# THESIS

# THE EFFECTS OF IMMUNIZATION AGAINST BONE MORPHOGENETIC PROTEIN-15 AND GROWTH DIFFERENTIATION FACTOR-9 ON OVARIAN FUNCTION IN THE MARE

Submitted by

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# ABSTRACT


## THE EFFECTS OF IMMUNIZATION AGAINST BONE MORPHOGENETIC PROTEIN-15 AND GROWTH DIFFERENTIATION FACTOR-9 ON OVARIAN FUNCTION IN THE MARE

The Bureau of Land Management (BLM) claims the current wild horse and burro population is nearly three times what the rangeland can support. Unmanaged, the horse population doubles every four years, which is detrimental for wild horses, wildlife, and rangeland. The BLM is investigating means of population control for these horses. Currently, no contraceptive vaccine exists capable of inducing permanent sterility in mares. This project serves as the first half of a two-year study investigating the effect of vaccination against Bone Morphogenetic Protein 15 (BMP-15) and Growth Differentiation Factor 9 (GDF-9) on follicular growth and ovulation rates in mares. In mice, rats, sheep, cattle, humans, and deer, these growth-factors have been shown to be critical regulators of follicular development and ovulation rate. Mutations involving expression of either *BMP-15* or *GDF-9* either increase ovulation rates or induce sterility in investigated species, indicating their importance in fertility and their potential value as a target for contraceptive use.

Since the role of these growth factors has been proven to be critical for normal follicular development in other species, we hypothesize that vaccination against BMP-15 and/or GDF-9 will prevent ovulation and/or accelerate the depletion of the oocyte reserve in the horse. For this project, 30 mares were randomly assigned to one of three groups (n=10/group). Mares were vaccinated with either BMP-15 or GDF-9 peptides conjugated to keyhole limpet hemocyanin (KLH) and

Seppic Montanide™ Pet Gel A polymeric adjuvant, or a control of phosphate buffered saline and adjuvant. The horses received a primary vaccination and three booster injections at weeks 0, 6, 12, and 18.

Mares were evaluated three days a week during the breeding season for follicular size and date of ovulation via transrectal ultrasonography. Abnormal ovulations and follicular development were noted. Estrous behavior and sexual receptiveness to a stallion were evaluated using a six-point teasing scale during a rail teasing technique with a stallion three times a week. In order to determine individual antibody responses to the immunizations, blood samples were collected every two weeks, with sera from the samples used for enzyme-linked immunosorbent assay (ELISA).

Evaluation of antibody responses demonstrated the majority of BMP-15 treatment mares elicited a consistently high response to the BMP-15 vaccine. However, only two mares in the GDF-9 treatment group demonstrated a consistently high antibody response to the GDF-9 treatment. No difference in ovulation rate ($P$=0.66) was noted in the GDF-9 group when compared to controls (10.8 and 10.0 ovulations respectively). However, the number of ovulations in the BMP-15 group was decreased ($P$=0.02; 4.9 ovulations) compared to the control group. Both treatment groups demonstrated differences in the average size of ovulatory follicles ($P$<0.001) when compared to controls. On average the last recorded size of ovulatory follicles prior to ovulation measured 21.3 mm, 27.8mm, and 35.7mm in the BMP-15, GDF-9 and control groups respectively. Upon evaluation of teasing records, the both the BMP-15 and GDF-9 vaccinated mares displayed estrus behavior less frequently than controls following the second vaccination ($P$=0.05 and 0.03 respectively). This indicates altered estrous behavior in both BMP-15 and GDF-9 vaccinated mares.

In the first year of this study, vaccination against BMP-15 successfully altered ovarian function in the mare by decreasing the ovulation rate and the size of ovulatory follicles. This altered ovarian function was also indicated by an alteration in estrous cycle behaviors in BMP-15 treated mares. Although GDF-9 did not appear to alter ovulation rate, the decrease in average size of follicles at ovulation and altered estrous behavior indicates further research is required to determine if greater effects are observed in subsequent years. Overall, altered ovarian function in both the BMP-15 and GDF-9 groups shows promise that vaccination against these growth factors could potentially serve as a contraceptive for use in controlling wild horse populations.

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## INTRODUCTION

The growing overpopulation of wild horses and burros in the United States continues to be detrimental to the health of the rangelands, ecosystems, and U.S. economy. The Bureau of Land Management (BLM) Wild Horse and Burro Program is tasked with maintaining wild horse populations at a reasonable and sustainable level. However, there is no current, publically-acceptable means of population control that is able to considerably reduce the current overpopulation of these animals. One method of controlling growth of this population is through the use of contraceptives. Currently, there is no single-use contraceptive for use in wild horses capable of inducing long-term sterility in mares. One potential means of inducing permanent sterility is targeting the regulation of ovarian follicular growth to prevent ovulation and/or accelerate depletion of the oocyte reserve. This comes as a challenge, however, since the exact mechanisms that control follicular growth are not completely understood. Two known regulators of follicular growth are Bone Morphogenetic Protein-15 (BMP-15) and Growth Differentiation Factor-9 (GDF-9), which are oocyte-specific growth factors (Aaltonen et al., 1999; McGrath et al., 1995). Presently, these growth factors and their effects on follicular growth have been investigated in species including sheep, mice, rats, humans, cattle, deer, and possum; however, their roles have not been researched in horses (Bodensteiner, 1999; Dube et al., 1998; Eckery et al., 2002; Laitinen et al., 1998; Wang and Roy, 2003). Once it is better understood what role these growth-factors play in folliculogenesis in horses, there may be potential to target these proteins as a means of long-term fertility control.

## THE BUREAU OF LAND MANAGEMENT: WILD HORSE AND BURRO PROGRAM

### *Overview*

As per the Wild Free-Roaming Horses and Burros Act of 1971 (Public Law 92-195), the BLM has been tasked with managing wild horses and burros that inhabit public lands of the United States. As mandated by U.S. Congress, this act defines wild horses and burros as living symbols of the West and emphasizes the importance of their continued presence in the country (*Public Law 92-195*, 1971). Therefore, the BLM developed the Wild Horse and Burro Program to ensure proper protection and management of these wild herds. Importantly, per the Federal Land Policy and Management Act of 1976, the BLM is also responsible for maintaining viability and ecological balance of U.S. rangelands, which serve multiple purposes including habitat for wild horses and other species of wildlife, grazing and forestry services, and public recreational use (*Public Law 92-195*, 1971). Therefore, management of wild horses and burros has been adapted to maintain a thriving wild horse and burro population while still protecting the viability of the rangeland (Kendall, 2010).

### *Wild Horse Population Control Methods*

In an effort to maintain a thriving ecological balance in rangelands, the BLM has determined an Appropriate Management Level (AML) for wild horses and burros, which is the maximum number of animals that the rangeland can adequately support. This is the maximum population that would ensure both wild horses and burros and the rangeland ecosystems would thrive (Kendall, 2010; National Research Council, 2013). These management levels are determined through evaluation of demands of multiple land uses as well as available forage and water resources throughout the rangelands. The AML for total population of wild horses and burros is 26,715 animals. As of March, 2017, current population of wild horses and burros is 72,647

individuals, more than double what the rangeland can adequately support (BLM.gov, 2017). Due to mandates laid out in the Wild Free-Roaming Horse and Burro Act, wild horses and burros are protected from slaughter and hunting practices, and any horses removed from the range may only be privately adopted or placed into holding facilities under the care of the BLM. These management regulations, combined with a lack of natural predators and high fertility of the species, allow wild horse and burro populations to double every four years and generate a challenge in controlling population numbers (BLM, 2005). The vast overpopulation of these animals is severely detrimental to the viability of the rangeland and overall well-being of the horses and burros. It also comes at a very high cost to the U.S. Government and tax-payers.

As mentioned, wild horses and burros collected off rangelands cannot be sold at public sale or auction, and must be instead offered for adoption. Unfortunately, demand for these animals has not met the supply of wild horses available. In 2016, only 2,900 animals were adopted. With 3,500 to 8,000 animals being removed from the range each year, and tens of thousands living in holding facilities across the U.S., this number of adopted animals is highly insufficient to control overpopulation (BLM.gov, 2017).

Unadoptable animals collected from rangelands live in either long- or short-term holding facilities. These off-range facilities are supported by the BLM, and care of these horses totals more than $49 million dollars each year, which consumes 63% of the Wild Horse and Burro Program annual budget. As of March, 2017, over 46,000 horses and burros are maintained in these facilities and populations are nearing maximum capacity. With the majority of the horses living on these facilities for the remainder of their lifetime, averaging a cost of $48,000 per individual, these off-range sites are not a feasible means of population control (BLM.gov, 2017). Instead, it is in the best interest of the BLM and U.S. tax payers to pursue a method of decreasing the rate of

population growth in these herds which will reduce the number of animals collected from rangelands in the future, lessening the strain on the rangelands and costs of the off-range facilities.

### *Contraceptive Use in Wild Horses*

In an effort to control the rate of population growth of wild horses and burros, the BLM has investigated implementation of several contraceptive techniques. According to the BLM, for a fertility control method to be utilized in wild horse populations, it must be effective, remotely delivered, reversible, safe for use in pregnant animals, and relatively inexpensive. Furthermore, it should not be detrimental to the health or herd dynamics of horses and should not impact unintended populations or species (BLM, 2005). Currently, there is no contraceptive for use in wild horses that satisfies all of these criteria.

In the 1980's, scientists developed an injectable testosterone propionate agent to utilize in harem stallions as a means of chemical sterilization. The agent had proven successful in decreasing sperm motility and therefore reducing contraception rates (Garrott et al., 1991). Use of this agent in wild horse herds came with many drawbacks including a shift in foaling season from spring to late summer or fall and a potential for the drug to impact other unintended species (Garrott et al., 1991). From a management aspect, administration was difficult since it required repeated capture and immobilization of harem stallions. Therefore, this contraceptive technique was abandoned for use in wild horse populations. Similarly, use of progesterone and estrogen implants in mares showed promise in preventing ovulation for 28 months, resulting in decreased pregnancy rates. However, this strategy was suspended given the invasive techniques required (National Research Council, 1991). Other possible female contraceptive techniques were not successful in wild herds due to their invasive nature, including ovariectomy and intrauterine devices (IUD) (Killian et al.,

2006, 2004; Rodgerson and Loesch, 2011). Therefore, focus has moved towards less invasive, remotely-delivered immunocontraceptives to reduce wild horse population growth.

