figure 7a). Peak anti-GnRH antibody response during phase two occurred 5 weeks following heterologous boost (54 weeks after Ad-GnRH prime) and had a PBIS value of

0.8778 which is 2.4 times higher than the peak anti-GnRH response that occurred during phase one (Figure 7b).

The third highest antibody response during both phase one and phase two occurred in Mare 13. During phase one this mare developed anti-GnRH antibodies that peaked 6 weeks after homologous Ad-GnRH priming with a PBIS value of 0.0.02811 (Figure 8a). During phase two, this mare's peak anti-GnRH antibody response occurred 5 weeks after heterologous boost (54 weeks after Ad-GnRH prime) as for mares 16 and 214, with a PBIS value of 0.81905 which is 2.9 times greater than peak production during phase one (Figure 8b).

The fourth highest antibody response during both phase one and phase two occurred in Mare 15. During phase one this mare developed anti-GnRH antibodies that peaked 25 weeks after Ad-GnRH priming with a PBIS value of 0.0.16604 (Figure 9a). During phase two, this mare's peak anti-GnRH antibody response was gradual, and occurred 16 weeks after heterologous boost (65 weeks after Ad-GnRH prime), with a PBIS value of 0.5477 which is 3.2 times greater than the mare's peak anti-GnRH antibody response during phase one. A 3.5 fold reduction in anti-GnRH antibody response occurred between 16 and 18 weeks after heterologous protein antigen boost (between 65 and 67 weeks after Ad-GnRH prime) . At week 67, this mare's PBS value decreased to 0.01278 (Figure 9b). This rapid decrease was observed concurrently with a reduction in anestrus index score to less than 10 (Figure 11).

The lowest antibody response occurred in mare 17. During phase one this mare developed anti-GnRH antibodies that peaked 8 weeks after Ad-GnRH priming, with a PBIS value of 0.0585 (Figure 10). This mare was removed from the research project and euthanized for reasons unrelated to this study.

7.2 Progesterone

Phase One (Week 0-46): Ad-GnRH prime day 0, Ad-GnRH boost week 4

Serum progesterone concentration for all mares displayed cyclical changes throughout the study period that reflected a normal interovulatory interval (Figures 6a,7a,8a,9a,10). Furthermore, following exposure

to a stallion, all mares showed diestrus behavior when serum progesterone concentration was greater than 2ng/ml, and estrus behavior when serum progesterone concentration was less than 2ng/ml.

Phase Two (Week 47-70): Heterologous boost with protein antigen at week 49

At the beginning of phase two, serum progesterone concentration for all mares displayed cyclical changes that reflected a normal interovulatory interval. Three weeks following the heterologous boost (52 weeks after Ad-GnRH priming), serum progesterone concentrations for all treatment mares were below 2ng/ml. Serum progesterone concentration for this group of mares remained below 2ng/ml for the remainder of the study period (figures 3b, 6b,7b,8b,9b). Serum progesterone concentrations for protein vaccine control mares reflected normal cyclicity throughout the study period (figures 4a,4b).

7.3 Interestrus Interval

Phase One (Week 0-46): Ad-GnRH prime day 0, Ad-GnRH boost week 4

Four of the five treatment mares displayed normal interestrus intervals (IEI), with a mean IEI of 23 ± 2 days, which was not different from control mares (mean IEI 22 ± 2 days) (P=0.462). One treatment mare (Mare 214) experienced two prolonged luteal phases of 70 days and 91 days respectively. This mare was determined as an outlier because these interestrus intervals were greater than 33 days, which is 1.5 times the interquartile range above the third quartile of the data. Data for this mare was not considered for statistical analysis of IEI.

Phase Two (Week 47-70): Heterologous boost with protein antigen at week 49

At the beginning of phase two, prior to heterologous boost with a protein antigen, four of 5 treatment mares displayed normal IEIs (25 ± 4 days). The same mare that had experienced prolonged luteal activity during phase one (mare 214) also experienced prolonged luteal activity that extended into phase two.

The mare's luteal phase lasted 85 days and occurred from week 38 through week 50. The duration of the luteal phase was greater than 68 days, which is 1.5 times the interquartile range above the third quartile of the data. Again, because this mare was determined to be an outlier, data for this mare was not considered for statistical analysis of IEI.

Four weeks following heterologous boost, treatment mares ceased displaying predictable estrus that would allow for calculation of IEI. At each observation period, teasing behavior became erratic. Mares would display behaviors consistent with estrus (score 3, 4) and anestrus/diestrus (1, 2) during the same observation period. IEI for treatment mares could not be calculated for the remainder of the study period (weeks 53-70). One mare that appeared to return to cyclicity (Mare 15) based on anestrus index score did not complete one full IEI before the completion of the study to allow for calculation of one IEI.

Protein vaccine control mares exhibited normal interestrus intervals (27 ± 3 days) that were not different from interestrus intervals determined from control mares during phase one of the study.

7.4 Anestrus Index

Phase One (Week 0-46): Ad-GnRH prime day 0, Ad-GnRH boost week 4

Anestrus index for treatment mares was calculated from measures of mare cyclicity taken from weeks 0-25, and again from weeks 35-46. All vaccinated mares remained cyclic following homologous Ad-GnRH prime and boost vaccination (anestrus score < 10). Four of five control mares exhibited normal reproductive cyclicity throughout the time period for which their cyclicity was monitored (weeks 11-25). One control mare (mare 11) cycled normally until week 16, after which the mare became anestrus (anestrus scores maintained above 10) for the remainder of the observation period for Ad-GnRH control mares. This occurred during late September to early October and may reflect normal seasonal transition.

Phase Two (Week 47-70): Heterologous boost with protein antigen at week 49

All treatment mares exhibited normal reproductive cyclicity at the beginning of phase two (anestrus score <10), prior to heterologous boost. Four weeks following heterologous boost (week 53), all treatment mares became acyclic (anestrus index score>10). All mares remained anestrus until week 68, after which one mare (Mare 15) returned to cyclicity based on the anestrus index score (anestrus index score <10) (Figure 11). This mare developed a large dominant follicle >30mm, uterine edema score >1, estrus behavior score >2, and progesterone was <0.2ng/ml. The mare did not ovulate before the end of the study period. Protein vaccine control mares exhibited normal reproductive cyclicity (anestrus index score <10) for the duration that their cyclicity was monitored (weeks 49-70).

7.5 Vaccine Associated Side Effects

There were no systemic vaccine reactions observed following Ad-GnRH prime, boost, or heterologous boost. There were no vaccine site reactions observed following initial Ad-GnRH priming. At week 4, the day following homologous Ad-GnRH boost, three of five treatment mares developed a non-painful 1-3cm raised nodule at the injection site which resolved without treatment within three days. Following heterologous protein antigen boost, two of four treatment mares developed a small (<2cm) raised, non-painful nodule that resolved without treatment within 3 days. An representative example of an injection site nodule can be seen in figures 12. An injection site nodule was first detected for mare 15 following Ad-GnRH boost one day post vaccination (figures 12a). An injection site nodule was first detected for mare 16 following heterologous boost one day post injection (figure 12b).

8. Discussion

Homologous prime-boost immunization against GnRH using the experimental Ad-GnRH vaccine resulted in the production of anti-GnRH antibodies in all treated mares. Heterologous prime-boost vaccination resulted in greater production of anti-GnRH antibodies compared with that of initial homologous prime-

boost vaccination. The present study used only five mares during phase one, and four treatment mares during phase two. In spite of the limited number of mares, it is clearly shown that immunization of mares against GnRH utilizing a vaccine strategy that incorporates Ad-GnRH prime and a heterologous protein antigen boost protocol results in the production of anti-GnRH antibodies and concurrent suspension of reproductive cyclicity and estrus behavior.

