

SUPPRESSION OF CYCLICITY AND ESTROUS BEHAVIOR IN MARES
THROUGH IMMUNIZATION AGAINST A RECOMBINANT GONADOTROPIN-
RELEASING HORMONE ANTIGEN

by

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Abstract

Mares in estrus can exhibit erratic and fractious behavior that may adversely affect their ease of handling. Current treatments for suppression of estrus in the mare include an oral progestin, Regu-mate® (Hodgson et al., 2005), uterine insertion of a glass ball (Nie et al., 2003), fetal crushing through rectal palpation (Lefranc, 2004) and surgical sterilization (McCue, 2003). Although effective, all of these procedures are problematic for different reasons. Immunization against an endogenous hormone critical to fertility is an attractive alternative in suppressing estrus; especially if it is less intrusive and reversible. This strategy has been demonstrated by targeting gonadotropin-releasing hormone (GnRH) in gilts (Esbenshade and Britt, 1985), ewes (Clarke et al., 1978), mares (Garza et al., 1986), and heifers (Johnson et al., 1988).

The antigen developed for this study was produced using the sequence of the bacterial protein, glutathione-S-transferase (GST) linked to three in-tandem repeats of the mammalian GnRH gene coding sequence (GST-GnRH3). Six reproductively sound mares, between the ages of 3 and 8 years, were used in this study. The anti-GnRH group (n=4) received 1 mg of GST-GnRH3 in 2ml of incomplete Freund's adjuvant (IFA) as the primary injection. Four weeks later, mares received a single booster injection of 0.5mg of GST-GnRH3 in 1ml of IFA. The control mares (n=2) received similar doses of GST protein only, in identical injection volumes of IFA as the anti-GnRH group. The entire duration of the study ran for 22 weeks from early May through September.

Ovaries were monitored three times weekly to track follicular growth and ovulation via transrectal ultrasonography. In addition, all mares were exposed to a stallion twice weekly and observed for estrous behavior. Weekly blood samples were collected to evaluate progesterone levels and serum binding of GnRH.

The GST-GnRH3 antigen suppressed follicular activity in all treatment mares within 45 days following the second injection. Estrous behavior was suppressed in all but one mare in the anti-GnRH group. When exposed to the stallion, this mare displayed strong estrous behavior for seven weeks despite her lack of ovarian cyclicity. Follicular activity and estrous behavior remained normal in one of the control mares (avg. cycle length = 20 days). For the final 10 weeks of the study, however, the other control mare developed large follicles but failed to ovulate according to the ultrasound data. This mare did not display estrous behavior during this period, and her progesterone levels remained greater than 2 ng/ml for most of the final 10 weeks of the study. Approximately 2 weeks after the booster injection all anti-GnRH mares had progesterone levels of <1ng/ml. GnRH antibody binding peaked two weeks following booster immunization in all treated mares and remained undetectable in control mares throughout the study.

The GST-GnRH3 treatment induced GnRH binding, suppressed follicular activity and reduced progesterone concentrations in all four mares. Although estrous behavior was abolished in 3 of the 4 treated mares, one did continue to demonstrate estrous behavior in the presence of a stallion. This dissociation of ovarian activity and estrous behavior was evident in our study with a limited number of animals, but the vaccine does show promise in reducing unwanted estrous behavior.

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Dedication

I would like to dedicate this thesis to my parents. I cannot even begin to express my appreciation for your constant guidance, love and support throughout my life. Dad, you influenced my love for agriculture and taught me one of the most important concepts to pursuing a life involved in agriculture, to work hard! Mom, thank you for EVERYTHING! You have taught me to be passionate about everything I do and you truly are my best friend.

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CHAPTER 1 - Review of Literature

Estrous Cycle of the Mare

Horses are seasonal polyestrous breeders and mares in the Northern Hemisphere, in general, have estrous cycles beginning early spring and continuing through mid-fall. However, the reproductive cycle of the mare is more variable than any other domestic species and anestrus is not necessarily consistent throughout the winter months (Ginther, 1992). One of the most important environmental signals for controlling estrus and anestrus is photoperiod. During short day months the pineal gland secretes melatonin suppressing estrus in the mare, in contrast to long day months when melatonin secretion is low and allowing for persistent waves of follicular development.

The mare's estrous cycle is approximately 21 days long beginning with 5-7 consecutive days of sexual receptivity (standing estrus). Ovulation usually occurs on days 3, 4, or 5 of the estrous cycle with behavioral estrus subsiding 24 to 48 hours after ovulation. A follicle stimulating hormone (FSH) surge at 1-2 weeks prior to ovulation initiates the growth and selection of the dominant follicle. Luteinizing hormone (LH) secretion in the mare is unique to other domestic species. In contrast to a quick burst, LH concentrations gradually increase during the follicular phase reaching maximum concentrations 1-2 days after ovulation and then slowly decline over the next 4-6 days (Ginther, 1992).

Gonadotropin-Releasing Hormone (GnRH) is composed of a highly conserved 10 amino acid sequence (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) and is a fundamental regulator of reproductive function through initiation of the hypothalamic-

pituitary-gonadal axis (Schally, 1975). Neurosecretory cells located in the medial basal hypothalamus synthesize and release GnRH into the hypothalamo-hypophyseal portal circulation enabling small quantities of hormone to be deposited in the primary portal plexus of the pituitary stalk (Senger, 2004). GnRH is rapidly transferred to a second capillary bed within the anterior pituitary and binds to GnRH-receptors expressed by the pituitary gonadotropes.

GnRH hormone-receptor binding initiates synthesis and release of gonadotropin hormones through signal transduction. Phosphorylation of guanosine diphosphate (GDP) on the intracellular G protein-coupled receptor alpha subunit (G_α) initiates activation of the phospholipase C (PLC) signaling pathway. PLC hydrolyses the phosphodiester link in phosphatidylinositol 4,5 bisphosphate (PIP_2), cleaving it into second messengers inositol triphosphate (IP_3) and transmembrane diacylglycerol (DAG). IP_3 quickly diffuses through the cytosol to the endoplasmic reticulum (ER) where it binds to a ligand gated calcium (Ca^{2+}) channel allowing release of Ca^{2+} into the cytosol. DAG activates protein kinase C (PKC) stimulating synthesis of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Release of LH and FSH into the circulation results from the influx and accumulation of calcium as well as PKC (Hirota et al., 1985).

Gonadotropins bind to receptors expressed on cells of developing follicles to stimulate follicular growth and ovulation. Granulosa cells in developing follicles synthesize and secrete the steroid hormone estrogen, which in turn acts on the hypothalamus to increase synthesis and secretion of GnRH, causing the LH surge

required for ovulation. After ovulation the corpus luteum develops and will secrete progesterone which inhibits GnRH concentrations.

Estrus Prevention

Typically it is a producer's goal to enhance the fertility in livestock species. This is obviously important for breeding stock, but in other production situations suppression or abolition of estrus and its associated behavior is advantageous. For example, there are several negative economic factors associated with heifers entering the feedlot pregnant including reduced average daily gain, decreased dressing percentage and the risk of calving in the feedlot (Sewell, 1993). Owners of performance, ranch, show or racing horses tend to devalue mares due to aggression and inattentive behavior exhibited during the 5-7 days of estrus (N. Edward Robinson, 2008). Handling of some mares in estrus can be difficult and dangerous due to their aggressive and obstinate demeanor. While estrous behavior is most apparent when the mare is in close proximity to a stallion, aggression and decreased response to commands can be demonstrated under any circumstance during estrus. The peak of sexual receptivity is characterized by lowering of the pelvis and spreading hind limbs, lifting of the tail, exposing the perineal region by rhythmic eversion of the clitoris, and frequent urination (Crowell-Davis, 2007). At these times it is impractical and potentially dangerous to use these animals, as they often attempt to bite or kick other horses. In an effort to eliminate these problems several methods of suppressing estrus and estrous behavior in mares have been explored.

Progesterone Treatments

The most common method of suppressing estrus in mares is by the use of progesterone and synthetic progestins. High levels of progesterone suppress the release of hypothalamic GnRH which reduces pituitary output of LH and FSH thereby suppressing follicular growth and estrogen concentrations known to induce estrous behavior. Progesterone, in an oil base, was shown to effectively suppress cyclicity and estrous behavior in mares when injected intramuscularly at a dose of 100 mg daily or 400 mg every other day (Loy and Swan, 1966). However, weekly administration can cause moderate to severe site reactions and muscle soreness. Medroxyprogesterone acetate (MPA) was investigated as another off-label use of progesterone for suppressing estrus in mares (Gee et al., 2008). In this study, an initial dose (1.6 g) of compounded MPA with subsequent weekly doses of 400 mg did not suppress estrous behavior or follicular development in any of the treated mares. Progesterone-estradiol benzoate (Synovex-S® implants; Syntex Animal Health, Des Moines, Iowa), labeled for use in cattle to promote feed efficiency and weight gain, were investigated as a method of suppressing estrus in mares (McCue, 1997). A normal dose of Synovex-S® consists of 8 implants equivalent to 200 mg of progesterone and 20 mg of estradiol benzoate. In this study estrous activity was not suppressed in mares receiving 0, 8, 32 or 80 implants 5 days post-ovulation. Alternatively, oral administration of the synthetic progesterone Regu-mate® (Altrenogest, Intervet Inc, Millsboro, DE) is labeled for use to help maintain pregnancy and delay estrus in cycling mares, and is widely used for performance and show horses. Altrenogest has shown to be 94% effective at suppressing estrous behavior and follicular development when administered daily for 15 days (Webel and Squires, 1982). Of the

mares that displayed estrus during the treatment period, some displayed estrus for the first 5-8 days while others displayed sporadic estrous behavior or remained in estrus the entire duration of the treatment. Daily doses of Regu-mate® (0.44 mg/kg) placed on the back of mare's tongue can be difficult to administer, expensive (approximately \$2.50 - \$4.00 a day) and must be handled with care as it is readily absorbed through skin which could cause a disruption of menstrual cycles of women.