Currently, two immunocontraceptives show promise in controlling wild horse and burro populations. First porcine zona pellucida (PZP), which induces an immune response to zona pellucida proteins surrounding oocytes. With this, zona pellucida-sperm binding receptors become blocked by antibodies and fertilization cannot occur (Kirkpatrick et al., 1996). This vaccine can be administered either by hand or remotely via darting. PZP is ideal in that it is minimally invasive, does not cause abortions in pregnant mares, will not impact unintended species, and is about 90% effective in reducing pregnancy rates if sufficient booster vaccinations are administered (Kirkpatrick and Turner, 1990; Kirkpatrick and Turner, 1985). PZP targets zona protein 3, a protein exclusive to the zona pellucida, which makes the vaccination highly specific (Mask et al., 2015). Still, PZP vaccine does not come without drawbacks, including the need for multiple booster injections, negative side-effects associated with the adjuvant previously used in vaccine formulation, and continued estrous cycling influencing herd dynamics (BLM, 2005).

Another immunocontraceptive being investigated for use in wild horses and burros is GonaCon, an anti-gonadotropin releasing hormone (GnRH) vaccine that causes the mares' immune system to mount a response to endogenous GnRH, preventing release of other hormones required for folliculogenesis and ovulation (Miller et al., 2008). This vaccine carries the same positives and negatives as PZP in that it can be remotely administered and has few negative side-effects but also requires booster immunizations in order to maintain its efficacy after one to two years (Killian et al., 2004). Given that these immunocontraceptives require booster injections, they are not ideal for use in larger populations of wild horses and burros since location and identification

of individual animals is infeasible. Therefore, there is a continued need for a long-term contraceptive for controlling population growth of these wild herds.

## IMMUNIZATION OVERVIEW

In order for an immunocontraceptive to be successful, it must stimulate the immune system to react to an endogenous component, such as the case with GonaCon. To do this, the naturally-occurring protein must be conjugated to an antigen that will appear foreign to the individual. When the body encounters a foreign antigen, antigen-presenting cells of the innate immune system will phagocytize and present the antigen to the adaptive immune system. These antigen-presenting cells include macrophages and dendritic cells. Naïve lymphocytes, B- and T-cells, of the adaptive immune system examine short segments of the antigen, called epitopes. If a naïve cell recognizes the antigen, it will become an effector lymphocyte (Murphy et al., 2008). When B-cells become activated they differentiate into plasma cells. Once differentiated, plasma cells begin making antibodies specific to the antigen being presented. These antibodies circulate throughout the body and neutralize any antigens present (Murphy et al., 2008). Some activated B-cells differentiate into memory cells that mount a faster immune response in the future when the same antigen is seen again. Normally, self-proteins do not initiate an immune response and antibodies are not created (Murphy et al., 2008). However, if these proteins are conjugated to a foreign antigen, the body will recognize them as foreign and mount an immune response in the same manner that would occur for a non-self antigen. Once these antibodies to the self-protein are created by the plasma cells and they enter circulation, they neutralize any specific self-proteins they encounter and inactivate it.

In order to improve immunogenicity of these vaccines, carrier proteins are used in vaccine formulation. Although their immunogenic properties are not completely understood, it is hypothesized that carrier proteins improve immunogenicity by providing antigens for T-cell

recognition (Siegrist, 2013). One carrier protein commonly used is keyhole limpet hemocyanin (KLH). The KLH protein is very large and found in a species of marine mollusk discovered to have significant immune stimulatory properties when used in mammals (Curtis et al., 1970; Harris and Markl, 1999). Given the immunostimulatory properties of KLH and its low toxicity, it is ideal in enhancing immunogenicity of small peptides in animals and has been frequently selected for use in vaccine research (Harris and Markl, 1999).

Another strategy to increasing immunogenicity of vaccines is through the use of an adjuvant. Adjuvants are combined with the target antigen to increase immunogenicity by converting protein antigens of interest into particulate matter that is more easily phagocytized by the antigen-presenting cells (Spickler and Roth, 2003). In veterinary vaccines, the two most common adjuvants used are oil and aluminum based (Audibert and Lise, 1993; Cox and Coulter, 1997); however, another more recent advancement in the field of adjuvants is the use of polyacrylic polymer dispersions (Vialle et al., 2010). Montanide™ Gel is a solution of sodium polyacrylate micronic particles dispersed in water and has been proven safe and effective for use in several species, including the horse (Deville et al., 2011; Parker et al., 2009). The small dispersal size of about 1µm allows optimal phagocytosis of the antigen by antigen presenting cells while also remaining highly efficient in adhering antigens to its surface. Additionally, the dispersion solution allows for sustained antigen release and enhanced recruitment of the innate immune system, making it an ideal adjuvant for increasing immunogenicity (Vialle et al., 2010).

## THE EQUINE ESTROUS CYCLE

### *Overview*

Mares are seasonally polyestrous, long-day breeders (Ginther, 1974; Hughes et al., 1975). The equine estrous cycle has an average duration of 21 days and is defined as the length of time

between one ovulation and the next. This cycle consists of two primary phases, estrus, or follicular phase, and diestrus or luteal phase (Ginther, 1974; Hughes et al., 1975). Estrus commences with regression of the corpus luteum from the previous ovulation, resulting in plasma progesterone levels falling below 1 ng/mL in blood (Asa et al., 1983; Crowell-Davis, 2007). The length of estrus varies with an average length of 4-7 days (Ginther, 1993; Hughes et al., 1975). During this time, gonadotropin releasing hormone (GnRH) levels within blood circulation increase in the absence of progesterone, triggering follicle stimulating hormone (FSH) and luteinizing hormone (LH) to be released by the pituitary gland (Evans and Irvine, 1976; Seeburg et al., 1987). These hormones, along with others, then trigger the growth and maturation of ovarian follicles which leads to an increase in estrogen produced by the growing follicles (Fortune, 1994; Gastal et al., 1997; Ginther, 2005). About 48 hours prior to the end of estrus, an increase in circulating LH as well as acquisition of LH receptors by granulosa cells of the dominant follicle results in ovulation (Xu et al., 1995). Following ovulation, theca and granulosa cells within the ovulated follicle luteinize to form a corpus luteum (CL) and begin to produce progesterone (Ginther, 2005, 1993; Roberto da Costa et al., 2005). This results in a decrease in estrogen and a transition into diestrus. Throughout diestrus, which persists for about 15 days, luteal cells within the CL continue to secrete progesterone, altering GnRH secretion. Toward the end of the diestrus period, progesterone levels in circulation begin to drop as a result of decreased production within the CL (Ginther, 2005; Meinecke et al., 1987). Next, if the mare is not pregnant, the endometrial secretion of prostaglandin ($PGF_{2\alpha}$) into peripheral circulation triggers luteolysis causing a marked decrease in progesterone production, allowing commencement of a new estrous cycle (Douglas and Ginther, 1972; Ginther, 2005).

### *Estrous Cycle Behavior*

Both estrogen and progesterone influence the mare's sexual receptiveness and behavior. These social behaviors communicate to the stallion when the mare is receptive to breeding. In addition, these actions influence herd dynamics and hierarchy within feral horse populations (Asa et al., 1979). When a mare is in estrus, she is said to be receptive to the stallion. Under the influence of estrogen, and in the absence of progesterone, mares exhibit distinct behaviors such as tail raising, clitoral winking, assuming a squatting posture, and showing an overall interest in the stallion (Asa et al., 1979; Curry et al., 2007; Ginther, 1979; Hughes et al., 1975). When in diestrus, the mare is under the influence of progesterone from the active luteal cells within the CL. At this time, there is also a lack of circulating estrogen due to the absence of a large follicle. In response to these hormonal changes, the mare will be averse to the stallion's advances to breed. She will display aggressive behavior by pinning her ears, swishing her tail, and attempting to kick or bite the male (Asa et al., 1979; Crowell-Davis, 2007; Curry et al., 2007; Ginther, 1979; Hughes et al., 1975).

## OVARIAN FOLLICULAR GROWTH

Mares are born with a finite number of oocytes contained within primordial follicles (Deanesly, 1975; Matzuk, 2002). Primordial follicles are comprised of an oocyte and a single layer of pre-granulosa cells. Primordial follicles remain quiescent until they are recruited to grow (Ginther et al., 1997). Progression from a primordial follicle to a pre-ovulatory follicle is characterized by a multi-step process. During the first step, called recruitment, primordial follicles transition into primary follicles at which point the layer of pre-granulosa cells develops into a single layer of granulosa cells surrounding the oocyte (Monniaux et al., 1997). Recruitment is irreversible and follicles must either continue through maturation process and ovulate, or undergo

programmed cell death, or atresia. Throughout the reproductive life span, about 99% of recruited follicles will undergo atresia (Chun and Hsueh, 1998; Gastal et al., 1997). Because of this, recruitment is highly regulated to prevent premature depletion of the oocyte reserve. Following recruitment, primary follicles develop into preantral follicles with increasing numbers of surrounding granulosa cells. These beginning stages, up through early antrum formation, are gonadotropin independent and are highly regulated by paracrine and autocrine actions (Dong et al., 1996; Fortune, 1994). Next developing follicles begin to form an antrum, or fluid filled cavity, at which time they become antral follicles. During this time, granulosa cells become gonadotropin dependent (Binelli and Murphy, 2010; Matzuk, 2002). At this stage, follicles grow at a similar rate and are not limited by availability of gonadotropins (Gastal et al., 1997; Ginther et al., 1997). In mares, when a follicle reaches 22 mm in diameter it is said to be the dominant follicle. Once one follicle becomes larger, smaller follicles undergo atresia due to increased LH concentrations and limited availability of FSH. Available FSH is limited due to an abundance of FSH receptors on the dominant follicle, allowing for increased acquisition of the gonadotropin by that follicle (Gastal et al., 1997; Ginther et al., 1997; Pierson and Ginther, 1987). With estrogen concentrations driving increased LH secretion, the dominant follicle becomes receptive to LH just prior to ovulation. This receptivity to LH as well as cellular changes influenced by estrogen production leads to ovulation of the dominant follicle (Monniaux et al., 1997).