Despite a significant increase in antibody response following homologous Ad-GnRH prime-boost, reproductive cyclicity was not suppressed and vaccinated mares continued to show normal estrus and ovarian activity. A possible explanation is that antibody production did not reach a required threshold that would adequately impede GnRH receptor binding. This notion is supported by what was observed for protein vaccine control mares. It is known that homologous prime-boost vaccination with Equity® at the labeled dose and administration schedule results in neutralizing antibody production, suspension of cyclicity and suppression of estrus behavior [74]. In the current study, the continued cyclicity that occurred in protein vaccine control mares confirms that these mares received a sub-effective antigenic dose required for suppression of the HPG axis. The mean peak antibody response that occurred in treatment mares during phase one was similar to the maximum antibody response that occurred for protein vaccine control mares during phase two (PBIS 0.2087 and 0.19166 respectively). This suggests that an antibody threshold greater than what occurred is required for suppression of reproductive cyclicity. During phase two, treatment mares responded to heterologous boost with suspension of cyclicity (anestrus index score >10) and suppression of estrus behavior. This confirms that neutralizing antibodies increased above a threshold required HPG suppression. The time of suspension of cyclicity occurred four weeks after heterologous boost (53 weeks after Ad-GnRH prime). Anti-GnRH antibody response was not measured at week 53 because anti-GnRH antibody was assayed every other week. Values for PBIS for treatment mares measured on week 52 were within the interval of 0.4855 and 0.7067. Values for PBIS that were measured the week following suspension of reproductive cyclicity (week 54) were within the interval of 0.50299 and

1.23665. This suggests that a minimum anti-GnRH antibody threshold for suspension of reproductive cyclicity and suppression of estrus behavior exists within an interval that is represented by a PBIS value between 0.4855 and 1.23665. Direct antibody titers were not determined in this study. Evaluations of titers retrospectively based on known PBIS values for given time frames following vaccination would help to further characterize the anti-GnRH antibody response and determine an anti-GnRH antibody titer that correlates with suppression or reproductive cyclicity and estrus behavior.

An additional consideration relates to the avidity of the antibody response that occurred following homologous Ad-GnRH vaccination. Previous studies have demonstrated that antibody avidity to free GnRH plays an important role when evaluating vaccine efficacy. Ferro and colleagues immunized male Sprague-Dawley rats with modified dimerized GnRH peptides. Their studies showed that antibody subclasses IgG1 and IgG2a displayed the highest avidity for free GnRH [111, 112]. The antibody subclass was not evaluated in this present study, and to the authors knowledge, has not been evaluated in other studies on the immune response of mares to GnRH immunization. Evaluation of anti-GnRH antibody avidity in mares may help to characterize their immune response and provide valuable insight for vaccine development. A further consideration with regard to characterization of the immune response is the use of an appropriate immunostimulant. Because GnRH is identified by the immune system as an alloantigen, it must be presented to the immune system with a specific antigen capable of provoking the appropriate signals to promote an appropriate immune response and breakdown of tolerance of the GnRH antigen. If alloantigens are detected in the presence of pro-inflammatory signals or other events that promote APC maturation, an immune response to the alloantigen can ensue. Many different carrier proteins have been proven successful for induction of immunity against GnRH [75, 78, 113, 114]. The Ad-GnRH vaccine used in this study contained the carrier protein bacterial leukotoxin (Leukotoxin A1 gene *Pasteurella haemolytica*). Design, production, purification and testing of this adjuvant as a carrier protein for GnRH was first described in the late 1990's. When tested in mice and pigs, treated animals responded by

producing high antibody titers to GnRH, a dramatic reduction in pituitary gonadotropin secretion, and reduced sex steroid secretion and gonadal size [115]. Robbins *et al*. evaluated the response of cats to immunization against GnRH using a vaccine with this same carrier protein. The vaccine consisted of 8 tandem repeats of GnRH fused to each terminus of a 52 Kda fragment of leukotoxin A (*Pasteurella haemolytica*). Vaccinated cats experienced a sustained antibody profile, and it was suggested that this response occurred as a result of the use of multiple copies of the GnRH antigen ligated to this leukotoxin carrier peptide [116]. The vaccine used for GnRH immunization of mares in this study was similar in that it consisted of 8 copies of GnRH fused to each terminus of bacterial leukotoxin.

While vaccinated mares responded immunologically (anti-GnRH antibody production), they did not experience a concurrent suppression of cyclicity. The immune response evoked may have been insufficient to inhibit GnRH activity as a consequence of inadequate antigenic dose. It has been shown that the dose of infectious units of an adenoviral vector can have a significant influence on consistency and strength of the immune response [117]. Dean *et al*. evaluated the immunogenicity of an Ad5 vector encoding mycobacterial antigens using a heterologous prime-boost vaccination regime in cattle [117]. Cattle were primed with live attenuated Mycobacterium bovis vaccine (Bacillus Calmette–Guérin vaccine) and the Ad5 vectored vaccine served as the heterologous boost. In this study, it was determined that the optimum dose of 2.0E9 ifu of the Ad5 vector modified to express mycobacterial antigens, given by the intradermal route conferred the most consistent and strongest immune response compared with lower doses that were evaluated. The infectious titer dose used to vaccinate mares in this study was computed by the accepted biopharmacological method of calculation based body surface area. Scaled from the mouse, a dose of 4.64E10-2E11 ifu was calcuated. Further studies in mares evaluating variation in dose or route of administration would provide insight into the optimum immunization strategy that would provide an enhanced immune response to homologous Ad-GnRH immunization.

It has also been demonstrated that the order of heterologous prime-boost administration may be important [118]. Using a murine model, it was shown that a DNA-prime and protein-boost regimen using murine herpes simplex virus (HSV)-2 gD antigen was required to induce T-helper 1-type cell proliferative responses and antibody levels greater than when administered in the reverse order. Protein-prime and DNA-boost failed to enhance the immune response when compared with homologous protein-protein vaccination [119]. Further research evaluating alternate order of protein-prime and Ad-GnRH-boost and further characterization of the immune response may offer some insight to the specific immune cell interactions and costimulatory factors that occur following heterologous GnRH immunization in the mare. Another consideration with regard to vectored GnRH vaccine efficacy is the choice of the vaccine vector. Based on previous work, adenoviral vectors seem to be able to prime and boost B cell responses more effectively than other vectors such as modified vaccinia virus [120]. It has been suggested that this may be due to prolonged, high level antigen expression following adenoviral vector vaccination, which favors B cell priming [120]. Because vectored GnRH vaccines are so novel, characterization of molecular pathways in immune cell signaling remain to be fully elucidated. Viral vectored immunocontraception is studied in wild pest species, such as the mouse, rabbit, and fox [121]. Research in these species, using canine herpes virus, myxoma virus, and cytomegalovirus respectively have utilized species specific viruses that express zona pellucida antigens. While these studies provide “proof of concept” for viral vectored immunocontraception, they utilize antigens that are not highly conserved between species. This was an attempt to avoid effects on fertility of non-target species, but also to avoid undesirable side effects such as premature termination of pregnancy, and alterations in sexual and social behavior within dominance hierarchies [121]. Modification of estrus behavior was a primary objective in this study, and therefore a non-replicating Ad-GnRH vaccine would provide a viable option for control of estrus in domesticated mares of known pregnancy status.