Oxytocin Treatments

Oxytocin during the equine luteal phase is not thought to be secreted by the CL to the same degree as other domestic species (Stevenson et al., 1991). However, it was reported that repeated injections of oxytocin during the midluteal phase was effective at prolonging the luteal phase in the mare (Stout et al., 1999; Vanderwall, 2007). A recent study demonstrated that injections of oxytocin every 12 hours from days 7-14 post-ovulation prolonged the CL and held progesterone levels above 1ng/ml for approximately 60 days (Vanderwall, 2007). More research is needed to improve our understanding of the impact that oxytocin has on estrus suppression and associated behavior in mares.

Uterine Glass Ball

Uterine insertion of a glass ball (UGB) has been a technique used to suppress behavioral estrus in the mare by prolonging the luteal phase and sustaining high progesterone levels. While the method of action by which the luteal phase is prolonged is unclear, the UGB may mimic a conceptus and movement around the uterus inhibits endometrial release of prostaglandin preventing luteolysis (Nie et al., 2003). A study of the efficacy of UGB treatment found that insertion of a ball approximately 35 mm in

diameter 24 hours post ovulation prolonged luteal function for up to 87 days post-ovulation in 39% of mares. A smaller 25 mm UGB was less effective as it was expelled in 50% of the mares tested, while no expulsion occurred in mares receiving a 35 mm UGB (Nie et al., 2003). Insertions of UGBs are effective at maintaining progesterone levels above 1ng/ml, and may serve as an alternative to daily administration of progesterone. This method is an easy, inexpensive and reversible method of suppressing estrus in the mare. However, the low efficacy rate, the possibility of microbial contamination and the need for monitoring cyclic activity to determine the time of insertion followed by removal of the ball makes it an unattractive method for many horse owners.

Surgical Sterilization

A more common procedure used to suppress estrous behavior and prevent pregnancy, compared to use of a UGB or manually reducing pregnancy, is surgical sterilization or ovariectomy. It is speculated that in most domestic species initiation of estrous behavior is due to ovarian steroid hormones, and by removing the ovaries such behavior would be eliminated. However, studies have shown that this is not always true and that continuous estrous behavior in the absence of ovarian activity has been observed in some ovariectomized, anovulatory, or mares with small inactive ovaries (Asa et al., 1980; Dalin et al., 2002; Hooper, 1993; Kamm and Hendrickson, 2007). It was also reported that the probability of an ovariectomized or anovulatory mare showing estrus behavior is higher on any given day than it is for an intact mare during the ovulatory season (Asa et al., 1979). This frequent behavior qualifies these females as good candidates for use as jump mares on breeding farms but can be problematic for a

performance horse owner. Irregular estrous patterns observed in anovulatory and ovariectomized mares could be the result of specific social compositions of mares teased in groups or due to individual mare preference for certain stallions (Asa et al., 1980). Moreover, estrous behavior in mares with inactive ovaries may be initiated by the release of estrogenic steroids from the adrenals (Asa et al., 1980; Crowell-Davis, 2007), suggesting estrous behavior displayed by ovariectomized or anovulatory mares may be due to low progesterone levels and circulating adrenal estrogens. Due to the unpredictable behavior after surgery and irreversibility of this method, it is not an ideal procedure for owners of mares that would like to reduce or eliminate typical behaviors associated with mares in estrus.

Immunocontraception

Immunization against an endogenous hormone critical to fertility is referred to as immunocontraception. The method by which immunocontraception initiates an immune response and suppresses estrus is through initiation of humoral immunity involving the proliferation of B immune cells and their production of antibody proteins. Upon primary immunization, a short burst of the immunoglobulin IgM is secreted by B cells as the initial response to neutralize the newly recognized antigen. The IgM response is associated with the action of innate immune cells which process and clear the antigen before presenting antigen to B cells to stimulate production of specific immunoglobulins. Subsequent exposure to the same antigen results in a secondary immune response involving the secretion of the memory immunoglobulin IgG. This secondary immune response is slightly delayed and IgG is secreted at a much higher concentration and for

longer periods of time than IgM secretion to ensure the binding, destruction and clearance of antigen.

In order to elicit an immune response to an endogenous reproductive hormone, a mimicking antigen must be linked to a foreign carrier protein. Antibodies will be produced against the foreign protein-hormone complex. These hormone-specific antibodies generated will cross react with the endogenous hormone and prevent it from reaching its target tissue. This methodology is an attractive alternative to other methods of reducing fertility and has been demonstrated to suppress estrus in horses by targeting the reproductive hormone gonadotropin-releasing hormone (Dalin et al., 2002; Elhay et al., 2007; Garza et al., 1986; Imboden et al., 2006; Tshewang et al., 1997).

Typically, the source of protein hormone used as antigens are chemically synthesized or purified from tissues and then chemically conjugated to a foreign carrier molecule. One disadvantage of this procedure is the variation of immunogenicity in antigen preparations as a result of the purification and conjugation procedures (Grieger and Reeves, 1990). As an alternative, the DNA coding sequence for a specific peptide can be linked to a bacterial carrier molecule DNA sequence through recombinant DNA engineering.

Anti-GnRH Vaccine in the Mare

An early study investigated the effects of a GnRH conjugated to bovine serum albumin vaccine on the secretion of LH and FSH (Garza et al., 1986). Mares received an immunization of 4.0 mg of the GnRH conjugate in Freund's complete adjuvant, followed 4 weeks later by a 2.0 mg dose of antigen in Freund's incomplete, which was then administered every 6 weeks until ovariectomy at week 30. Findings from this study

concluded that this anti-GnRH vaccine successfully suppressed plasma concentrations of progesterone, LH and to a lesser extent FSH while increasing tritiated GnRH binding after the third immunization.

Recently, commercial conjugate anti-GnRH vaccines such as Improvac® (Pfizer Animal Health P/L, West Ryde, NSW, Australia) and Equity™ (Pfizer Animal Health P/L, West Ryde, NSW, Australia) have been evaluated as effective methods of suppressing estrus in the mare (Elhay et al., 2007; Imboden et al., 2006). Improvac®, commonly used in Australia to treat against boar taint, was administered to 9 mares on weeks 0 and 4 (Imboden et al., 2006). Follicular development and progesterone concentrations were suppressed following the second injection, however sporadic estrous behavior of the Improvac® treated mares was observed throughout the duration of the study. Similarly, two injections of Equity™ administered 4 weeks apart successfully suppressed follicular development and progesterone levels, but was not capable of suppressing estrous behavior in all mares (Elhay et al., 2007). Equity™ is currently approved for use in fillies and mares to suppress estrous behavior and activity in Australia.

Recombinant DNA Technology

The use of recombinant DNA technology eliminates the need for tissue purification of protein and conjugation steps (Johnson et al., 1988). Recombinant DNA is the incorporation of two or more sources of DNA to construct a new recombinant molecule. This technology is an attractive alternative to conjugation of tissue-purified protein due to the similarity and repeatability between batches of recombinant antigen. A recombinant glutathione S- transferase-GnRH vaccine (Koster and Grieger, 1995) was

effective in abolishing estrous cycles in yearling beef heifers (Holladay et al., 2003). Use of recombinant DNA technology in a vaccine against GnRH was described by Zhang et al. (1999) employing ovalbumin as the carrier protein source. This recombinant GnRH vaccine was shown to be effective at suppressing follicular development in mice (Zhang et al., 1999), rats (Conforti et al., 2007), rams (Ulker et al., 2005) and cattle (Geary et al., 2006; Hernandez et al., 2005; Sosa et al., 2000; Stevens et al., 2005).

CHAPTER 2

Suppression of cyclicity and estrous behavior in mares through immunization using a recombinant GnRH antigen.

Introduction

Typically it is a producer's goal to enhance the fertility in livestock species. This is obviously important for breeding stock, but in other production situations suppression or abolition of estrus and its associated behavior is advantageous. For many horse owners, mares are less valuable than geldings as working or performance horses due to gender-related behavior problems such as aggression, focused attention to surroundings, and a decreased response to commands. Ranch or show mares often exhibit behavioral problems during estrus that may adversely affect their ease of handling. At these times it is impractical and potentially dangerous to use these animals, as they often attempt to bite or kick other horses.