Follicular growth is highly regulated by endocrine, paracrine, and autocrine actions to ensure longevity of the oocyte reserve. However, numerous aspects involved in this regulation are not completely understood. It has been identified, however, that recruitment of follicles to mature from the primordial to primary stage is predominantly regulated by oocyte-secreted factors (Binelli and Murphy, 2010; Buccione et al., 1990; Eppig, 2001). The majority of these factors are members

of the TGF-β superfamily. This family of extracellular signaling proteins are crucial regulators of cell proliferation and differentiation (Massague and Hata, 1997). Some of these family members are expressed in mammalian oocytes including Growth Differentiation Factor-9 (GDF-9), and Bone Morphogenetic Protein (BMP) -4, -6, -7, and -15 (Carabatsos et al., 1998; Dong et al., 1996; Drummond, 2005; Galloway et al., 2000). Both BMP-15 and GDF-9 are critical for female fertility, and mutations affecting expression of these growth factors have been found to significantly alter fertility in multiple species (Di Pasquale et al., 2004; Hanrahan et al., 2004; Yan et al., 2001). BMP-15 and GDF-9 have therefore become the focus of many fertility and sterility studies. To date, no research has been done to determine the effects of BMP-15 and GDF-9 in the mare.

## BONE MORPHOGENETIC PROTEIN-15

### *Overview*

Mature BMP-15 protein exists as a 125 amino acid sequence and is most closely related to GDF-9 in the TGF-β superfamily (Dube et al., 1998). Both GDF-9 and BMP-15 are synthesized as preproproteins and can form homodimers with themselves or heterodimers with each other (Liao et al., 2003). Research indicates that GDF-9:BMP-15 herterodimers have a greater effect on regulation of follicular growth than either protein homodimer (Peng et al., 2013). BMP-15 is expressed exclusively in oocytes of most species, and is involved in nearly every step of folliculogenesis (Dube et al., 1998; McGrath et al., 1995; Otsuka et al., 2000) It is first present in primary follicles in several species including mice, rats, humans, and sheep (Aaltonen et al., 1999; Dube et al., 1998; Galloway et al., 2000; Laitinen et al., 1998). In bushtail possum, however, it is detected earlier in primordial follicles (Eckery et al., 2002). This growth factor regulates follicular growth primarily through its effects on granulosa cell function. This oocyte secreted factor stimulates mitosis and proliferation of granulosa cells in follicles and stimulates granulosa cell

expression of kit ligand (Hayashi et al., 1999; Otsuka et al., 2000; Otsuka and Shimasaki, 2002). Kit ligand is an important stimulator of theca cell proliferation and involved in transition of primordial to primary follicles (Driancourt, 2000). Additionally, kit ligand inhibits oocyte expression of BMP-15, resulting in a negative feedback loop (Otsuka et al., 2000). BMP-15 also suppresses FSH receptor mRNA expression, which thereby alters expression of steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage enzyme (P450scc), and 3$\beta$-Hydroxysteroid dehydrogenase (3$\beta$-HSD). This altered expression limits the follicle's responsiveness to FSH while stimulating granulosa cell proliferation in early follicle maturation (Otsuka et al., 2001; Otsuka and Shimasaki, 2002). FSH related suppression also decreases FSH induced progesterone production and LH receptor expression in granulosa cells. Allowing for granulosa cell proliferation while inhibiting premature luteinization (Otsuka et al., 2001, 2000). Throughout follicular growth, BMP-15 inhibits apoptosis of cumulus cells surrounding the oocyte, and aids in cumulus cell expansion just prior to ovulation through enhanced expression of epidermal growth factor (EGF)- like growth factors and EGF receptors (Hussein, 2005; Su et al., 2010; Yoshino et al., 2006). Overall, BMP-15 is a critical regulator of folliculogenesis through its promotion of follicle growth while also regulating the number of follicles developing at one time by limiting follicle responsiveness to gonadotropins (Shimasaki et al., 2004).

### *BMP-15 Mutations in Other Species*

In sheep, several mutations affecting *BMP-15* expression have been identified. Heterozygotes for the mutation have increased ovulation rates while homozygotes are made infertile (Bodin et al., 2007; Davis, 1991; Davis et al., 1992; Galloway et al., 2000; Hanrahan et al., 2004). The first mutation was noted in Inverdale sheep by Davis et al. (1991) during their investigation of highly-proliferative sheep with high incidences of twinning. At that time, they

identified an X-linked mutation that would later be mapped to the location of the chromosome encoding for *BMP-15* (Galloway et al., 2000). Similar mutations encoding for the same *BMP-15* gene in Hanna, Belclare, and Lacaune breeds were discovered to have similar effects on fertility (Bodin et al., 2007; Davis et al., 1994; Hanrahan et al., 2004). Ovaries of sheep with homozygous mutations are extremely abnormal in that they are significantly smaller than those with one or no copies of the mutation, and are devoid of ovulated follicle remnants or normal follicles beyond the primary stage of development. These ovarian phenotypes are termed streak ovaries (Braw-Tal, 1993; Davis et al., 1994). These naturally occurring mutations peaked interest in the role of BMP-15 in fertility and follicular development.

Several *BMP-15* mutations have also been identified in women with fertility issues, and has therefore been found to play a critical role in human fertility. Women carrying these mutations experience premature ovarian failure and are infertile (Di Pasquale et al., 2006, 2004; Dixit et al., 2006). Additionally, ovaries of females with this mutation are abnormal streak ovaries as seen in homozygous mutant sheep (Di Pasquale et al., 2004). Interestingly, a study by Di Pasquale et al. (2004) found that these patients were heterozygous for the $BMP\text{-}15^{Y235C}$ mutation and were infertile, which is notably different the increased fertility of heterozygote *BMP-15* mutant sheep (Di Pasquale et al., 2004).

To determine what effects these mutations elicit in species other than sheep, Yan et al. (2001) developed *BMP-15* knockout mice and determined that female homozygous mutant mice were sub-fertile, but not completely infertile as expected. Homozygous mutant mice ovulate 33% fewer oocytes than wild-type females. Homozygous mutants have a higher incidence of abnormal follicles with oversized oocytes and below average numbers of cumulus cells surrounding the oocyte, which is most likely the cause of decreased ovulation rates. Additionally, there is no

increase in fertility of heterozygous knockout mice as observed in heterozygous mutant sheep. Differences in effects of altered *BMP-15* expression appears to be different between species, with BMP-15 being a crucial modulator of fertility in sheep and humans, but a non-crucial fertility regulator in mice. There is some speculation that these differences relate to altered BMP-15 roles in mono-ovulatory and poly-ovulatory species (Galloway et al., 2002; Otsuka et al., 2011; Yan et al., 2001).

## GROWTH DIFFERENTIATION FACTOR-9

### *Overview*

Mature GDF-9 protein is a 135 amino acid chain that is secreted as a preproprotein (Liao et al., 2003; Mcpherron and Lee, 1993). It is involved in nearly every step of follicular growth, beginning with primordial stage in cattle, sheep and possum and at primary stage in humans, mice, and rats (Aaltonen et al., 1999; Bodensteiner, 1999; Dube et al., 1998; Eckery et al., 2002; Laitinen et al., 1998; Wang and Roy, 2003). Like BMP-15, GDF-9 plays a crucial role in follicular development, specifically through its proliferative actions on granulosa, cumulus, and theca cells while inhibiting cell differentiation. To do this, GDF-9 inhibits FSH actions by inhibiting LH receptor expression and decreasing FSH derived steroidogenesis (Orisaka et al., 2006; Spicer et al., 2008, 2006; Vitt et al., 2000). Additionally, GDF-9 stimulates expression of FSH receptors on granulosa cells to increase viability of follicles during FSH limited development while also stimulating inhibin production to limit FSH production (Hayashi et al., 1999; Orisaka et al., 2006). In rats, apoptosis is decreased as a result of GDF-9 expression, but this effect was not observed in cattle (Hussein, 2005; Orisaka et al., 2006). In pre-ovulatory follicles, GDF-9 promotes cholesterol biosynthesis in cumulus cells to sustain the oocyte and drives cumulus cell expansion through

upregulation of hyaluronan synthase 2 (HAS2) and cyclooxygenase 2 (PTGS2) (Elvin et al., 1999a; Su et al., 2007).

### *GDF-9 Mutations in Other Species*

Several studies have been conducted to investigate effects of GDF-9 in knockout mice (Carabatsos et al., 1998; Dong et al., 1996; Elvin et al., 1999b; Yan et al., 2001). Collectively, mice homozygous for the knockout are infertile with folliculogenesis halted at the primary follicle stage (Dong et al., 1996; Yan et al., 2001). Primary follicles have abnormally large oocytes, partially due to an upregulation of kit ligand (Carabatsos et al., 1998; Elvin et al., 1999b) However, granulosa cells surrounding the oocyte are unable to proliferate and the oocyte is unsuccessful in converting precursors to theca cells. Therefore, the follicle remains in primary stage and eventually becomes unviable (Carabatsos et al., 1998; Elvin et al., 1999b). Once the oocyte is no longer functioning, granulosa cells undergo differentiation and form a structure similar to a small corpus luteum (Elvin et al., 1999b). Due to failure of these follicles to mature past the primary stage, ovaries of these double knockout mice are very small in size (Yan et al., 2001). Interestingly, mice that are heterozygous for *GDF-9* knockout mutation are phenotypically normal and do not demonstrate a change in fertility (Dong et al., 1996). This is a notably different effect than is described in sheep who are heterozygous for naturally occurring *GDF-9* mutations.