This study provides “proof of concept” that mares can mount an immune response following homologous prime-boost Ad-GnRH vaccination. Moreover, this immune response can be enhanced with heterologous boost using a sub-effective dose of a protein conjugated GnRH vaccine. As mentioned previously, further study is required regarding Ad-GnRH vaccine dose, characterization of the immune response to immunization, and order of administration of vaccine constructs with heterologous vaccination regime. Despite the observed immunological response to vaccination in both phases of the study, only during phase two, following heterologous boost, did mares become acyclic. At a latitude of 38° North, the mean date of anestrus onset in young mares (less than 5years of age) is November 6th (range October to January), with an average duration of anestrus being 176 days ± 30 days. Mature mares at this same latitude (>10 years of age) generally continue to exhibit estrous cycles at the time that younger mares become seasonally anestrus, with the mean time of onset of anestrus occurring during the first week of January [122]. As a result, mature mares experience a relatively shorter duration of anestrus (75 ± 18 days). All mares used in this study were mature (> 10 years of age), and therefore it is expected that these mares would have maintained cyclicity throughout the study period if not vaccinated, given that the study period ended in October. Additionally, all mares from phase one (both treatment and control), and the protein vaccine control mares from phase two maintained cyclicity throughout each study phase. Anti-GnRH antibody production did not wane in mares that remained anestrus, providing further evidence that the maintenance of an anestrus state in treated mares was a direct result of immunization against GnRH. The duration of suppression of cyclicity could not be fully evaluated in this study because three of the four vaccinated mares were still acyclic at the completion of the study. The fourth mare (mare 15) was no longer acyclic, based on anestrus index score, at the completion of the study. In this mare, a return to cyclicity occurred concurrently with a marked reduction in detectable anti-GnRH antibodies. This provides evidence that heterologous immunization against GnRH, utilizing Ad-GnRH-prime and protein-GnRH-boost does induce ‘temporary’ suppression the HPG axis. The increase in uterine edema, follicle diameter,

and estrus behavior is suggestive of a reduction in inhibition of the HPG axis. This finding provides evidence that GnRH immunization strategies that incorporate Ad-GnRH vaccination may be temporary, and will allow vaccinated mares to retain breeding potential, which is pivotal in performance horses. Further research that incorporates larger group sizes, and measures of fertility such a pregnancy rates and live foal rates are required to confirm that vaccine induced effects are temporary and do not alter future fertility.

Despite all mares becoming anestrus within four weeks of heterologous boost, estrus behavior was not completely abolished. When presented to a stallion, mares would sporadically display behaviors that communicate receptivity to a stallion (eversion of the clitoris, urinating) concurrently with signs of anestrus (indifferent to the presence of the stallion). Estrus behavior unaccompanied by major follicular activity is common in anestrus mares and has been referred to as "unseasonal estrus"[1]. The phenomenon of unseasonal estrus has been studied in both anestrus mares and ovariectomized mares. In these mares, it is likely that estrus behavior is expressed as a result of androgen production from the adrenal gland. It is suspected that androgens (androstenedione, dehydroepiandrosterone, testosterone) have an FSH regulatory role in females but the exact role in eliciting unseasonal estrus behavior remains to be elucidated [1]. Support for the notion of adrenally derived androgenic influence of estrus behavior has been proven via the administration of dexamethasone to ovariectomized mares to suppress adrenal hormone synthesis. Following treatment, there was suppression of estrus behavior in treated mares [123]. Stimulation of the mare's adrenal glands using a synthetic adrenocorticotropic hormone fragment increased circulating plasma concentrations of testosterone but not estradiol-17β [124]. It has also been shown that at a dose of 200 micrograms/kg body weight, in a single injection of testosterone propionate on day five of progestagen feeding elicits estrus behavior in approximately 70% of mares [125]. This research suggests that even in the face of increased progestagens, supraphysiological doses of testosterone can elicit estrus behavior. Additionally, testosterone levels ranging from 15-70 pg/ml are

highest during estrus in most mares, with a second peak occurring 11 days before ovulation [126]. Furthermore, it is well known that progesterone suppresses estrus behavior and its absence is permissive of estrus behavior [127]. Despite this, immunization against GnRH is still attractive because the estrus behavior displayed by mares during anestrus is usually of lower intensity and is usually only displayed in the presence of a stallion. The probability of anestrus mares displaying estrus behavior on any given day is only approximately 50% [127].

A recent survey investigated the effect of immunization against GnRH using Equity® on horses with unwanted behavior. Following vaccination, 84% of the mares displayed a decrease in the expression of behaviors deemed undesirable by the observers [128]. One of the challenges in reviewing research in which behavioral criteria alone is used to define estrus is that it is not adequately stated and standardized [1]. For this reason, in the current study, an anestrus index was developed that incorporated a number of physiological and behavioral parameters to determine a state of anestrus or estrus/diestrus. Calculation of this index provided objective data with which to draw conclusions and accounted for multiple factors that influence mare reproductive physiology. The value of anestrus index was highlighted in this study with reference to the behavioral and physiological response to vaccination by mare number 214. The mare experienced two prolonged, successive interestrus intervals. This mare also experienced the highest antibody response of all treated mares during both phase one and phase two of the study. Had IEI alone been used to evaluate vaccine efficacy, these results would have appeared supportive of a highly effective physiological response to Ad-GnRH homologous immunization. With the added consideration of progesterone concentration and sonographic findings, this mare maintained an anestrus index of two or less, which is consistent with either diestrus or estrus.

Based on systemic progesterone concentrations and sonographic findings, mare 214 experienced repeated prolonged luteal phases due to persistent CL function. In normal cyclic mares, luteolysis begins on average at day 14 post ovulation and occurs for a duration of 22.9 ± 0.9 hours [129]. A CL that persists

beyond 14 days in a non-pregnant mare has be described as a persistent CL [130]. In the absence of uterine pathology or early embryonic death, a persistent CL may be considered “spontaneous” (7). Spontaneous CLs have been attributed to alterations in the signal for $PGF_{2\alpha}$ release from the uterus [131]. This condition can affect up to 6% of interovulatory intervals during the ovulatory season, and up to 28% during the transition into the anovulatory season [131, 132]. Body mass index, body weight, height, age, and mare type do not influence the frequency of persistent CL function [133]. Before inclusion in the study, all mares were confirmed to cycle normally, with an interovulatory interval between 20 and 24 days. The relationship, if any, between GnRH immunization and persistent CL activity is not clear.

A weakness in the application of anestrus index scoring is that the method does not account for the transitional period of the mare’s reproductive cycle. For example, based on anestrus index score, mare 15 had returned to cyclicity, despite the fact that the mare had not ovulated within the study period. The behavioral and sonographic findings, coupled with serum progesterone concentration <2ng/ml was consistent with that observed in a mare that is in the resurging phase that occurs as a gradual return to ovulatory status [1]. It can only be assumed that mare 15 would have ovulated a dominant follicle, developed a CL, and experience a normal luteal phase which is the more commonly recognized indicator of a return to cyclicity [75, 79].

Lastly, all mares experienced only minimal side effects following vaccination with Ad-GnRH, and again following heterologous boost. The only observed side effects occurred locally at the injection site and were observed following both Ad-GnRH, and protein-heterologous boost. Following Ad-GnRH boost, and protein-GnRH-boost three of five mares and two of four mares respectively developed a small injection site nodule that resolved within 3 days without treatment. This finding was interesting due to the reported high incidences side effects following vaccination with peptide-based GnRH vaccines. Reported effects include raised nodules, swelling and pain at the injection site [74], stiffness of the neck, pyrexia, and

apathy [75]. The side effects observed in this study were not so severe that they would be expected to interfere with mare athleticism and performance.