Current treatments for suppressing estrus in the mare include the oral progestin, Regu-mate® (altrenogest, Intervet, Inc. (Hodgson et al., 2005), uterine insertion of a glass ball to suppress behavioral estrus (Nie et al., 2003), fetal crushing through rectal palpation (Lefranc, 2004), oxytocin treatments (Vanderwall, 2007) and surgical sterilization (McCue, 2003). However, all of these procedures are problematic for different reasons. Regumate must be administered daily, fetal crushing is uncommon and often viewed as ethically unacceptable, and surgical sterilization is effective but

irreversible. An effective, non-invasive and reversible method of sterilization would be ideal for the horse owner, whether their mares are used for pleasure, show, or ranch and feedlot work.

Immunization against an endogenous hormone critical to fertility is referred to as immunocontraception. This methodology is an attractive alternative to other methods of suppressing fertility and has been demonstrated by targeting the reproductive hormone gonadotropin-releasing hormone (GnRH) in gilts (Esbenshade and Britt, 1985), ewes (Clarke et al., 1978), mares (Garza et al., 1986), and heifers (Johnson et al., 1988).

Gonadotropin-releasing hormone is a hypothalamic peptide that causes release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland (Hafez and Hafez, 2000). Since the primary roles of FSH and LH are to cause follicle growth and ovulation, altering the function of GnRH will disrupt reproductive cycles. However, in order to immunize animals against an endogenous “self” protein such as GnRH, the peptide must first be linked to a foreign carrier molecule which will induce an immune response. Typically, the source of protein hormone for use as antigens are chemically synthesized or purified from tissues, and then chemically conjugated to a carrier molecule. One disadvantage of this procedure is the variation of immunogenicity in antigen preparations as a result of the purification and conjugation procedures (Grieger and Reeves, 1990). As an alternative, the DNA coding sequence for GnRH can be linked to a bacterial carrier molecule DNA sequence through use of recombinant DNA engineering. This methodology eliminates the need for tissue purification of protein and conjugation steps. The use of recombinant DNA technology to produce fusion proteins can also provide a consistent source of antigen. Previous

studies in our laboratory reported the development of a recombinant GnRH vaccine (Koster and Grieger, 1995) that was effective in abolishing estrous cycles in yearling beef heifers (Holladay et al., 2003). In mares it would be ideal to give vaccinations against GnRH at selected times of the year to suppress unwanted estrous periods. Since immunocontraception has been shown to be reversible in cattle (Grieger and Reeves, 1990; Sosa et al., 2000), this may be very beneficial in the equine industry where estrus could be suppressed early in a mare's life, with the option of breeding her when she is older. The objective of this study was to test the use of a recombinant GnRH vaccine as a method of inducing temporary sterility in mares.

Materials and Methods

Plasmid Construction

The construction of the expression plasmid GST-GnRH3 was previously described by Holladay and coworkers in 2003 (Figure 1). The commercial expression vector (pGEX-4T-3; GE Healthcare, Piscataway, NJ) was engineered to include three tandem copies of the coding sequence for GnRH in-frame and downstream of the glutathione-S-transferase coding sequence. The expression plasmid created was named pGEX-GnRH3. In the current study the plasmid was transformed into *E. coli DH5-α* cells, an attenuated laboratory strain used for plasmid replication and protein expression. Transformed cells were streaked onto Luria-Bertani (LB) ampicillin agar plates and grown overnight at 37°C. Resulting colonies were cultured and plasmid DNA was isolated using QIAprep® Spin Miniprep Kit (Qiagen, Valencia, CA). Purified plasmid DNA was digested with restriction endonuclease BamH I (New England Biolabs, Ipswich, MA) or both BamH I and EcoR V (New England Biolabs). Digests were evaluated on a 2% agarose gel containing ethidium bromide to verify predicted insertion size.

The control vaccine was generated using GST protein from the pGEX-4T-3 vector. Vector DNA was digested with BamH I to confirm absence of GnRH3 insert.

Protein Expression

A 25 ml aliquot of an overnight culture of plasmid-transformed DH5-α cells was used to inoculate 500 ml of LB ampicillin broth. The 500 ml flasks remained under constant shaking at 37°C until A₆₀₀ was between 1.0 - 2.0 relative absorptive units. When

the culture reached desired confluence, isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce protein expression.

A series of four different treatments were investigated to determine which would render the greatest yield of soluble fusion protein (GST-GnRH3). Incubation time and solubilizing buffer were the two factors tested. Time of IPTG incubation was either overnight at room temperature or for 3 hours at 37°C. The solubilizing buffer consisted of either lysozyme alone or lysozyme plus sarcosine (Sambrook and Russell, 2001). Each treatment culture was replicated twice.

In treatments using sarcosine buffer, the culture containing IPTG was left to shake overnight at room temperature or for 3 hours at 37°C. After either 3 hours or an overnight incubation time, cultures were centrifuged at 3300xg for 20 minutes at 4°C, supernatant was discarded and a 1/20 volume of Gibco DPBS was used to resuspend the remaining pellet. Cell walls were disrupted by adding 1 mg/ml lysozyme to the resuspended pellet and placed on ice for 30 minutes. The cell lysate was centrifuged at 10,000 xg for 10 minutes at 4°C, the supernatant was removed and saved while the pellet was solubilized in an 8 ml detergent solution containing 1.5% N-lauroylsarcosine, 25 mM triethanolamine and 1 mM EDTA (pH 8.0). The resuspended pellet was mixed on a rocker for 10 minutes at 4°C then centrifuged at 10,000 xg for 10 minutes at 4°C. The remaining supernatant was combined with the supernatant from the initial detergent-free lysate supernatant and filtered through a 0.45 micron filter.

In treatments without sarcosine buffer, the culture containing IPTG was left to shake overnight at room temperature or for 3 hours at 37°C. After allowing the correct incubation time, cultures were centrifuged at 3300 xg for 20 minutes at 4°C, supernatant

was discarded and a 1/20 volume of Dulbecco's Phosphate Buffered Saline (DPBS; Invitrogen, Carlsbad, CA) was used to resuspend the remaining pellet. Cell walls were disrupted by adding 1mg/ml lysozyme to the resuspended pellet and placed on ice for 30 minutes. Triton X-100 at a 1% concentration was added to the cell lysate and shaken vigorously. DNase (Sigma, St. Louis, MO) and RNase (Sigma) were then added to the detergent and lysate at a final concentration of 5µg/ml and mixed on a rocker for 10 minutes at 4°C. Centrifugation at 3,000 x g for 30 minutes at 4°C was performed and supernatant collected. Dithiothreitol at a final concentration of 1mM was added to the supernatant then filtered through a 0.45 micron filter.

For production of the control vaccine (GST-only) a 3 hour IPTG incubation without sarcosine buffer was used.

Protein Purification

Purification of the GST-GnRH3 protein was done with glutathione bound GSTrap FF columns (1 ml; GE Healthcare) in conjunction with a peristaltic pump. Each treatment and control vaccine protein was filtered through a 0.45 micron filter and assigned to an individual column. The columns were equilibrated with 5 ml of binding buffer (Gibco DPBS) before the sample was applied at a flow rate of approximately 0.7 ml/min. The columns were then washed with 10 ml of binding buffer at an increased flow rate of approximately 1.3 ml/min and the protein was eluted with a buffer consisting of 50 mM Tris-HCl and 10 mM reduced glutathione (pH 8.0; Sigma). Samples were collected in 1ml aliquots and protein concentration quantified using a spectrophotometer (Nano Drop ND-1,000). Total protein isolated from the GSTrapFF columns was calculated individually for each treatment type.

Approximately 15 mg of GST-GnRH3 protein and 17 mg of GST protein was transferred into a dialysis membrane (12,000-14,000 molecular weight cut off) and stirred slowly in 4 L of filtered water at 4°C overnight. The samples were then dried using a FTS systems (Stone Ridge, NY) model FD-20-54 lyophilizer and condensed with a TDS-4A DuraStop tray dryer.

Western Blots

Western blot analysis was used to confirm size and specificity of recombinant protein. Bio-Rad precast gels (12% gradient) were loaded with 10 µg of GST-GnRH protein, electrophoresed at 200 V for 35 minutes, and stained with Coomassie blue to determine size of bands. An identically loaded gel was electrophoretically transferred to a nitrocellulose membrane for western blot analysis. After a 1 hour 100 V transfer, the membrane was cut in half and blocked for 30 minutes with 1X casein. One half of the membrane was probed with goat anti-GST antibody at 1:2000 and the other half was probed with rabbit anti-GnRH at 1:100. Both halves were left to incubate overnight. Membranes were washed with 1X casein for 15 minutes prior to a 30 minute incubation with either a 1:500 dilution of biotinylated anti-goat antibody or a 1:200 dilution of biotinylated anti-rabbit antibody. The membranes were each washed in 1X casein and incubated with prepared Vectastain ABC Reagent (Vector Laboratories, Burlingame, CA) for 30 minutes with subsequent washing in 1X casein. Membranes were placed in a TMB substrate solution peroxidase (Vector Laboratories) until desired color development occurred; development was stopped with a distilled water rinse and membranes were left to air dry.