A mutation affecting expression of *GDF-9* in sheep was identified by Hanrahan et al. (2004) to have similar effects on fertility as the previously mentioned BMP-15 mutations. Breeds of sheep that carry this mutation are the Belclare and Cambridge. Within these breeds, sheep that are homozygous for this mutation, labeled FecG, are infertile, while heterozygotes have increased fertility (Hanrahan et al., 2004). This phenomenon is also observed in Thonka sheep that possess a different genetic mutation that also affects *GDF-9* expression (Nicol et al., 2009). Upon

morphological evaluation of ovaries of homozygous ewes, Nicol et al. (2009) identified very few follicles that had matured past the primary stage, with the majority of them being abnormal. Abnormalities included abnormally large oocytes within immature follicles, inappropriate arrangement of granulosa cell layers surrounding these oocytes, and clusters of granulosa-like cells without oocytes present. Granulosa cells in primary follicles showed little to no evidence of proliferation and there was no evidence of theca cell recruitment (Nicol et al., 2009). Morphological observations in homozygous mutant ewe ovaries are similar to those of *GDF-9* knockout mice described above, indicating the critical role of GDF-9 in fertility across various species.

In humans, alterations in *GDF-9* expression may alter fertility in a similar manner as it does in sheep. Several *GDF-9* mutations have been identified both in women bearing dizygotic twins and women with premature ovarian failure (Dixit et al., 2005; Palmer et al., 2006). Although research in this area is limited, it is hypothesized that reduced or absent expression of *GDF-9* may either increase or decrease ovulation rates depending on a heterozygous versus homozygous carrier phenotype, similar to the effects observed in sheep and mice (Otsuka et al., 2011; Palmer et al., 2006).

## IMMUNIZATION AGAINST BMP-15 AND GDF-9 IN OTHER SPECIES

Given that both BMP-15 and GDF-9 play such vital roles in fertility, several studies have investigated the effects of vaccination against these growth factors to determine potential for a vaccination to serve as a contraceptive as well as to prove the importance of these growth factors in female fertility. The first immunizations against GDF-9 or BMP-15 were investigated in sheep. The majority of ewes vaccinated with either a short peptide of BMP-15 or GDF-9 conjugated to KLH experienced decreased ovulation rates with the majority of animals being completely

anovulatory (Juengel, 2002; McNatty et al., 2007). Of the ewes that were not anovulatory, there was a significant increase in ovulation rates compared to controls (McNatty et al., 2005). Sheep in both BMP-15 and GDF-9 vaccinated groups had high incidences of abnormal ovarian morphology, including little to no presence of follicles past the primary stage of growth, above average oocyte size compared to stage of folliculogenesis, and high incidences of abnormal granulosa cell clusters and unruptured but luteinized follicles. Additionally, treated ewes had small ovaries and irregular expression of estrous cycle behaviors (Juengel, 2002; McNatty et al., 2007).

Studies investigating effects of immunization against GDF-9 and BMP-15 in deer and cattle have yielded more confounding results. In deer, immunization against BMP-15 and GDF-9 yielded no change in fertility rates in the first year in either treatment group (Eckery et al., 2014). However, in years two and three, BMP-15 immunized deer had increased fecundity and GDF-9 immunized deer were infertile. Juengel et al. (2009) vaccinated cattle with GDF-9 or BMP-15 peptide conjugated to KLH and reported that GDF-9 vaccination had no effect on ovulation rates of treated cows, and BMP-15 vaccination either increased or decreased ovulation rates depending on the individual animal. Both treatments caused a decrease in both number and size of antral follicles (Juengel et al., 2009). Differences in observed vaccination effects may support the hypothesis that an incomplete suppression of either GDF-9 or BMP-15 expression by these immunizations may cause an increase in ovulation rates, which may explain why BMP-15 immunized deer had increased fertility, and BMP-15 vaccinated cattle displayed contrasting changes in ovulation rates.

## CONCLUSION

Due to an overpopulation of wild horses and burros in the U.S., the need for a long-term or permanent contraceptive is evident. One promising avenue to develop this fertility control

method in horses is immunization against oocyte-specific growth factors. Being that these targets are oocyte-specific, they are ideal candidates as immunocontraceptives that would limit unintended effects in other body systems. Also, by targeting factors that regulate depletion of the finite oocyte reserve, it is possible to achieve permanent sterility in vaccinated animals. However, limited knowledge of the regulation of this reserve and follicular growth limit the possibilities of a successful contraceptive. Also, lack of research in oocyte-secreted factors, namely BMP-15 and GDF-9, in the horse further compounds this issue. Successful immmunocontraceptive research and natural mutations in other species that affect expression and function of BMP-15 and GDF-9 in other species show promise that similar results can be replicated in the immunization of horses against either BMP-15 or GDF-9 as well. Therefore, the goal of this study was to determine the effects of immunization against these growth factors in the horse. We hypothesize that immunization against GDF-9 and/or BMP-15 will prevent ovulation and/or accelerate depletion of the oocyte reserve in mares.

# CHAPTER II: THE EFFECTS OF IMMUNIZATION AGAINST BONE MORPHOGENETIC PROTEIN-15 AND GROWTH DIFFERENTIATION FACTOR-9 ON OVARIAN FUNCTION IN THE MARE


## SUMMARY

Currently, there is no contraceptive vaccine that can cause permanent sterility in mares. This study investigates the effect of vaccination against oocyte-specific growth factors, Bone Morphogenetic Protein 15 (BMP-15) and Growth Differentiation Factor 9 (GDF-9), on equine ovarian function. We hypothesized that immunization against these growth factors would prevent ovulation and/or accelerate depletion of the oocyte reserve. For this study, 30 mares were randomly assigned to three groups (n=10/group) and vaccinated with BMP-15 or GDF-9 peptides conjugated to KLH and adjuvant, or a control of phosphate buffered saline and adjuvant. Horses received vaccinations at weeks 0, 6, 12, and 18. Ovarian activity and estrous behavior were evaluated three days a week via ultrasound and exposure to a stallion. Upon evaluation of ovulation rate, the GDF-9 group did not demonstrate a difference ($P$=0.66) in ovulation rate when compared to controls (10.8 and 10.0 ovulations respectively), but the number of ovulations in the BMP-15 group was decreased ($P$=0.02; 4.9 ovulations). Average follicle size prior to ovulation was decreased in both treatments ($P$<0.0001) compared to controls. Estrous cycle behavior was altered in both the BMP-15 and GDF-9 groups compared to controls after the second vaccination ($P$=0.05 and 0.03 respectively). Although further research is required to determine the continued effects of vaccination against GDF-9 on ovulation rates, these results show promise that vaccination against BMP-15 and GDF-9 could potentially serve as a contraceptive in wild horse populations.

# INTRODUCTION

Current wild horse and burro population in the United States is nearly three times what the rangeland can support, which is detrimental for wild horses, wildlife, and rangeland (BLM citation). The Bureau of Land Management (BLM) is investigating the use of contraceptives in decreasing wild horse population growth and a long-term or permanent contraceptive vaccine would be ideal. However, there is currently no vaccine available for inducing permanent sterility in mares following a single vaccination. One area of interest in contraceptive research is regulation of ovarian follicular growth. Through understanding what factors regulate follicular growth and the depletion of the oocyte reserve, there is potential to target these regulators to prevent ovulation and/or accelerate the depletion of the oocyte reserve, thereby inducing sterility. This comes as a challenge, however, since the exact mechanisms that control follicular growth, especially in the beginning stages of follicular maturation, are not completely understood. Two known regulators of follicular growth are Bone Morphogenetic Protein-15 (BMP-15) and Growth Differentiation Factor-9 (GDF-9) (Dong et al., 1996; Galloway et al., 2000). These growth factors are exclusive to the oocyte in most species, making them an ideal target for contraceptive research (Juengel and McNatty, 2005). Currently, none have investigated the role of BMP-15 and GDF-9 in equine ovarian function. Mutations affecting expression of either *BMP-15* or *GDF-9* induce sterility in homozygous sheep, while heterozygotes have increased fecundity, indicating that altered expression of these growth factors have a drastic impact on fertility (Galloway et al., 2000; Hanrahan et al., 2004). These effects have been replicated through immunization against BMP-15 and GDF-9 in sheep, deer, and cattle and have produced varying results, with some treatments increasing fertility and others inducing sterility (Eckery et al., 2014; Juengel, 2002; Juengel et al., 2009). Therefore, this study aims to determine effects of immunization against BMP-15 and

GDF-9 on ovarian function in the mare. We hypothesize that vaccination against these oocyte-specific growth factors will either prevent ovulation or accelerate the depletion of the oocyte reserve. Mares were vaccinated with either BMP-15 peptide or GDF-9 peptide conjugated to carrier protein and adjuvant in treatment groups, or phosphate buffered saline and adjuvant in the control group. Ovarian activity was monitored via transrectal ultrasonography and estrous behavior was monitored via teasing with a stallion to evaluate alterations in ovarian function and estrous cyclicity.

## MATERIALS AND METHODS

### *Horse Care*

All horse use for this project was approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC #15-5984A) and were obtained from Abraham Equine Inc. (Canadian, TX). Mares (n=30) were housed at Colorado State University Equine Reproduction Laboratory (Fort Collins, CO). They were maintained on a dry lot pasture and fed grass-alfalfa mix with free choice salt and mineral supplement. All mares had normal reproductive histories and were of good physical health.

### *Experimental Design*

Mares were randomly assigned to one of three treatments (n=10/group). The three groups were identified as BMP-15, GDF-9, and control. Researchers were blinded to groups until termination of the project as to prevent biases in observation.

### *Immunization Protocol*

Horses in BMP-15 and GDF-9 groups were vaccinated with BMP-15 or GDF-9 peptides conjugated to keyhole limpet hemocyanin (KLH) in adjuvant while horses in the control group

were vaccinated with adjuvant and phosphate buffered saline. The BMP-15 peptide consisted of a 24 amino acid sequence (QAGSMGSEVLGPSREREGPESNQC) of the mature protein. The GDF-9 peptide is a 14 amino acid sequence (SEYFKQFLFPQNEC) of the mature protein (Celtek Bioscience, Franklin, TN (1st vaccination); Life Technologies Corporation, Carlsbad, CA (2nd, 3rd, 4th vaccinations)). Both sequences are 100% homologous to mature equine protein and 80% or 100% homologous to sequences used in studies in sheep and deer for BMP-15 and GDF-9 respectively. Keyhole limpet hemocyanin was used as a carrier protein to improve immunogenicity of peptides. Peptide/KLH conjugate was used in combination with Seppic Montanide™ Pet Gel A polymeric as the adjuvant. Each vaccination formulation contained 1000 µg of peptide-KLH conjugate in 2 ml volume. Vaccines were administered intramuscularly in the cervical musculature of the neck using a 20-gauge needle. The mares were vaccinated at weeks 0, 6, 12, and 18 with the first vaccination on February 4th, 2016.