9. Conclusions

This study demonstrates that mares are capable of developing an anti-GnRH antibody response to homologous immunization using a replication-defective E1/E3 deleted replication-defective adenovirus vector encoding GnRH peptide, bacterial leukotoxin, and T-helper epitopes. Homologues prime-boost vaccination of mares with Ad-GnRH at the dose and frequency used in this study does not result in suppression of reproductive cyclicity and estrus behavior.

This study demonstrates that heterologous prime-boost vaccination of mares using an Ad-GnRH prime and protein based GnRH vaccine boost results in an antibody response that suppresses reproductive cyclicity, and interferes with estrus behavior. Vaccine induced effects are observed within four weeks of heterologous boost and may be maintained for at least 12 weeks.

Vaccinated mares experience minimal side effects following vaccination with either Ad-GnRH or heterologous boost using a sub-effective dose (100µg protein conjugate) of a protein based GnRH vaccine. The only side effects occur locally at the injection site and include a small, raised non-painful nodule that resolves within 3 days of vaccination.

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Appendix

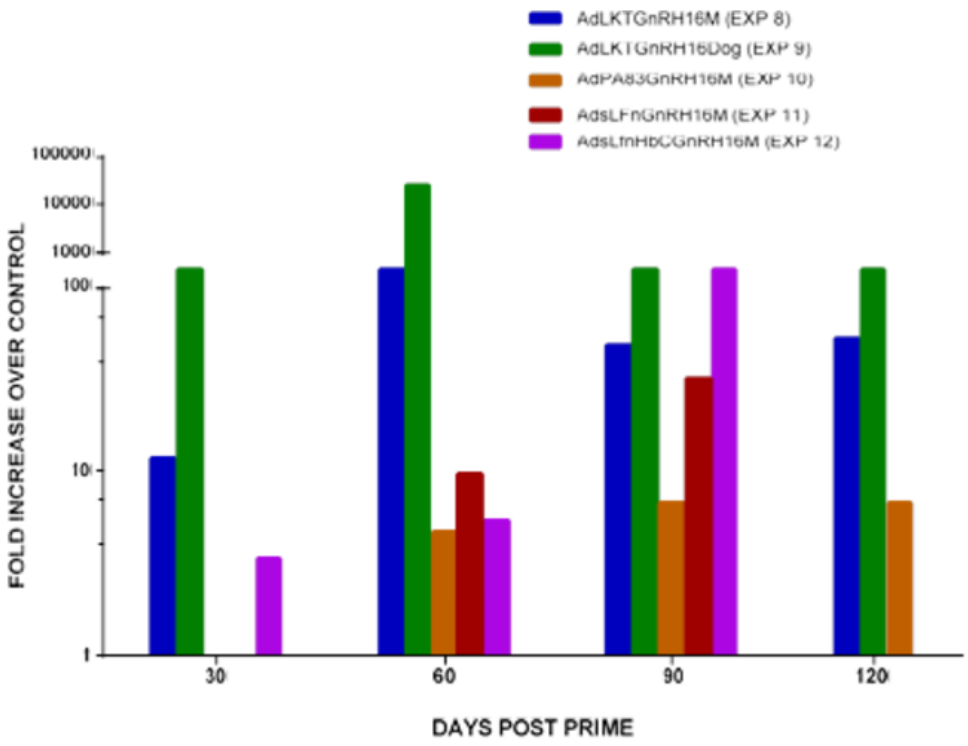
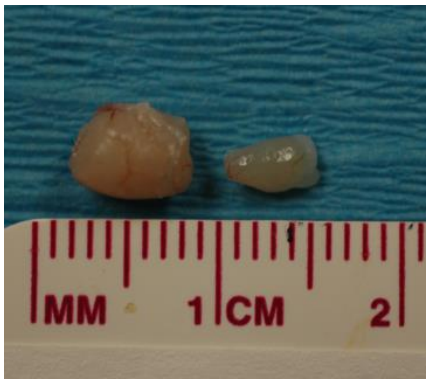
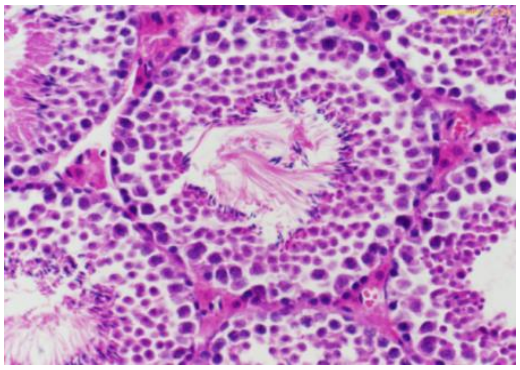


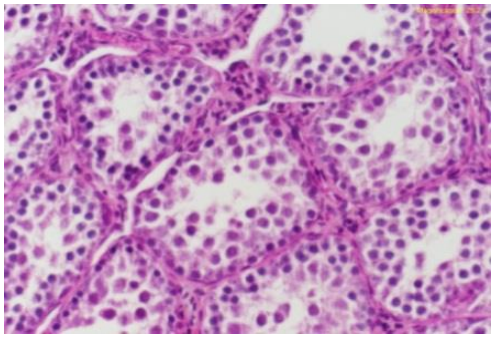
Figure 1. Fold increase over control (baseline) antibody titer in male mice at given time points following a single vaccination using 1x10^9 ifu of an experimental Ad-GnRH vaccine. Each bar represents an Ad-GnRH vaccine engineered to express 16 multimers of GnRH linked to one of five antigenic protein carriers. The highest antibody titer at all time points was produced in response to vaccination utilizing the bacterial leukotoxin (leukotoxin A1 gene of *Pasteurella haemolytica*) carrier protein, shown in green. (Unpublished data of a study by Baker *et al*., permission granted to include this figure).



2a



2b



2c

Figure 2. Gross testicular dysgenesis of an immunized mouse (right) compared with normal gonad (left)(2a). Normal mouse testicular histological structure showing well defined Leydig cells and active spermiogenesis (2b). Testicular dysgenesis showing loss of Leydig cells and aspermiogenesis in an immunized mouse (2c) (Unpublished data of a study by Baker *et al*., permission granted to include this figure).