Animals

All procedures used in the care, handling and sampling of animals in our study were approved by the Kansas State University Institutional Animal Care and Use Committee. Six reproductively sound Quarter Horse mares (ages 3 to 8 years) were used. Four were assigned to an anti-GnRH treatment (505.91 ± 6.89 kg) and two were assigned as controls (515.91 ± 19.55 kg). Ultrasonography (Sonovet 600; 5mHz linear transducer) of ovaries three times per week was used to demonstrate reproductive cyclicity of all mares for one month prior to the initial treatment. Following primary injection and throughout the end of the study all mares were continually scanned three times a week (Figure 2). In order to synchronize the estrous cycles for the initiation of treatments, one control mare was administered a 10 mg dose of Prostagmate (Agrilabs, St. Joseph MO) 8 days after a recorded ovulation and one anti-GnRH mare received Prostagmate (10 mg) 5 days post-ovulation. Human chorionic gonadotropin (hCG; 2500 IU; Intervet Inc, Millsboro, DE) was administered on day 22 of cycle to induce ovulation in another anti-GnRH treated mare followed by an injection of Prostagmate (10 mg) 5 days post-ovulation.

Mares were exposed to a stallion twice weekly to monitor estrous behavior in a group setting with the stallion located in a center tease pen. Each mare was assigned a teasing score of 1-5. A mare received a score of 1 if she showed no interest or exhibited aggressive behavior towards the stallion, and received a score of 5 for leaning into the stallion, squatting and urinating. Weekly blood samples were collected via jugular venipuncture into 10 ml vacutainers. Samples were collected for 22 weeks from May 9, 2008 to October 3, 2008. Following collection, samples were refrigerated and allowed to

clot overnight at 4°C. Samples were centrifuged and the serum stored at -20°C until assayed.

Immunizations

Mares in the anti-GnRH treatment group received an intramuscular injection of 1mg of GST-GnRH3 in 2 ml incomplete Freund's adjuvant (IFA; Sigma) in the right side of the neck. Four weeks later, mares received a second immunization of 0.5 mg of GST-GnRH3 in 1ml IFA in the right mid-gluteal region. The antigen and adjuvant dose was reduced in half for the second immunization due to adverse reactions observed following the primary injection. Control mares (n=2) received similar doses of GST protein only in identical injection volumes of IFA. Mares were observed twice daily for the development of injection site reactions (heat, pain, swelling) for 7 days following vaccination ; body temperatures of all mares were also recorded twice a day for one week following each immunization.

Hormone Assays

Serum binding of GnRH was analyzed using an enzyme-linked immunosorbent assay (ELISA) as described previously (Holladay et al., 2003). Nunc brand maxisorp 96-well microtiter plates (Fisher Scientific, Waltham, MA) were coated with 100 ng/well of GnRH peptide (Anaspec, San Jose, CA) in 0.01M carbonate-bicarbonate buffer (Sigma), shaken for 1 hour at room temperature and incubated at 4°C overnight. Plates were rinsed with wash buffer (Kirkegaard and Perry Labs, Gaithersburg, MD), blocked for 1 hour at room temperature with lab buffer (BSA, sodium azide, Tween 40, DTPA) and then rinsed with wash buffer. Serum diluted in lab buffer at 1:100 was added and

incubated for 1 hour before being rinsed with wash buffer. Binding was detected by adding 100 μ l of a 1:1000 dilution of goat anti-horse IgG horseradish peroxidase (HRP) conjugate (Bethyl, Montgomery, TX), incubated for an hour then rinsed with wash buffer. 100 μ l of the visualizing agent tetramethylbenzidine (TMB; BioFX, Owings Mills, MD) was added and left to incubate for 15 minutes before including 100 μ l of 450 stop reagent (BioFX). The plate was read at 450 nm using a Wallac 1430 multilabel counter. Plates were adjusted to eliminate plate variation using a common serum sample.

Progesterone concentrations were determined by radioimmunoassay as previously described (Stevenson, 1981). For each sample 20 μ l of serum was assayed in duplicate. The intra-assay CV was 7.6%.

Statistical Analysis

Statistical analysis on GnRH binding and progesterone concentration was performed with SAS 9.1 (SAS; SAS Institute, Cary NC) using PROC MIXED model. GnRH binding and progesterone means before and after second injection were compared with treatment as a fixed effect. Mares were initially included as a random effect but were not significant, therefore they were not included in the final model.

Results

Plasmid and Protein Analysis

BamH I and BamH I/EcoR V digests of the pGEX-GnRH3 plasmid resulted in the predicted band sizes of a 90 bp GnRH3 fragment (Figure 3). A BamH I digest of the plasmid produced products of approximately 4970 bp and 90 bp in length, which match the predicted size of the vector and GnRH3 insert, respectively (Figure 1). The BamH I/EcoR V digest resulted in products of approximately 3285 bp, 1785bp and 90 bp. There is a unique EcoR V site within the vector sequence which was used to further confirm the identity of the plasmid. A BamH I digest of pGEX-4T-3 vector used to produce control protein (GST only) resulted in a single predicted 4968 bp fragment (Figure 4).

In the two treatments tested to increase fusion protein yield, the amount of total protein collected between the two replications was similar. The 3-hour incubation without sarcosine buffer yielded the highest amount of protein (18.9 mg) from the GST columns. Overnight incubation without sarcosine buffer yielded 4.7 mg, overnight incubation with sarcosine yielded 1.5 mg, and the 3 hour incubation with sarcosine yielded 0.3 mg of protein from the GST columns.

The GST only vector for control mare immunizations was expressed using the 3-hour incubation without sarcosine buffer and resulted in 17.0 mg of protein.

Polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the GST-GnRH3 protein by size in order to assess molecular weight (Figure 5). As a control on the gel, 20 ug of bovine serum albumin (BSA) was loaded and displayed a major protein at approximately 70 kD. GST-GnRH3 lanes contained major proteins at approximately

25 kD and 30 kD. From the protein sequence, GST alone is approximately 26 kD while the GST-GnRH3 fusion protein is 29kD.

Western blot analysis was used to verify the specificity of the recombinant GST-GnRH3 protein (Figure 6). The anti-GnRH serum bound to bands in the area of 30 kD.

Follicular Development and Estrous Behavior

The GST-GnRH3 vaccine suppressed follicular activity in all treated mares following the second immunization (Figure 7). One of the anti-GnRH mares (T-2) displayed only small follicles (<10 mm) within the first two days following the second injection while the other three anti-GnRH mares (T-1, T-3, T-4) displayed only small follicles within 45 days after the second injection. Follicles and ovaries of all anti-GnRH mares remained small and inactive through the end of the study.

At time of the second injection two anti-GnRH (T-2, T-4) mares had large (> 40 mm) follicles present on their ovaries (Figure 7). One mare (T-2) ovulated this follicle 2 days after the injection and no estrous behavior or ovarian activity was observed from this point throughout the end of the study. The large follicle on the ovary of the other anti-GnRH mare (T-4) slowly regressed over 5 weeks. This mare began to display continuous estrous behavior 2 weeks following the second immunization which lasted for 7 consecutive weeks despite the lack of ovarian activity during the latter half of this period. The other two anti-GnRH treated mares (T-1, T-3) both had 28 mm and 23 mm follicles, respectively, at the time of the second injection.

Estrous behavior was suppressed in 3 of the 4 anti-GnRH mares following the second injection (Figure 8). One anti-GnRH treated mare (T-4) displayed strong estrous behavior for seven weeks following the second injection before behavior ceased. One of

the control mares (C-1) exhibited normal 21 day estrous cycles with normal estrous behavior throughout the study. The other control mare (C-2) cycled normally until the beginning of August in which large follicles developed but failed to ovulate. This control mare did not display estrous behavior and progesterone levels remained moderately high (4.6ng/ml) for 6 weeks towards the end of the study.

Serum Progesterone

Progesterone concentrations fluctuated between 0.29 ng/ml and 13 ng/ml depending on the stage of the estrous cycle during the first 5 weeks prior to primary immunization (Figure 9). Before the second injection mean progesterone concentrations of the anti-GnRH (4.06 ± 0.46 ng/ml) and control mares (4.00 ± 0.65 ng/ml) were similar; whereas, the mean progesterone concentration were significantly lower ($p < 0.01$) for the anti-GnRH mares (0.95 ± 0.38 ng/ml) as compared to the control mares (4.07 ± 0.54 ng/ml) following the second injection (Table 1). Mean progesterone concentrations of the control mares varied due to the stages of their estrous cycle, although levels remained above 2.5 ng/ml during the period in which one of the control mares (C-2) failed to ovulate (Figure 9). Three weeks after the second injection all anti-GnRH treated mares demonstrated anestrus-like progesterone profiles (< 1.0 ng/ml), which remained low throughout the final 9 weeks of the study.

Serum Binding of GnRH

Binding of GnRH with serum from both anti-GnRH (0.27 ± 0.12 A₄₅₀ nm) and control (0.20 ± 0.12 A₄₅₀ nm) mares was similar up to the second immunization.