### *Blood Sample Collection*

Blood samples were obtained every other week for 32 weeks in order to measure individual antibody responses. For each sample, 20 ml of jugular venous blood was obtained from each mare using a 20-gauge blood collection needle and two 10 ml blood collection tubes. Following collection, samples were incubated at room temperature for at least 2 hours to allow separation of sera and red blood cells. Samples were centrifuged at 2000 xg for 10 minutes to separate the blood components. Serum was pooled from collection tubes from each mare and aliquoted into 15 ml conical tubes. Serum was then centrifuged for 30 minutes at 5250 xg to eliminate debris. Samples were divided into 1 ml aliquots and stored at -80° C until further processing.

*Antibody Responses*

Serum was used to identify antibody responses to vaccination with either BMP-15 or GDF-9 using enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Santa Cruz Biotechnology, Inc.; Dallas, TX) were coated with 50 µl of a solution of 500 ng of BMP-15 peptide or GDF-9 peptide and carbonate bicarbonate buffer (Celtek Bioscience, Franklin, TN ($1^{st}$ vaccination); Life Technologies Corporation, Carlsbad, CA ($2^{nd}$, $3^{rd}$, $4^{th}$ vaccinations). Plates were incubated overnight at 4°C and washed three times with 300 µl PBST (0.01 M phosphate buffered saline (PBS) plus 0.05% Tween 20, pH 7.4) per well at room temperature. In each well, 200 µl of a solution of 20% SeaBlock (Thermo Fisher Scientific; Waltham, MA) and 5% Tween 20 in 0.01 M PBS was applied to block non-specific binding sites and plates were incubated for 1 hour at 24°C, followed by another three washes with PBST. Sera from mares in treatment and control groups were run in duplicate at a dilution factor of 1:5000 or 1:10,000 for GDF-9 or BMP-15, respectively, in 50 µl of 0.01 M PBS and incubated for 1 hour at 24°C. Plates were then washed three times with PBST. Secondary antibody, rabbit anti-horse IgG (Sigma-Aldrich; Saint Louis, MO) diluted 1:5000 in 0.01 M PBS (50 µl) was applied and incubated 1 hour at 25°C, followed by three washes with PBST. Bound anti-BMP-15 or anti-GDF-9 antibody was detected using 50 µl of horseradish peroxidase conjugated goat-anti-rabbit IgG (Sigma-Aldrich; Saint Louis, MO) diluted 1:5000 in 0.01 M PBS. Samples were incubated for 1 hour at 25°C and washed three times with PBST. Enzyme substrate (3,3',5,5'-tetramethylbenzidine (TMB) dihydrochloride in phosphate citrate buffer with urea $H_2O_2$; Sigma-Aldrich) was added to each well. After 6 minutes, 50 µl of 2 M sulfuric acid was added to terminate the reaction. Absorbance of each well was measured at 450 nm and plate background was corrected for by subtracting mean absorbance of all PBS wells from all assay plates. Antibody responses were reported as optical densities. Titer

thresholds for each dilution factor were calculated as the pre-vaccination sample mean plus three standard deviations. Samples with values above threshold value were classified as positive for BMP-15 or GDF-9 antibodies. Samples with values below the threshold value were classified as negative for BMP-15 or GDF-9 antibodies.

### *Ovarian Activity Records*

Transrectal ultrasonography was performed three times a week for 32 weeks using a Sonosite M-Turbo ultrasound system with a 5.0 MHz linear array transducer (Sonosite, Bothell, WA). During each exam, ovarian follicle diameters and appearances were recorded and any abnormal ovarian structures were noted, including hemorrhagic anovulatory follicles, persistent anovulatory follicles, and hemorrhagic corpora lutea. A hemorrhagic anovulatory follicle was defined as a follicle containing echogenic strands within the antral space, with no thickened follicular wall present. A persistent anovulatory follicle was defined as a follicle with a thickened, highly echogenic wall, with or without echogenic strands within the antrum. A hemorrhagic corpus luteum was noted by an incomplete luteinization of a corpus luteum, resulting in anechoic pockets within the echogenic luteinized body. Representations of each abnormal follicle or corpus luteum are demonstrated in Figure 1A-C. Uterine edema and fluid accumulation were recorded to monitor stage of estrous cycle and incidence of abnormal estrous cycles.

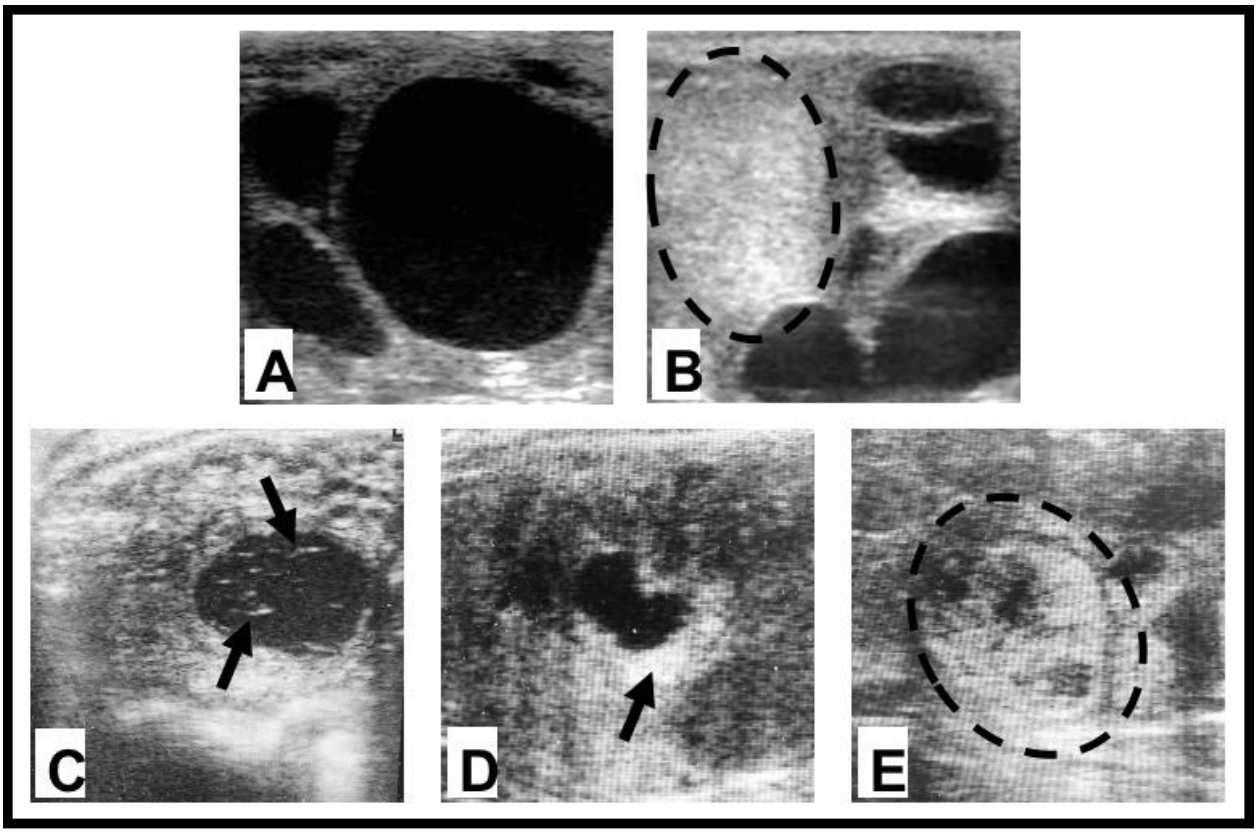



***Figure 1: Ultrasound Images of Normal and Abnormal Ovarian Structures***

This figure illustrates types of follicles and luteal structures observed during ultrasound of equine ovaries. Figure 1A depicts the appearance of a normal follicle. Figure 1B is an image of normal appearance of a corpus luteum. Figure 1C is denoted as a hemorrhagic anovulatory follicle, noted by the white strands (↑) within the black antral space. Figure 1D is a persistent anovulatory follicle, noted by the thickened, highly echogenic wall (↑). Figure 1E represents a hemorrhagic corpus luteum, denoted by the inconsistent echogenicity within the structure.

### *Estrous Cycle Behavior Records*

Throughout the breeding season, mares were monitored three times a week for 32 weeks to record their sexual receptivity to a stallion, potentially indicating their stage in the estrous cycle. Several stallions owned by the Colorado State University Equine Reproduction Lab were utilized in a rail-teasing scenario. For rail-teasing, the mares were arranged in a single file chute and the stallion interacted with each mare individually. Each mare was observed for her behavior and scored on a six-point sexual receptivity scale. A score of 0 indicated that the mare was combative

toward the stallion, which was indicated by pinned ears, a swishing tail, bared teeth and general discontent in the stallion's presence. A score of 5 indicated that the mare was very sexually receptive and would show all signs of estrus including squatting, posturing, urinating, and showing intense interest in the male prior to interaction with the stallion. Descriptions of scores 0 through 5 are outlined in Table 1. Estrous cycle behavior was analyzed using the tease scores recorded throughout the season. To compare days in estrus between groups, a score of 2 or greater was considered an "in estrus" observation.