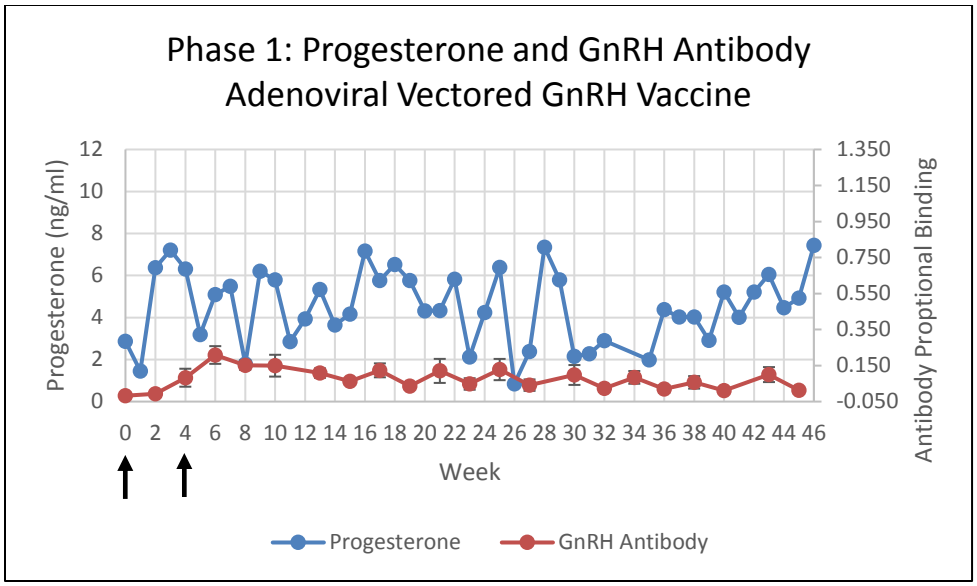


Fig 3a

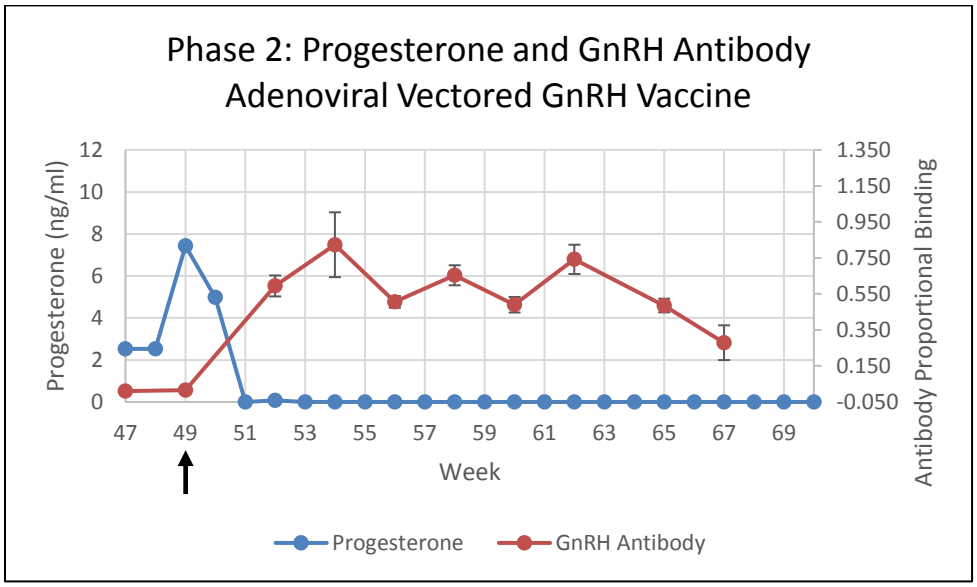


Fig 3b

Figure 3. Mean serum progesterone and GnRH antibody proportional binding during phase one (3a) and phase two (3b). Time of Ad-GnRH prime and boost indicated by arrow at week zero and week four during phase one. Heterologous boost with protein antigen GnRH vaccine indicated by arrow at week 49 during phase two.

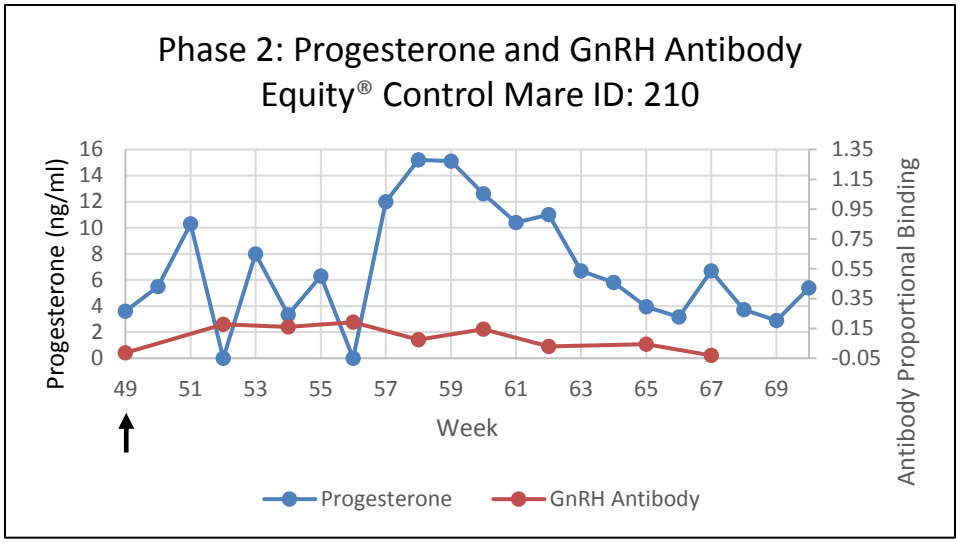


Fig 4a

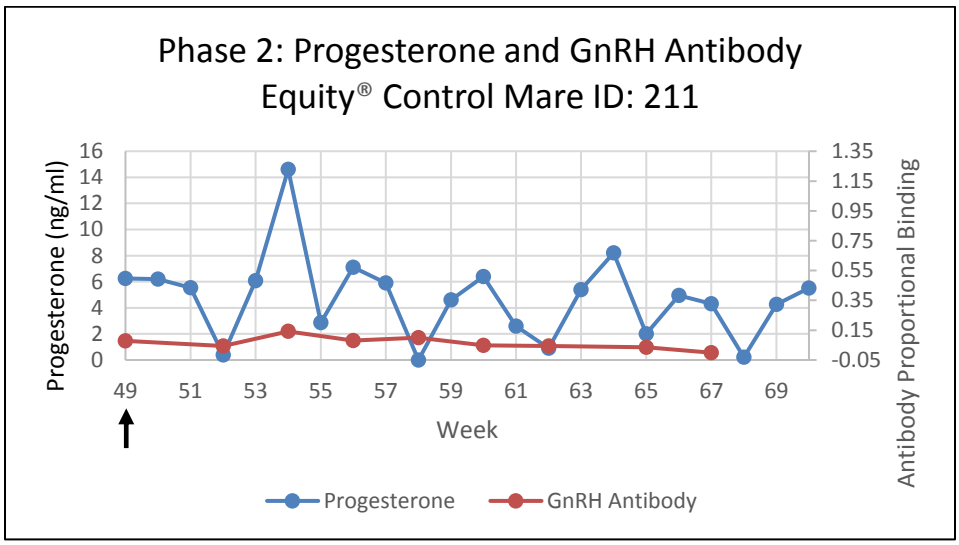


Fig 4b

Figure 4. Individual mare progesterone concentration and GnRH antibody proportional binding for antigen boost control mares throughout phase two. Time of protein antigen GnRH vaccine indicated by arrow at week 49.