However, following the second immunization binding of GnRH with serum from anti-GnRH mares ($0.59 \pm 0.11 A_{450nm}$) was significantly higher ($p < 0.01$) than binding of the control mares ($0.33 \pm 0.12 A_{450nm}$; Table 1). GnRH binding for anti-GnRH mares peaked at week 6; two weeks following the second injection (Figure 10).

Adverse Reactions

Adverse injection reactions were observed in all but one anti-GnRH mare as well as both control mares. Adverse reactions were defined as visible granulomas (Figure 11) and elevated temperatures ranging from 39°C to 40°C. Horses with fevers were treated twice daily with 0.5 mg/kg body weight of Banamine® (flunixin meglumine) until temperatures subsided. All fevers occurred within 7 days of immunization and subsided in 2-5 days. Granulomas appeared 24 hours post primary injection and all were reduced to only a small palpable knot within one week. No granulomas were found in the hind quarters after the second injection. Three months following the primary injection one of the anti-GnRH mares developed a granuloma in the region of the first injection (right neck) which was still visible at the end of the study.

Discussion

The pGEX-4T vector is commercially designed to produce fusion proteins through the use of GST-glutathione binding affinity. GST and GST fusion proteins can be recovered with elution by free reduced glutathione (Smith and Corcoran, 1994). Previously in our laboratory, one factor impeding the yield of GST-GnRH3 fusion protein was formation of insoluble proteins (inclusion bodies) after plasmid expression in *E. coli* (Holladay et al., 2003).

N-laurolysarcosine buffer was tested in the present study to reduce insoluble GST fusion proteins (Sambrook and Russell, 2001). Although, Hull et. al. (1992) saw improved recovery of soluble protein after using this buffer, this was not the case in the present study. It was found that the 3-hour incubation without sarcosine buffer preparation produced the highest fusion protein yields. It is possible that combining the soluble pellet resuspension with the sarcosine buffer treatment suspension reduced yield, increased precipitate, and subsequently decreased binding to the affinity column.

The recombinant GnRH vaccine suppressed cyclicity and serum progesterone in each of the anti-GnRH mares following the second injection through the remainder of the study. All anti-GnRH mares reached this state within 45 days following the second immunization. The ultrasound data confirmed that ovaries were in an anestrus state due to no evidence of a CL present and only small diameter follicles visible on the ovarian surface. Increased serum binding of GnRH following the second immunization demonstrated the presence of endogenous anti-GnRH antibodies. Presumably this binding prevented GnRH from binding to gonadotroph receptors in the anterior pituitary, thereby decreasing the release of LH and FSH.

The recombinant GnRH vaccine used in this study suppressed follicular activity and cyclicity in mares similar to a previous report for heifers (Holladay et al., 2003). This immunocontraception strategy was also effective in mares treated with conjugated GnRH vaccines (Dalin et al., 2002; Elhay et al., 2007; Imboden et al., 2006; Tshewang et al., 1997). GnRH vaccines have also been successful at reducing serum testosterone levels and sperm motility in sexually mature stallions (Turkstra et al., 2005) and colts (Dowsett et al., 1993). Moreover, this method of immunocontraception was proven to be reversible in studies which evaluated return to estrus or functional reproduction (Dalin et al., 2002; Dowsett et al., 1993; Elhay et al., 2007; Holladay et al., 2003; Imboden et al., 2006; Tshewang et al., 1997). In our study two of the anti-GnRH mares were available to be examined one year after the initiation of treatment. One of the mares had a CL while the other had a large follicle but no evidence of a CL at the time of ultrasonography. These results suggest a return to cyclicity is possible in the subsequent breeding season following vaccine treatment.

Continuous estrous behavior in the absence of ovarian activity was observed in a previous study using an anti-GnRH vaccine (Dalin et al., 2002) as well as in ovariectomized or anovulatory mares (Ginther, 1992). This behavior is hypothesized to be initiated by the release of estrogenic steroids from the adrenals (Crowell-Davis, 2007). However, in our current study one anti-GnRH mare (T-4) was developing a large follicle at the time of the second injection. Rather than ovulate, this large follicle regressed slowly until undetectable through ultrasonography and no significant structures were visible on the ovary throughout the rest of the study. Since this was the treated mare that displayed continuous estrous type behavior it is possible that this slowly regressing

follicle was a factor. The treated mare which received the second immunization just prior to ovulation established an anestrus like ovary and suppressed estrous behavior almost immediately following injection. Due to this finding, it may be best to immunize just prior to or following ovulation so that slowly regressing follicles do not present a source of estrogen.

It was unclear why one of the control mares built follicles which failed to ovulate during the latter part of the study, although after evaluating progesterone data it is possible that persistent luteal tissue was undetected with ultrasonography. This mare proved to be difficult to ultrasound throughout the study and the presence of luteal tissue was questioned during scanning. In a comparable study, Dalin and coworkers (2002) reported one mare that failed to ovulate three months after immunization and then displayed some cyclic activity 5 and 7 days prior to entering seasonal anestrus. We hypothesize that due to elevated progesterone levels and the absence of ovulation and estrous behavior the control mare in the present study sustained a persistent CL.

The adverse reactions to the immunizations reported here were similar to those observed in earlier reports (Dalin et al., 2002; Elhay et al., 2007; Imboden et al., 2006). Each of these studies investigated different GnRH conjugates and adjuvants and though the severity of reactions varied, they cause concern about the safety of GnRH vaccines. Adverse reactions in the mares in the present study were thought to be caused by the adjuvant or carrier protein as they occurred in both the anti-GnRH and control mares. Results from this study demonstrated a significant reduction in adverse reactions when both the adjuvant and antigen dose was reduced in half and then administered in the mid-gluteal region. This region may be the best site for injection due to the continuous

flexing and movement of this muscle reducing the possibility for the formation of a granuloma. It is also important to evaluate different adjuvants used as immunostimulators for this type of contraception. Researchers are currently investigating the use of CpG motif-based adjuvants in recombinant GnRH vaccines (Conforti et al., 2007) and synthetic GnRH vaccines which includes three linear repeats of GnRH, the hinge region of human IgG1 (hinge), and a T-helper epitope from the measles virus protein (MVP), conjugated to a purified recombinant heat shock protein 65 (Hsp65) of *Mycobacterium bovis*, as an alternative for common adjuvants (Jinshu et al., 2005).

In conclusion, the GST-GnRH3 vaccine was effective in suppressing follicular development and progesterone concentrations in mares by stimulating antibody production against GnRH. In this study with a limited number of animals, estrous behavior was suppressed in all but one treated mares. This type of vaccine is beneficial for suppressing estrous behavior in mares that are often exposed to a stallion during competition or working situations. Immunocontraception also provides the owner with the option of breeding the mare later in life. However, for this method to be safe, effective and practical, an optimal dose of antigen and adjuvant needs to be determined.