***Table 1: Description of Tease Scores***

This table outlines the descriptions of tease scores as indicated by the mare's behavior upon interaction with a stallion. Teasing scores are correlated to a status of either "in estrus" or "not in estrus" at each observation.

| Status | Tease Score | Description |
|---|---|---|
| Not in estrus | 0 | Combative with pinned ears, a swishing tail, bared teeth, and general discontent |
| Not in estrus | 1 | Indifferent toward stallion, neither combative or receptive |
| In estrus | 2 | Slightly receptive with raised tail but no urination or squatting posture |
| In estrus | 3 | Delayed teasing behavior, with signs of estrus (urination, squatting, raised tail, etc.) occurring only after individual interaction has ended |
| In estrus | 4 | Teasing behavior upon individual interaction, including urination, squatting, clitoral winking, posturing, and raised tail |
| In estrus | 5 | Intense teasing behavior (urination, squatting, raised tail, etc.) beginning prior to individual interaction with the stallion |

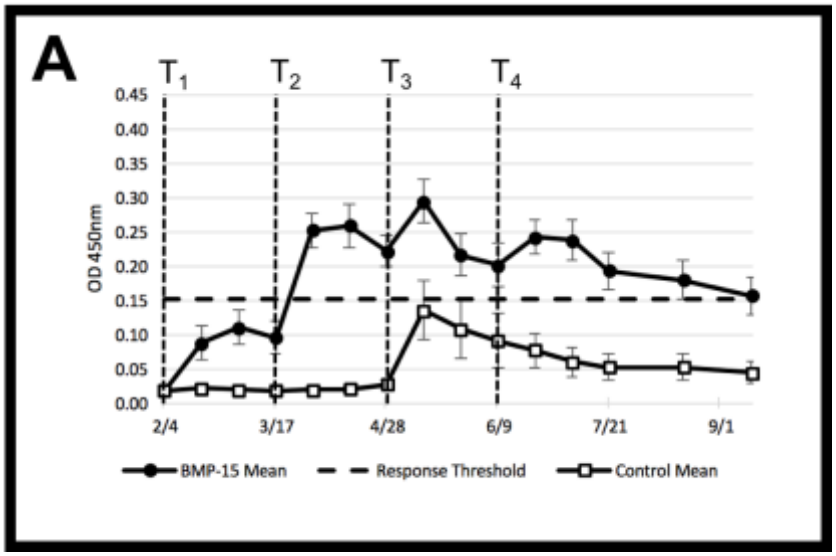
### *Statistical Analysis*

Ovulation rates and occurrence of abnormal follicles were compared using two-tailed, student's t-tests to indicate statistical significance between treatments (Microsoft Excel; Microsoft Office Software, Redmond, WA). All other statistical analyses were completed using SAS 9.4 (SAS Institute Inc., Cary, NC). Pre-ovulation follicle sizes were compared using Proc GLM with a Tukey-Kramer adjustment, with days to ovulation as a covariable. Total days observed in estrus was compared across the entire observation period and observations following the first booster vaccination. Both analyses were completed using Proc GENMOD using a Poisson distribution. A Wald Chi-Squared test (F-test) was used for evaluation. Statistical significance for all analysis was denoted as $P \leq 0.05$.

## RESULTS

### *Antibody Responses*

BMP-15 vaccinated mares elicited a positive antibody response according to an average optical density measurement above the antibody response threshold value following administration of the second vaccination. Average response remained above the positive threshold for the remainder of the study (Figure 2A). Average response of GDF-9 vaccinated mares only measured above the positive threshold after the third and fourth vaccination, falling below the threshold in the subsequent observation (Figure 2B). The control treatment group maintained a negative antibody response to BMP-15 and GDF-9 throughout the study.



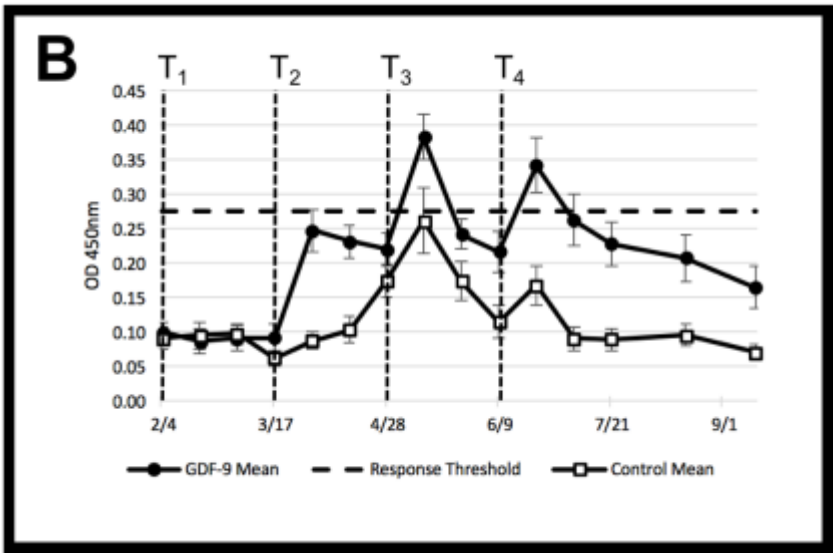




***Figure 2: Antibody Responses to BMP-15 and GDF-9 Peptide***

Mean antibody responses of treatment mares compared to controls, measured by optical density (OD) at 450nm. The response threshold, represented by the horizontal dashed line, is the pre-vaccination average plus three standard deviations. Vertical dashed lines indicate vaccination dates ($T_1$, $T_2$, $T_3$, $T_4$). Error bars represent the standard error of the mean. Figure 2A represents the BMP-15 vaccinated mares and controls. Figure 2B represents the GDF-9 treated mares and controls.

*Ovarian Activity*

Mares in the BMP-15 treatment ovulated fewer times (5.0 ovulations) than controls (10.0 ovulations; $P$=0.024; Figure 3). Four of the ten mares in the BMP-15 treatment ovulated only once or not at all. When evaluating incidence of ovulations in relation to antibody responses in each group, 92% of ovulations in the BMP-15 group occurred after the final vaccination. Ovulation occurrence in mares in the GDF-9 treatment (10.8 ovulations) was not different from control group (10.0 ovulations; $P$=0.66; Figure 3). In control and GDF-9 groups, ovulations occurred in the same frequency throughout the study. A graphical depiction of ovulation occurrence compared to antibody responses for each treatment is provided in Figure 4 and individual antibody responses compared to ovulation occurrence is located in Appendix I. The last recorded follicle size before ovulation was different in all treatment groups with an average measurement before ovulation of 21.3 mm for BMP-15 group, 27.8 mm for GDF-9 group, and 35.7 mm in controls ($P$<0.0001 for both treatments, Figure 5). Of the BMP-15 vaccinated group that ovulated several times, 50% of ovulations resulted from follicles measuring less than 30 mm in diameter compared to 9% of control ovulations and 5% of GDF-9 ovulations (Figure 6). There was a higher incidence of abnormal follicles, hemorrhagic or anovulatory, in both BMP-15 ($P$=0.010) and GDF-9 ($P$=0.017) treatments compared to control group. Mares that ovulated fewer than 2 times were excluded from this evaluation since they had a considerably lower occurrence of adequately sized follicles capable of becoming hemorrhagic or anovulatory. There was no difference in occurrence of hemorrhagic corpus lutea between groups (Figure 7).

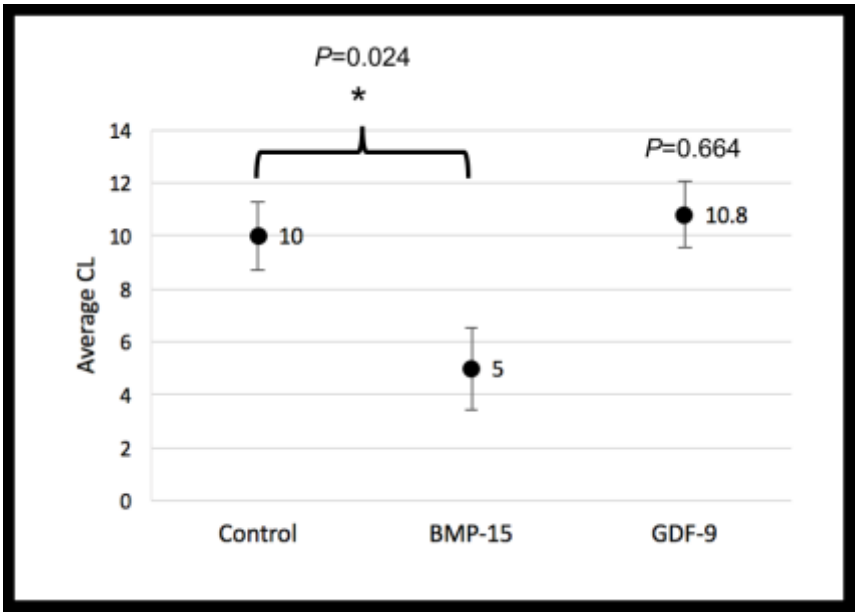



***Figure 3: Average Number of Ovulations***

Average number of ovulations indicated by the number of corpus lutea in each treatment group. Error bars indicate the standard error of the mean and significance with $P<0.05$ when compared to controls is denoted by an asterisk.