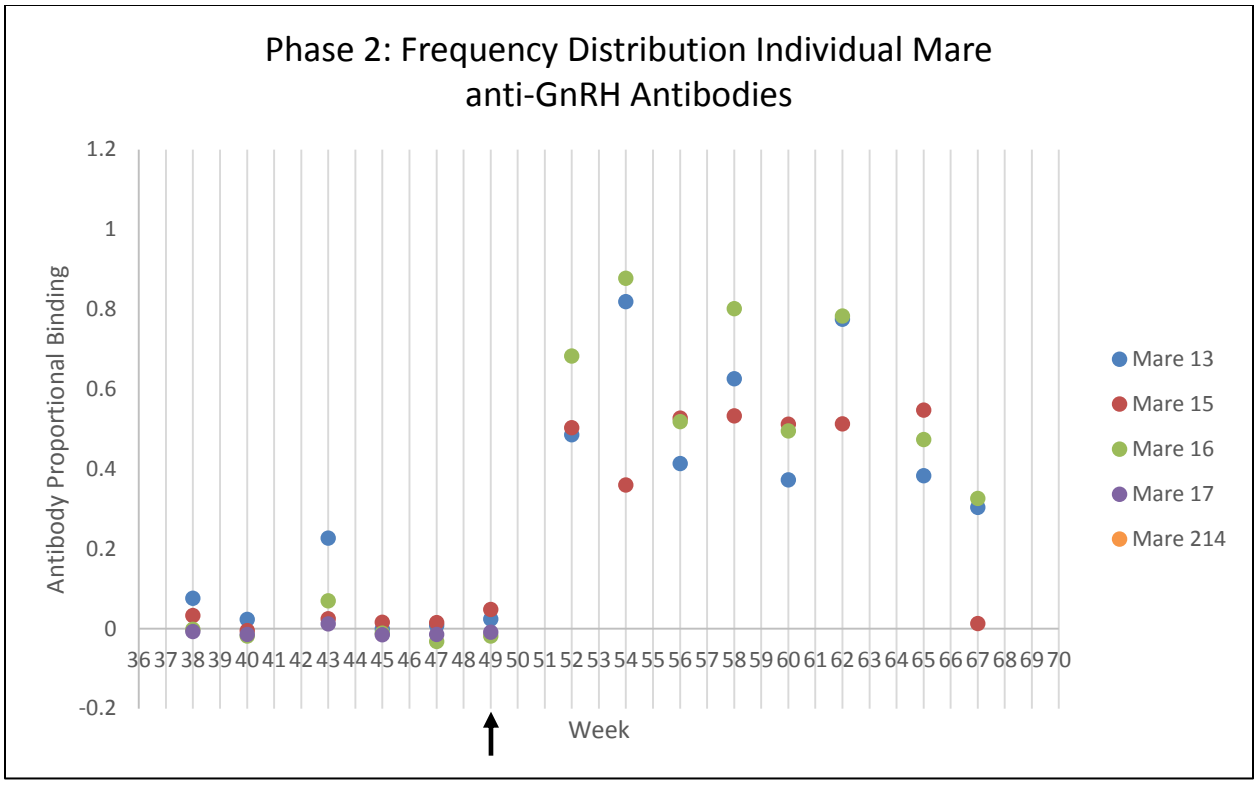


Figure 5. Frequency distribution of anti-GnRH antibody data for treatment mares during phase two. Note an increase in frequency distribution that occurred following heterologous boost with a protein antigen GnRH vaccine at week 49 (black arrow).

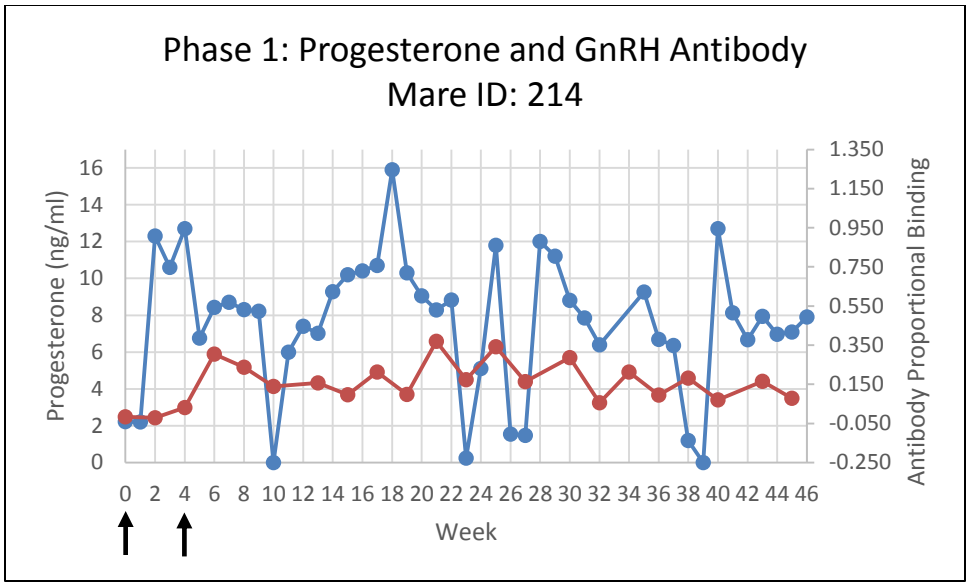


Fig 6a

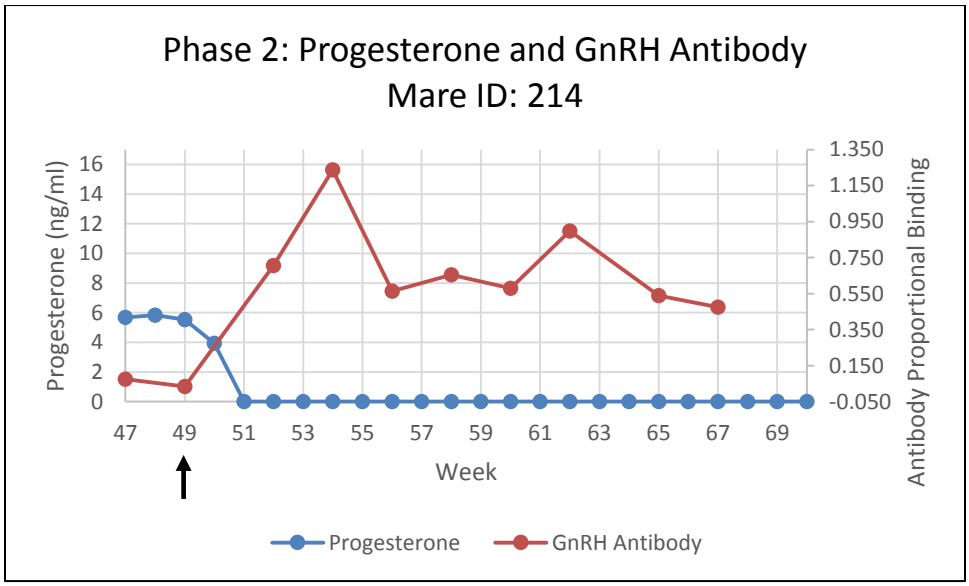


Fig 6b

Figure 6. Mare 214 progesterone concentration and GnRH antibody proportional binding. Ad-GnRH prime and boost indicated by arrows at weeks 0 and 4 respectively (6a). Heterologous boost with protein antigen GnRH vaccine indicated by arrow at week (6b).