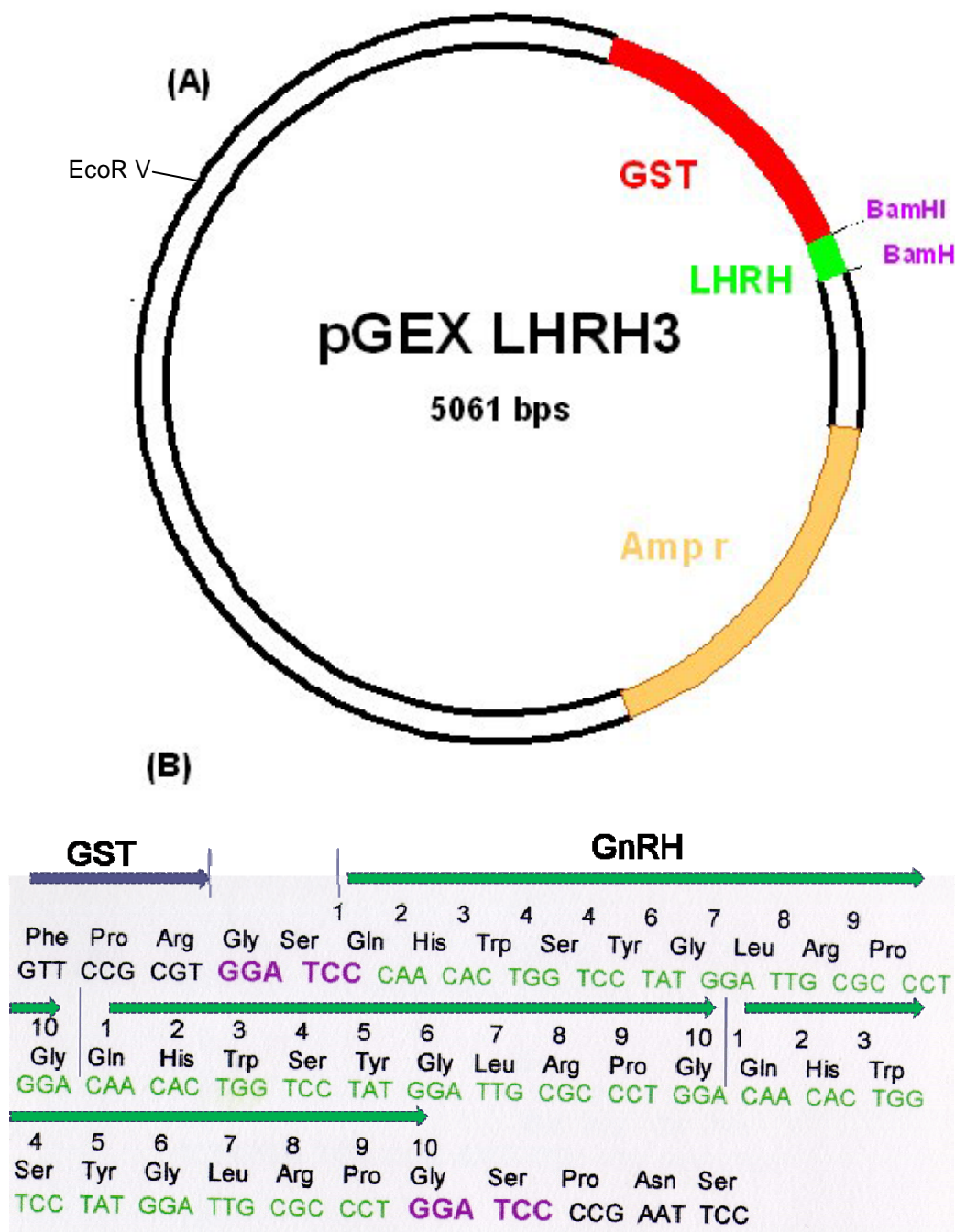


Figure 1. A map of the pGEX-GnRH3 protein expression plasmid.

(A) A 90bp coding sequence coding for 3 copies of GnRH was inserted at the BamH I restriction site of pGEX-4T-3. (B) The sequence of the coding region downstream of GST: The 3 tandem repeated coding region of GnRH is highlighted in green and the recognition site for BamH I in purple.

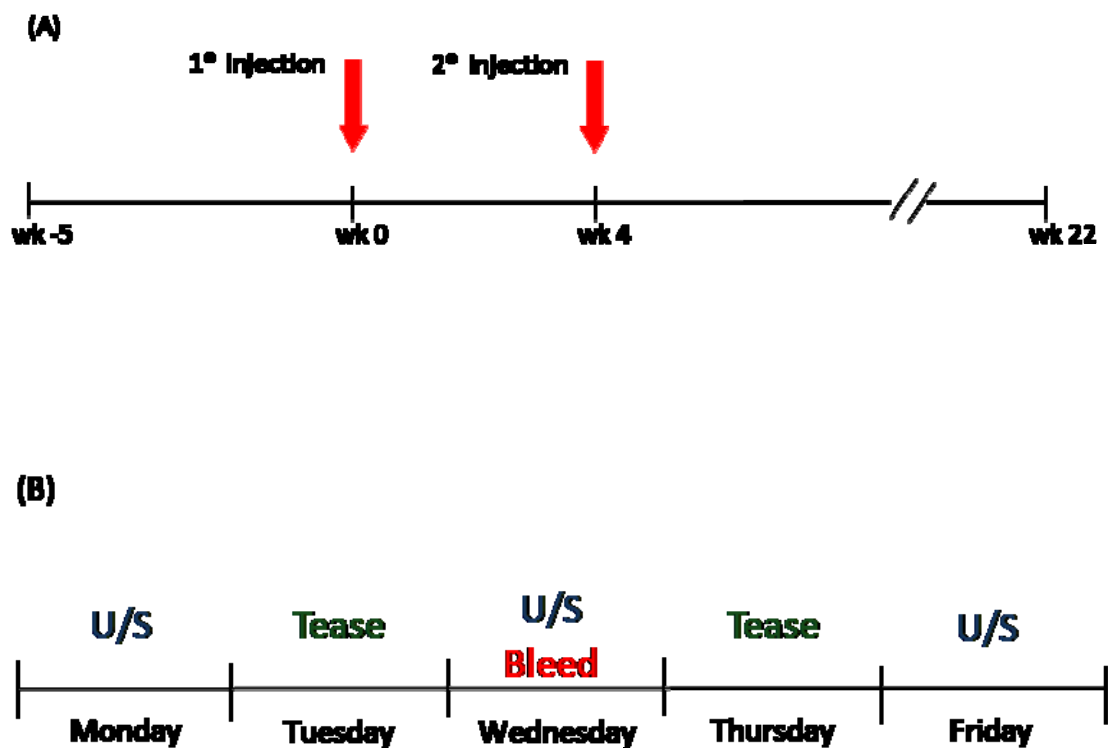


Figure 2. Experimental design.

(A) Immunization schedule. (B) Weekly data collections of ultrasound (U/S), teasing and bleeding.

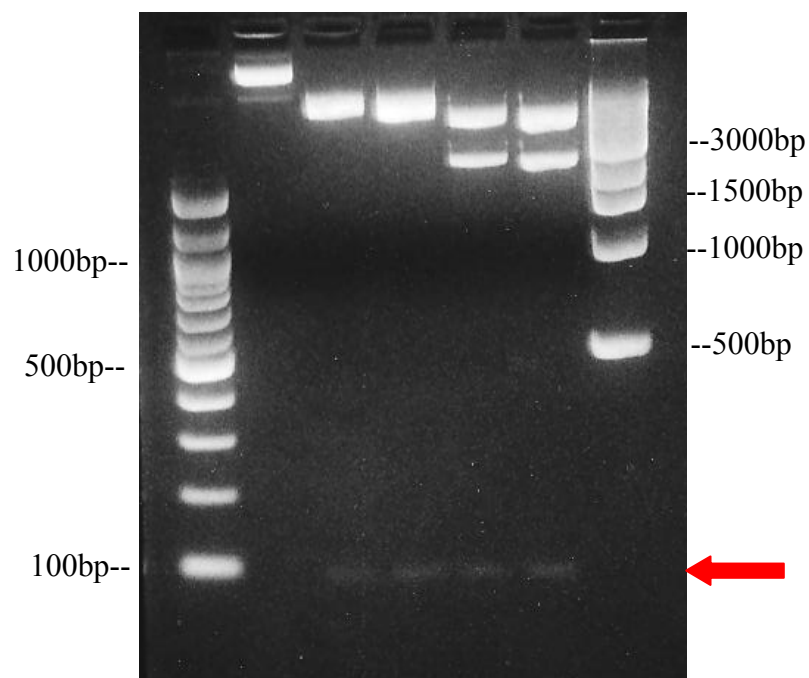


Figure 3. Restriction endonuclease digest of the pGEX-GnRH3 plasmid.

An ethidium bromide stained agarose gel loaded with 100bp DNA ladder (lane 1), the undigested pGEX-GnRH3 plasmid (lane 2), BamH I (lanes 3 and 4), BamH I and EcoRV (lanes 5 and 6) and a 1.0kb DNA ladder (lane 7). The bold arrow indicates the 90bp GnRH-3 BamH I fragment.

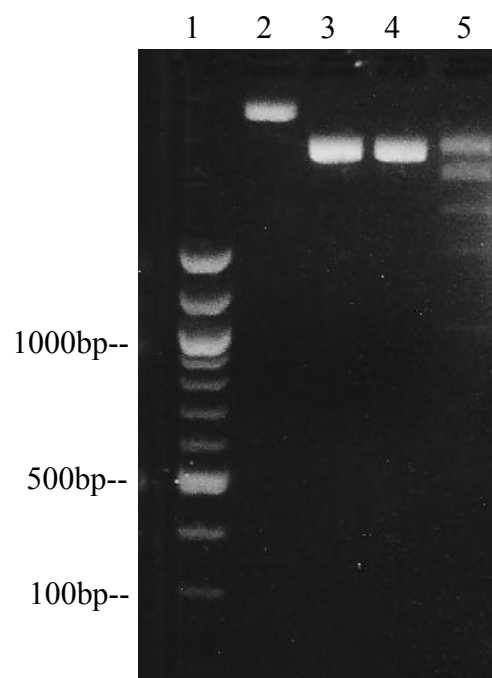


Figure 4. Restriction endonuclease digest of the pGEX-4T vector.

An ethidium bromide stained agarose gel was loaded with 100bp DNA ladder (lane 1), undigested pGEX-4T (lane 2), BamH I digest (lanes 3 and 4) and 1.0kb DNA ladder (lane 5).