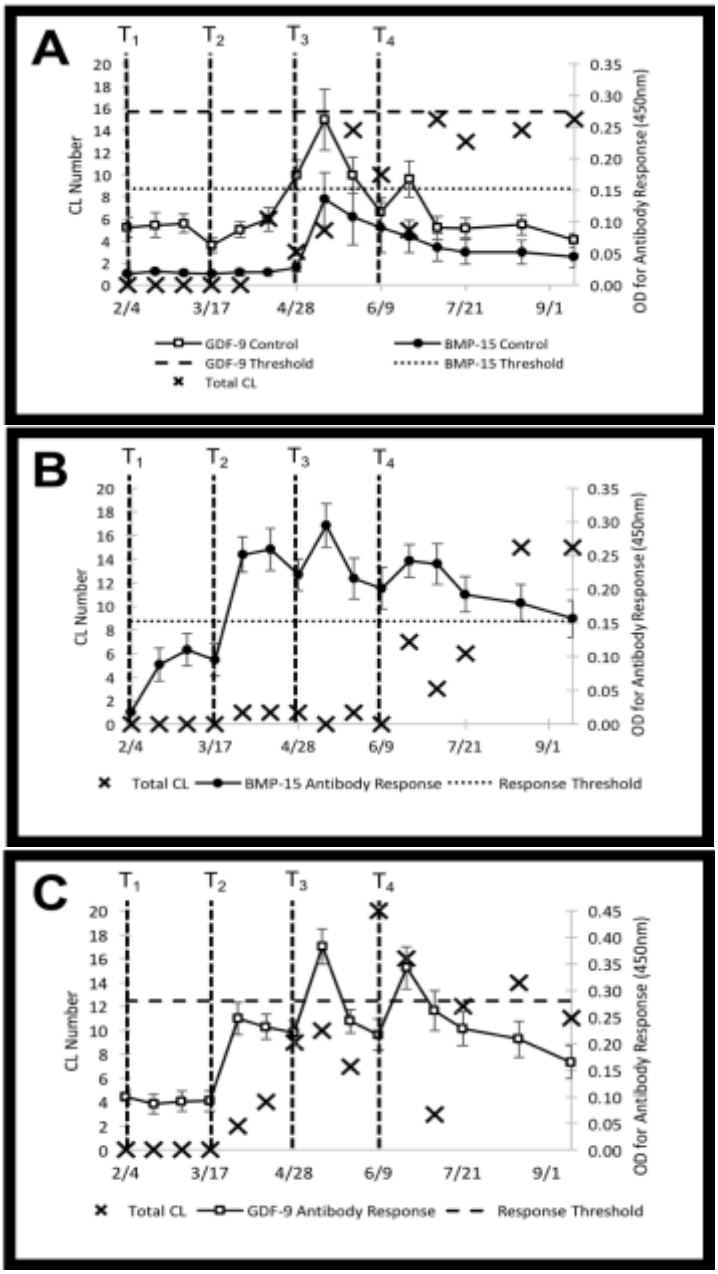



***Figure 4: Ovulations with Respect to Antibody Response***

Total ovulations per treatment between each antibody response measurement compared to the antibody levels, measured in optical density at 450nm. Ovulation number is recorded as total number of corpora lutea for all mares in each group. Vertical dashed lines indicate dates of vaccinations ($T_1$, $T_2$, $T_3$, $T_4$). Error bars indicate standard error of the mean. Horizontal dashed lines indicate antibody response thresholds for BMP-15 and/or GDF-9 peptides. Figure 4A represents ovulations from control mares with respect to their GDF-9 and BMP-15 antibody responses. Figure 4B represents ovulations from BMP-15 treated mares with respect to their BMP-15 antibody response. Figure 4C represents ovulations from GDF-9 treated mares with respect to their GDF-9 antibody response.

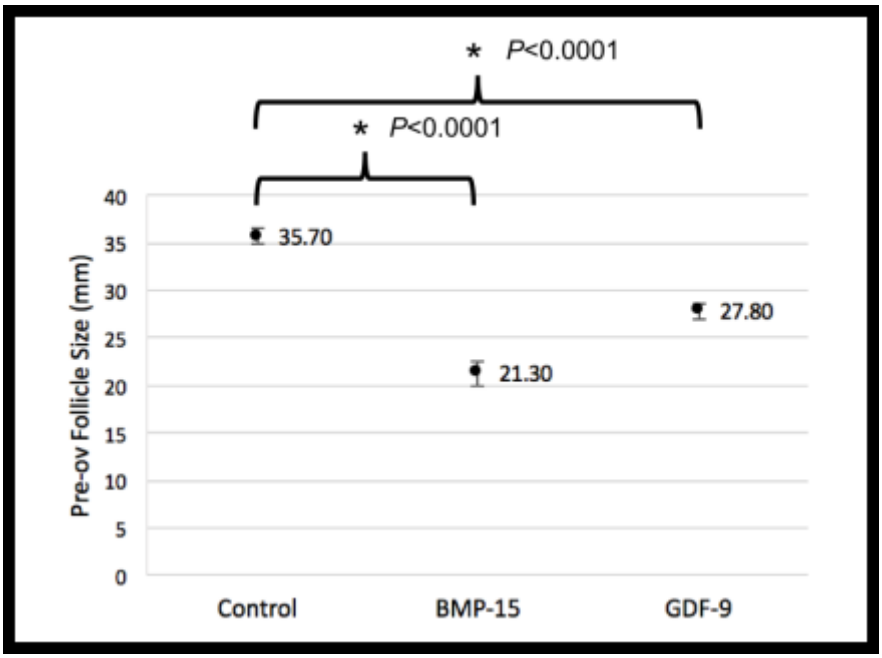



***Figure 5: Least Squares Mean of Follicle Size Before Ovulation***

Least squares mean pre-ovulation follicle size for each treatment, measured in millimeters. Significance with $P<0.0001$ when compared to controls is denoted for each value with an asterisk and error bars indicate standard error of the mean.

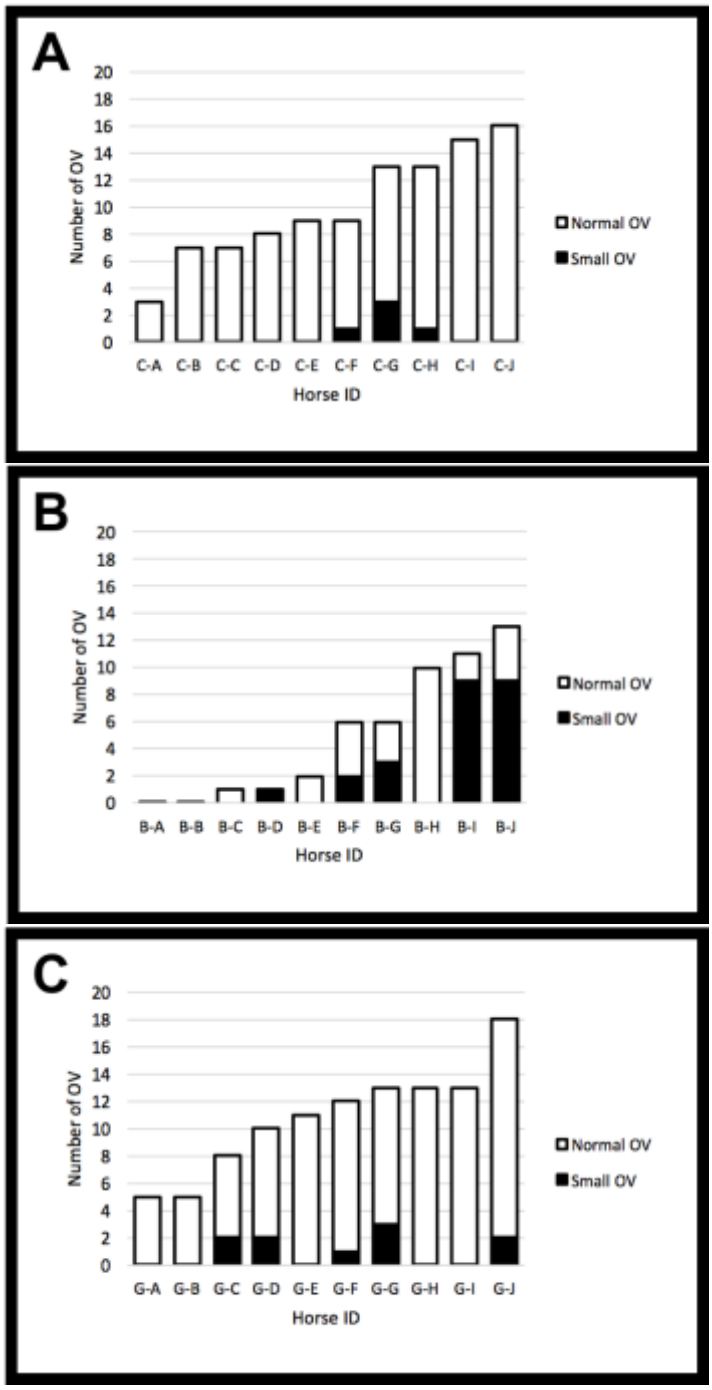



***Figure 6: Ovulation Occurrence Based on Follicle Size by Individual Mares***

Number of ovulations for each mare in each treatment categorized by either small or normal ovulations. Figure 6A represents ovulation occurrence for control mares. Figure 6B represents ovulation occurrence for BMP-15 treated mares. Figure 6C represents ovulation occurrence for GDF-9 treated mares.

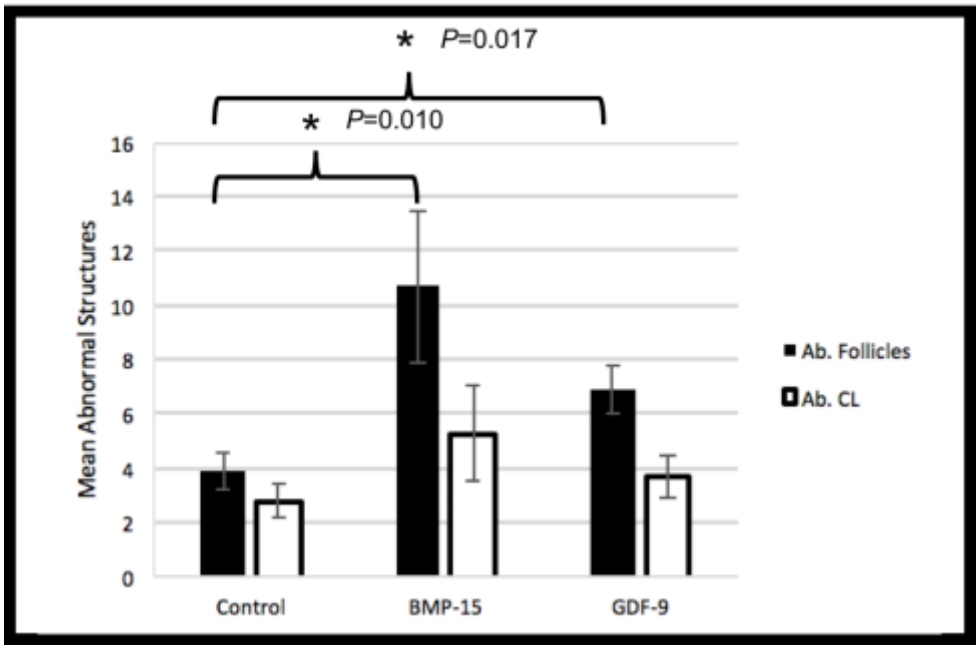



***Figure 7: Abnormal Ovarian Structures per Treatment***

Occurrence of abnormal follicles, hemorrhagic or anovulatory, and abnormal corpus lutea per treatment group. Significance of $P<0.05$ when compared to controls is denoted by an asterisk. Error bars denote standard error of the mean.