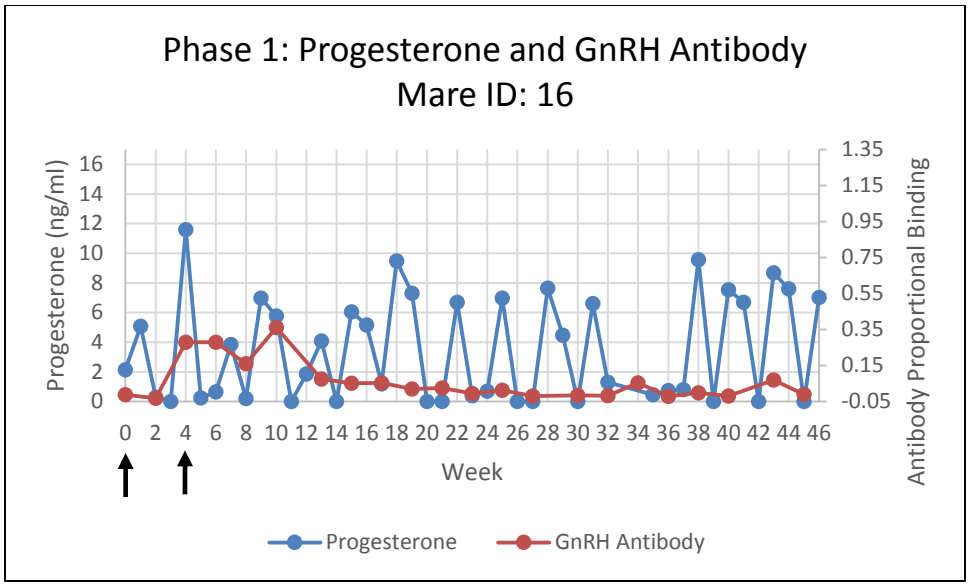


Fig 7a

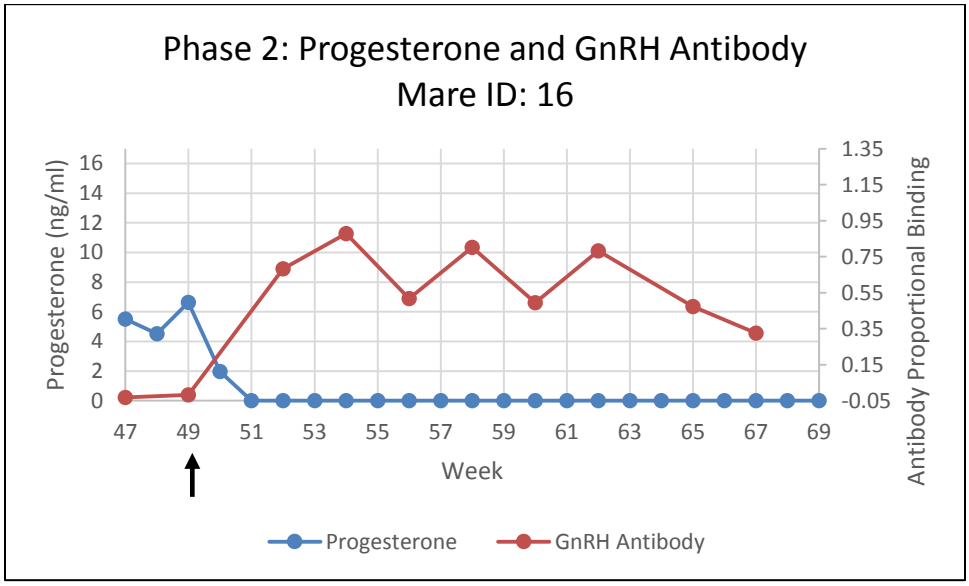


Fig 7b

Figure 7. Mare 16 progesterone concentration and GnRH antibody proportional binding. Ad-GnRH prime and boost indicated by arrows at weeks 0 and 4 respectively (7a). Heterologous boost with protein antigen GnRH vaccine indicated by arrow at week (7b).

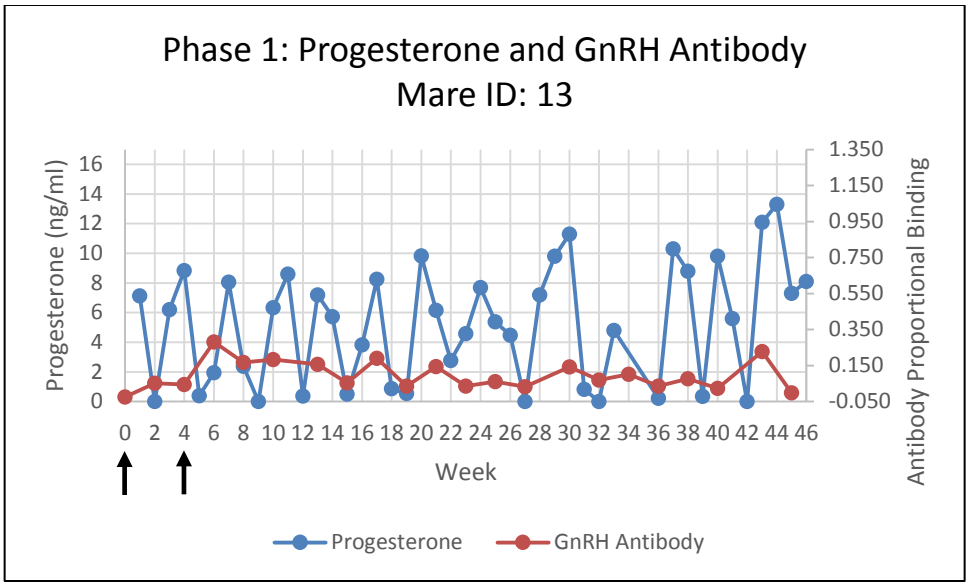


Fig 8a

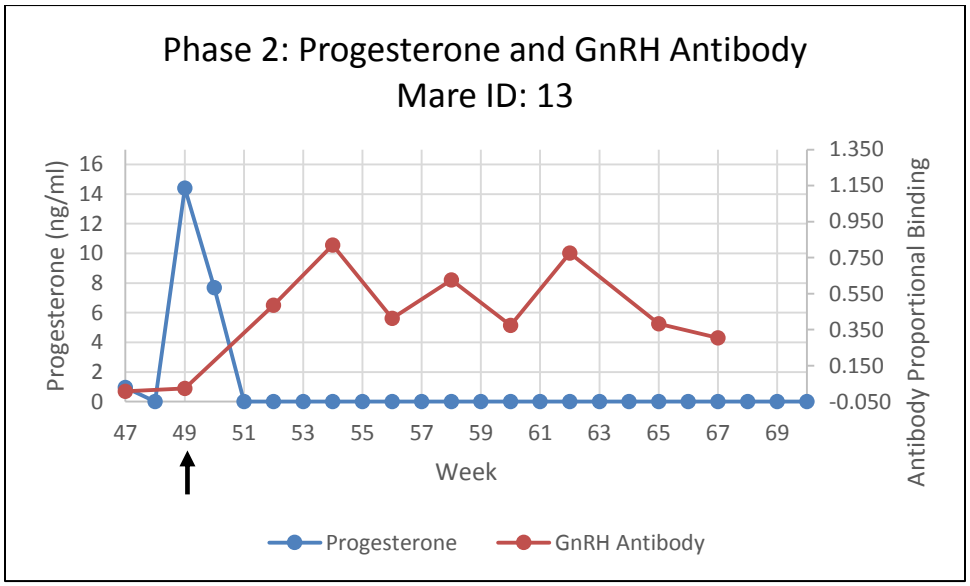


Fig 8b

Figure 8. Mare 13 progesterone concentration and GnRH antibody proportional binding. Ad-GnRH prime and boost indicated by arrows at weeks 0 and 4 respectively (8a). Heterologous boost with protein antigen GnRH vaccine indicated by arrow at week (8b).