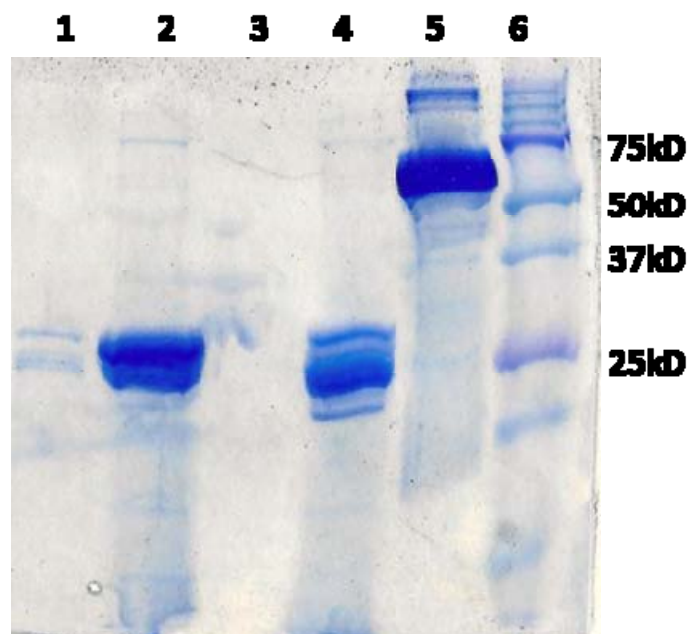


Figure 5. Coomassie blue stained gel of GST-GnRH3 fusion protein.

An acrylamide gel loaded with GST-GnRH3 protein from the 3-hour incubation without sarcosine treatment (lanes 1: 3mg, and lane 2: 6mg), overnight incubation with sarcosine treatment (lane 3), overnight incubation without sarcosine treatment (lane 4), 20 ug of BSA (lane 5), and molecular weight standards (lane 6).

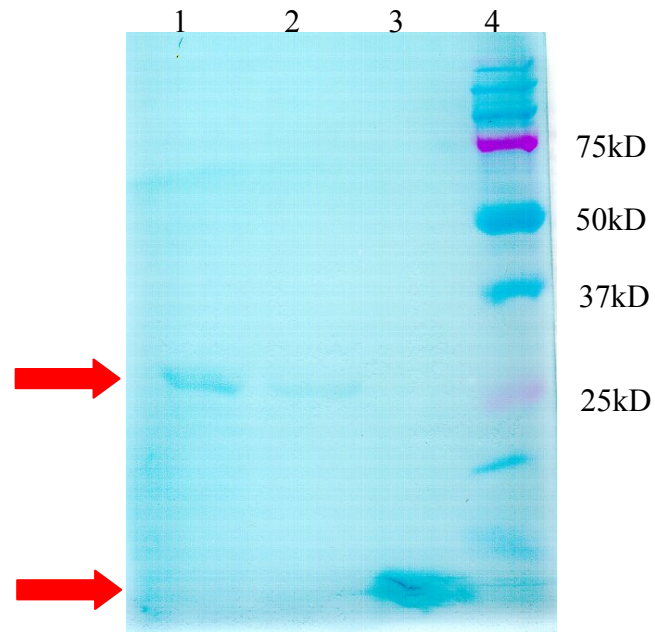


Figure 6. Western blot analysis of GST-GnRH3 fusion protein.

A PVDF membrane was probed with rabbit anti-GnRH. Lanes 1 and 2 were loaded with GST-GnRH3; lane 3 contained purified GnRH for control; and lane 4 was loaded with molecular weight standards. Bold arrows depict the purified GnRH band (lane 3) and the GST-GnRH3 band (lane 1).

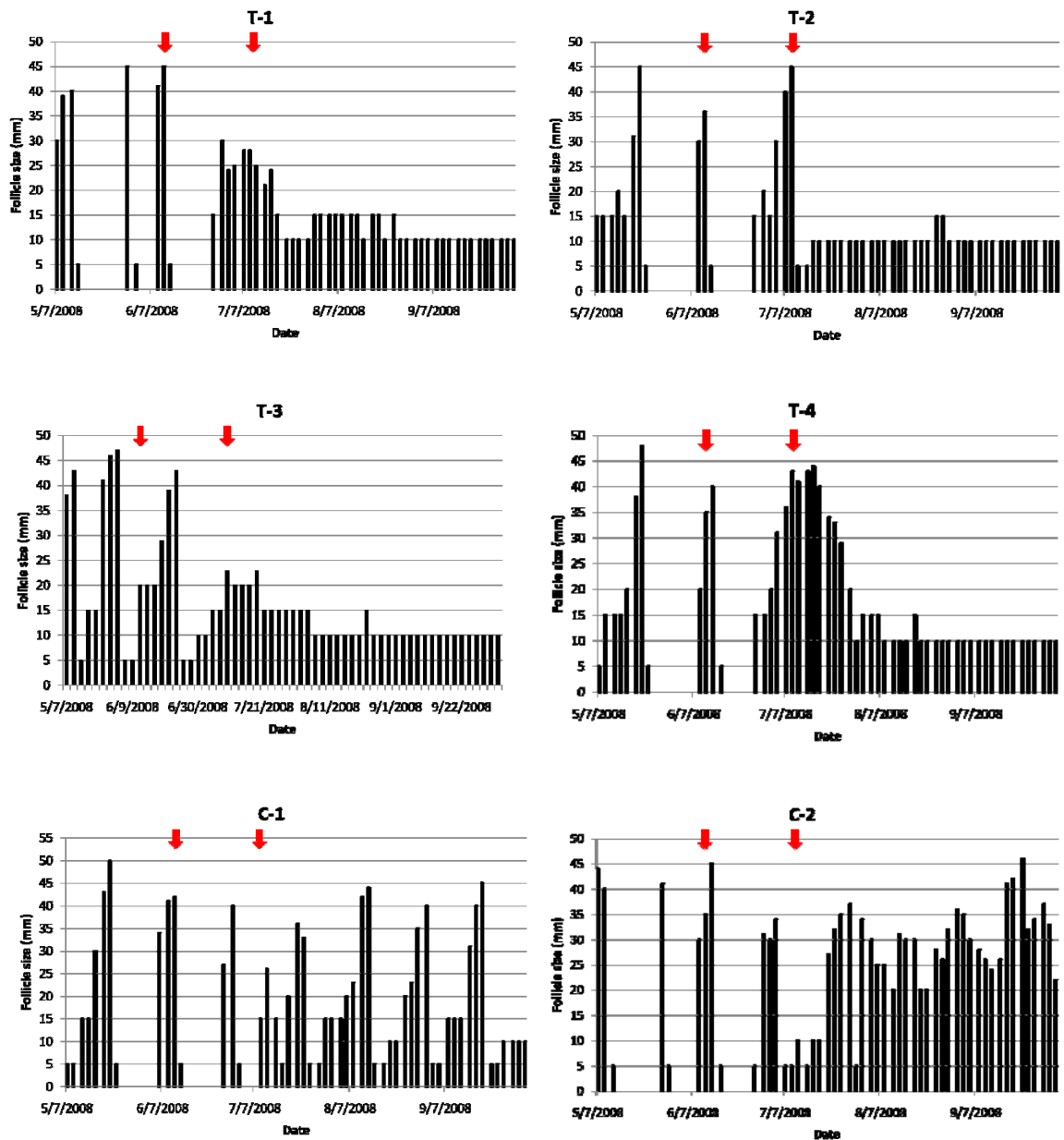


Figure 7. Ovarian ultrasonography data for individual mares.

T-1, T-2, T-3 and T-4 represent the four anti-GnRH treated mares, while C-1 and C-2 represent the two control mares. The arrows indicate times of the immunizations. Small follicles were categorized to be approximately ≤ 10 mm, medium follicles 11-15mm and large 16-20mm. A post ovulation CL was arbitrarily assigned a 5mm designation.