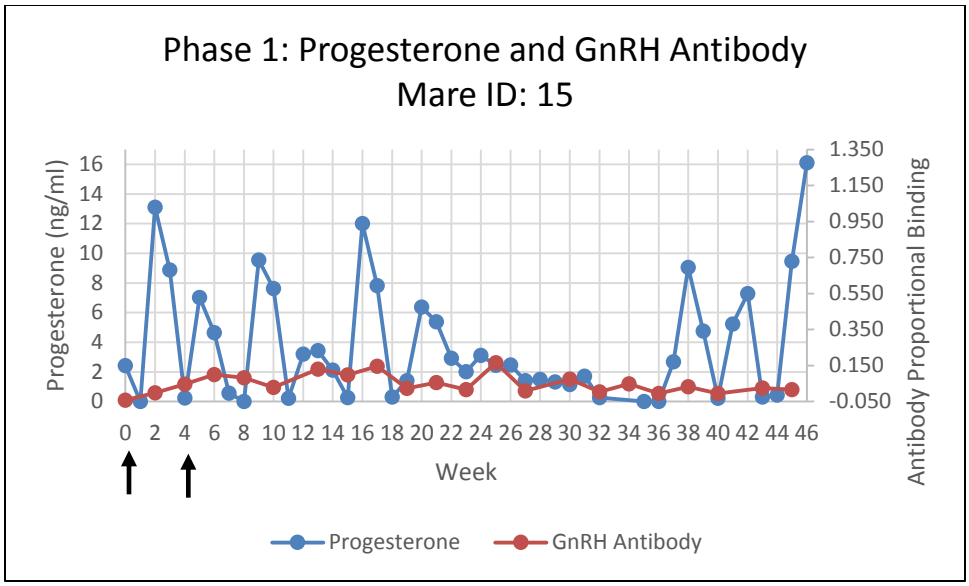


Fig 9a

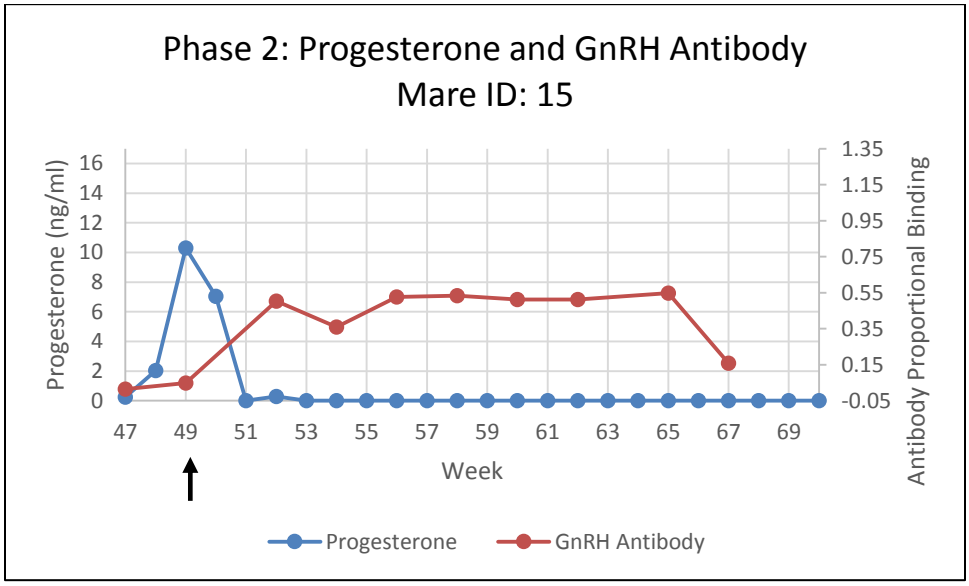


Fig 9b

Figure 9. Mare 15 progesterone concentration and GnRH antibody proportional binding. Ad-GnRH prime and boost indicated by arrows at weeks 0 and 4 respectively (9a). Heterologous boost with protein antigen GnRH vaccine indicated by arrow at week (9b).

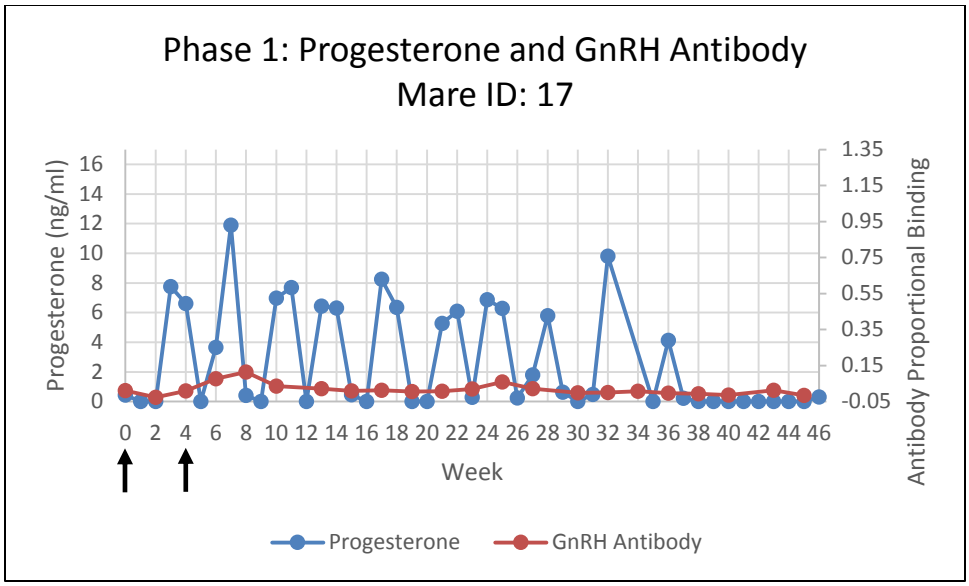


Figure 10. Mare 17 progesterone concentration and GnRH antibody proportional binding. Time of Ad-GnRH prime and boost indicated by arrow at week 0 and 4 respectively.

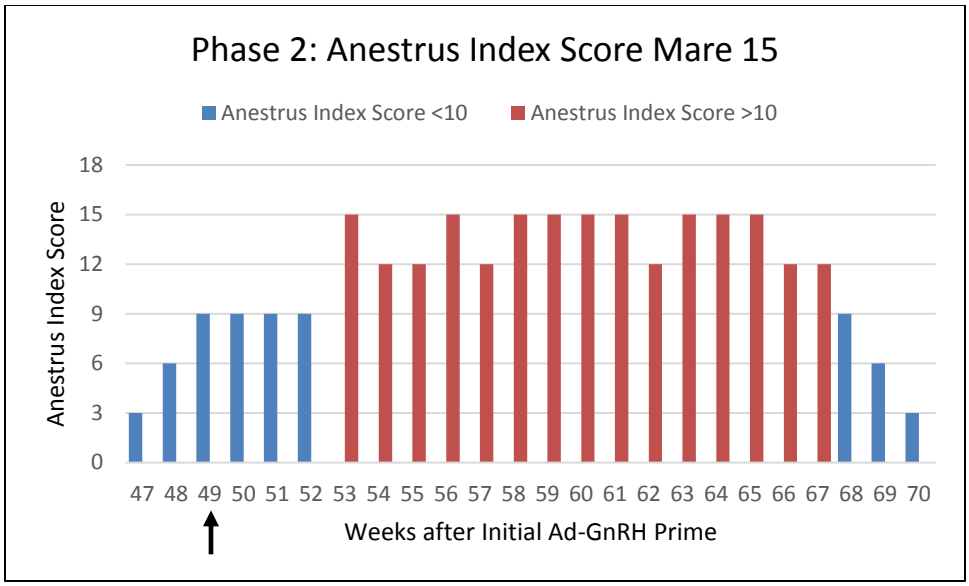


Figure 11. Anestrus Index Score for mare 15 that received homologous prime-boost using Ad-GnRH at weeks 0 and 4 respectively. Heterologous boost with a protein antigen GnRH vaccine occurred at week 49 and is indicated by the arrow. The mare became acyclic (Anestrus index score >10) 4 weeks following heterologous boost and returned to cyclicity (Anestrus Index score <10) 19 weeks following heterologous boost.

Fig 12a

Fig 12b

Figure 12. Following homologous Ad-GnRH boost, three of five treatment mares developed a non-painful 1-3cm raised nodule at the injection site. An injection site nodule for mare 15 one day post vaccination with Ad-GnRH (12a). Following heterologous boost, two of four treatment mares developed a small (<2cm) raised, non-painful nodule. An injection site nodule for mare 16 one day post heterologous boost (12b). All injection site reactions resolved within 3 days without treatment.