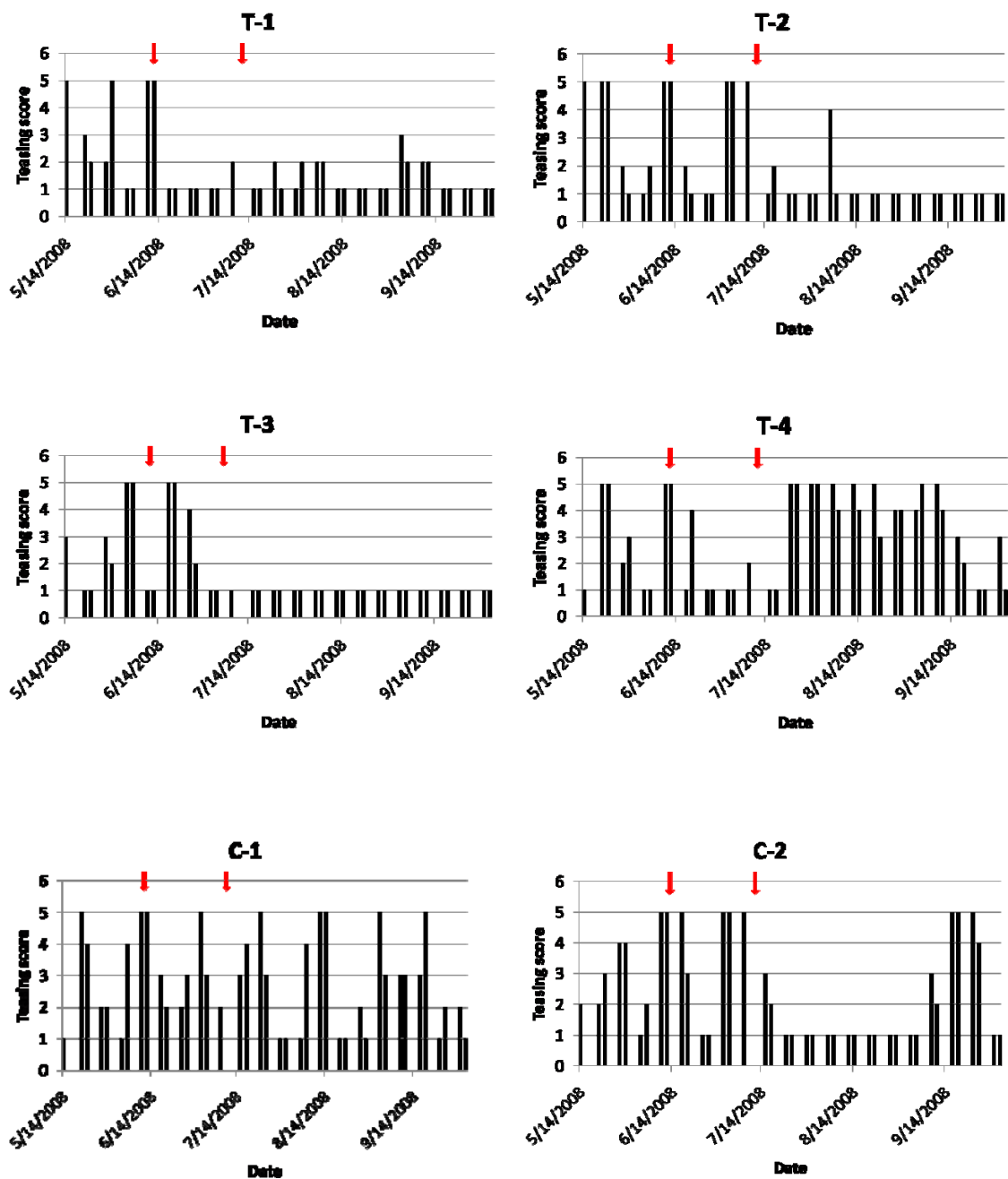


Figure 8. Teasing scores for individual mare after exposure to a stallion.
 Estrous behavior of anti-GnRH (T-1, T-2, T-3 and T-4) and control (C-1 and C-2) mares. Teasing behavior was evaluated when exposed to a stallion and mares were assigned a score of 1-5. Arrows indicate times of the immunizations.

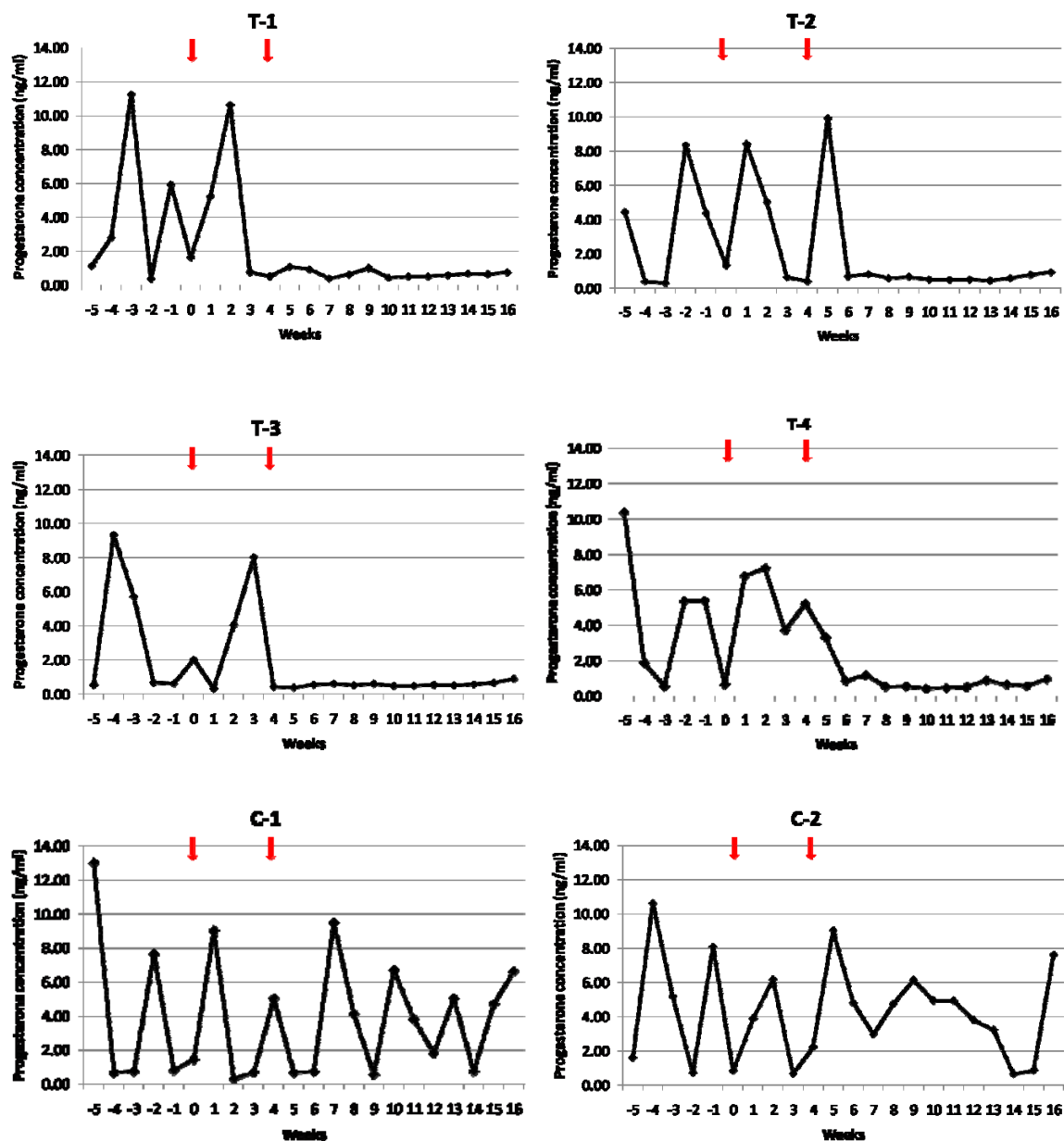


Figure 9. Serum progesterone profiles for individual mares.

The progesterone profiles of each anti-GnRH mare (T-1, T-2, T-3 and T-4) and control mares (C-1 and C-2). Arrows indicate times of the two immunizations.

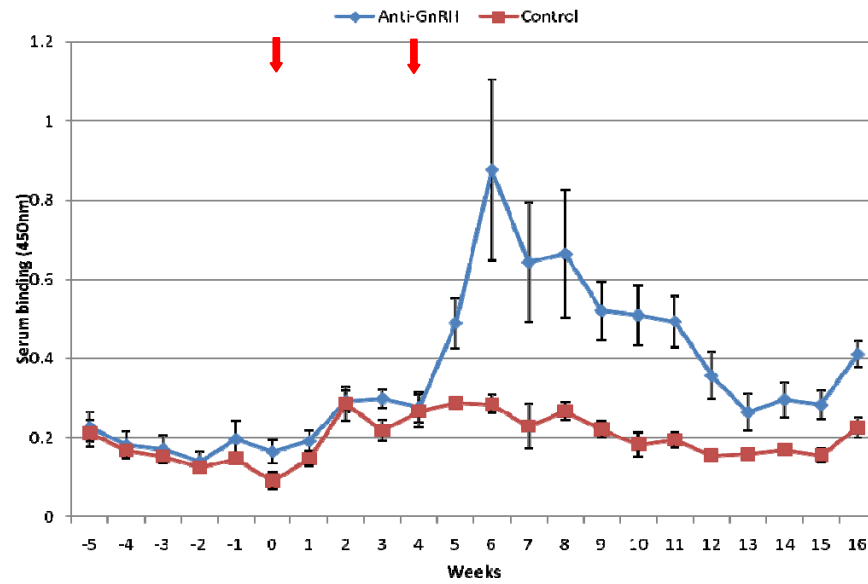


Figure 10. Serum binding of the GnRH.

The circles indicate the GnRH binding of serum from anti-GnRH mares (n=4) while the squares indicated GnRH binding of serum from control mares (n=2). Arrows indicate times of the immunizations.



Figure 11. Injection site granuloma from an anti-GnRH mare.
Photograph was taken two days after the primary immunization. The circled area represents the area of a granuloma.

Table 1. Mean serum progesterone concentration and GnRH binding.

Mean progesterone concentration and GnRH binding in Anti-GnRH immunized mares and Control mares before 2nd immunization (-5 to 3 wk) and after 2nd immunization (4 to 16 wk).

Treatment	2nd immunization	Progesterone (ng/ml)	GnRH binding (A₄₅₀)
Control (n = 2)	Before	4.00 ± 0.65^a	0.17 ± 0.04^a
	After	4.07 ± 0.54^a	0.21 ± 0.03^a
Anti-GnRH (n = 4)	Before	4.06 ± 0.46^a	0.21 ± 0.03^a
	After	0.95 ± 0.38^b	0.47 ± 0.02^b

*Significant differences (p<0.01) within columns with different superscripts.

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