### *Estrous Cycle Behavior*

Upon evaluation of the total days observed in estrus, neither the BMP-15 group or GDF-9 group ($P$=0.09, 21.4 days in estrus and $P$=0.12, 22.2 days in estrus respectively) differed from controls (25.7 days in estrus). When total days observed in estrus was evaluated following the second immunization, the total days in estrus for the BMP-15 group ($P$=0.05, 21.0 days in estrus) was decreased when compared to controls (25.7 days in estrus). Total days observed in estrus was decreased in the GDF-9 group from controls ($P$=0.03, 21.1 days in estrus).

## DISCUSSION OF RESULTS

Ovarian function was altered in both GDF-9 and BMP-15 treatments. Although GDF-9 treatment did not affect ovulation rates, altered estrous behavior, a decreased pre-ovulation follicle size and an increased incidence of abnormal follicles demonstrates that treatment does have an

effect on follicular growth. Variable antibody responses in the majority of GDF-9 treated mares may indicate a limited ability of the vaccine to successfully elicit an immune response to GDF-9 protein used in this study. Therefore, the peptide sequence used in vaccine formulation may not induce antibodies that readily bind the epitopes on the mature GDF-9 protein expressed in mares (Murphy et al., 2008). However, peptide sequence used in this study is 100% homologous to sequences used in other studies with successful immunization against GDF-9 in sheep and deer (Eckery et al., 2014; McNatty et al., 2007). Eckery et al. (2014) reported that deer immunized with the same GDF-9 peptide did not have altered fertility rates in the first year, but became infertile in the second and third years. Although that study did not record ovulation rates or abnormal ovulation occurrence in deer, it is possible that this same delay in effects will be observed in mares. This may be due to an incomplete deactivation of GDF-9 in the first year, but complete inactivation in the subsequent years. Additionally, despite no change in ovulation rates of GDF-9 treated mares, vaccination could still be successful in depleting oocyte reserve in these animals as indicated by an increased number of abnormal follicles reported. Since recruitment of follicles is an irreversible step, follicles that begin to grow but fail to reach ovulation as a result of becoming hemorrhagic or anovulatory still result in one less oocyte in the reserve (Gastal et al., 1997). Therefore, if GDF-9 treated mares are recruiting more follicles to grow, despite these follicles becoming abnormal, there would be accelerated depletion of oocyte reserve, potentially resulting in permanent sterility in the future. Further study is needed to confirm whether there is an increased depletion of the oocyte reserve in these treated mares.

The BMP-15 treatment induced a consistently positive antibody response after the first booster, indicating an appropriate immune response to the peptide used. Additionally, altered ovarian activity and decreased ovulation rates suggest that antibodies created in response to

immunization are effective in binding the BMP-15 protein in the mare. Results of this study for BMP-15 treatment are similar to those reported in other BMP-15 immunization studies in sheep and cattle, with an overall decrease in ovulations and some animals becoming completely anovulatory (Juengel, 2002; Juengel et al., 2009, 2004; McNatty et al., 2007). Four of the mares in this group ovulated only once or not at all, whereas mares with several ovulations had a high incidence of abnormally small ovulations. This is similar to reports by Juengel et al. (2009) that claimed some treated cattle failed to ovulate, while the animals with multiple ovulations appeared to have ovulated smaller than average follicles. Therefore, immunization against BMP-15 in horses appears to have a similar effect of ovarian function as cattle and sheep. It may be possible that abnormally small ovulations reported in mares treated with BMP-15 are not fertile ovulations since the average ovulatory follicle size in horses is 35-50 mm in diameter (Hughes et al., 1975). Follicles smaller in diameter may not yet have the appropriate ovulatory factors or contain oocytes that are mature enough to be fertile. If oocytes from small follicles that ovulate prematurely are not fertile, immunization against BMP-15 would be an effective contraceptive despite multiple ovulations occurring in treated horses. However, fertility trials in treated animals are required to draw this conclusion. Also, it is possible that the small luteal structures observed upon ultrasound examination are a result of a spontaneous luteinization of the follicle, without the occurrence of an ovulation or rupturing of the follicle. Such is the case in sheep immunized with BMP-15, with luteinized structures still containing an oocyte (McNatty et al., 2007). In these cases, follicles that appear to ovulate normally and become a corpus luteum may actually result from a spontaneous lutinization of the follicle, and do not result in a viable ovulation. Therefore, further investigation into the histological appearance of these structures on the ovaries of vaccinated mares is required

to determine whether recored corpora lutea result from a successful ovulation or from a spontaneous luteinization of an intact follicle.

High incidences of hemorrhagic and anovulatory follicles in BMP-15 treated mares also indicates altered follicular maturation, suggesting that rates at which follicles are recruited may also be affected; however, subsequent studies are required to determine this effect. In BMP-15 treated mares, 92% of ovulations occurred after the final vaccination when antibody levels were decreasing. These results suggest that there is an optimal antibody level required to induce an anovulatory state in mares, and when antibody responses are above that threshold, immunization against BMP-15 will be a successful contraceptive by preventing ovulation.

Following the second vaccination, both the GDF-9 and BMP-15 vaccinations altered estrous cycle behavior with vaccinated mares observed in estrus fewer days than control mares. This would be expected in the BMP-15 group since this treatment also decreased ovulation rates. Therefore, we would anticipate with fewer ovulations, mares would have fewer large follicles secreting estrogen, decreasing the effects of estrogen on inducing estrus behavior. Further research to determine the hormone levels in GDF-9 vaccinated mares may indicate altered progesterone or estrogen levels resulting from the higher incidence of abnormal follicles, which would explain the altered estrous behaviors in this group. The impact of altered behavior on wild horse herd dynamics as a result of contraceptive administration is a topic of consideration for the BLM (BLM, 2005). Further research must be conducted to determine the extent of decreased estrus behavior in both treatment groups; however, it can be argued that decreased incidence of estrus behavior is normal in wild horse herds since they most often become pregnant early in the season which would greatly decrease the frequency of estrus behaviors.

# CONCLUSIONS

Immunization against BMP-15 and GDF-9 both lead to an altered ovarian function in mares. Although GDF-9 treated mares did not demonstrate a decrease in ovulation rates in the first year, follicular maturation and estrous behaviors were altered, with increased incidences of abnormal follicles and smaller pre-ovulation follicle size. Further research is required to determine if the higher incidences of abnormal follicles leads to a decrease in follicular reserve and if ovulation rate is decreased in treated mares in subsequent years of this project. Immunization against BMP-15 significantly decreased the ovulation rate and ovulation size in treated horses, which highlights potential success of vaccination against BMP-15 as a contraceptive in mares. Investigation into the fertility of BMP-15 and GDF-9 immunized mares is required to determine if high incidence of abnormal follicles and abnormally small pre-ovulation follicle size contributes to a reduction in fertility or a depletion of the oocyte reserve.

# CHAPTER III: DISCUSSION

## INTERPRETATION OF RESULTS

These results provide valuable insight into the effects of vaccination against BMP-15 and GDF-9 on folliculogenesis and ovulation rates in mares, which has not been previously investigated. Analysis of these results and postulating how these effects may relate to known functions of both growth factors is valuable in understanding the role of BMP-15 and GDF-9 in equine reproduction.

Peptide sequences chosen for this research were selected for their homology to sequences used in other immunization studies in sheep and deer, with BMP-15 and GDF-9 peptides being 80% and 100% homologous, respectively, to previous studies (Eckery et al., 2014; Juengel, 2002; McNatty et al., 2007). Sequences of both mature proteins are highly conserved between species; however, there are some differences between the mature sequences in horses, sheep, and deer (Juengel and McNatty, 2005; Otsuka et al., 2011). Therefore, despite a peptide sequence being a reliable epitope in one species, that same sequence may not be a good epitope in another species. This is because proteins are highly complex, tightly folded complexes, and epitopes are often not continuous sequences. Rather they are discontinuous peptide series that are accessible to the antibody due only to the complex configuration of the protein (Murphy et al., 2008). Therefore, BMP-15 or GDF-9 proteins may be configured differently in each species due to their slightly different peptide sequences, giving every species unique epitopes for recognition by antibodies. Accordingly, a peptide sequence that induces an effect in one species may not induce the same response in another. This may explain the lack of an effect of GDF-9 treatment on ovulation rates in our study. Therefore, it may be beneficial to investigate the ability of other GDF-9 peptide

sequences to induce sterility, similar to the technique used by McNatty et al. (2006), which investigated the efficacy of nine different GDF-9 peptide sequences to determine their ability to elicit an antibody response and the effects on ovarian activity in sheep.

Since 92% of ovulations in BMP-15 vaccinated mares occurred after the last booster vaccination, it is important to consider effectiveness of immunization in eliciting a sustained positive antibody response. Overall, antibody responses for the BMP-15 group were positive for the study duration following the second immunization. Levels dropped, however, below an effective level in some mares following the last immunization, allowing for decreased efficacy and increased ovulation rates. In order for the vaccine to be effective without continued booster injections, it should elicit a sustained antibody response to keep circulating antibodies in high enough concentrations to maintain efficacy. Continued research should be considered to investigate methods to maintain a heightened immune response in treated mares, such as utilizing a stronger adjuvant that resists degradation and remains in the system longer.

Upon evaluation of the individual antibody responses compared to number of corpora lutea across the breeding season, two patterns can be identified in the BMP-15 treatment (Appendix I). In the control treatment, corpora lutea were recorded at relatively consistent intervals with only one to two CL occurring at a time, which is normal in horses (Ginther et al. 1974). However, some mares in the BMP-15 group, notably mares B-I (Figure 26), and B-J (Figure 27), frequently ovulated more than two follicles at one time. These mares are also noted to have a very high incidence of ovulations from abnormally small follicles (Figure 6). Therefore, it appears that although these mares ovulated several times throughout the season, ovulations were abnormally small, and occurred in sets of three or more, which is also abnormal. Also, BMP-15 vaccinated mares that ovulated once or not at all, notably mare B-A (Figure 18), B-B (Figure 19), and B-D

(Figure 21), maintained a relatively high antibody response, with an ovulation occurring only after antibody levels begin to fall. This indicates that when antibody responses are relatively high, BMP-15 vaccination inhibits ovulation in these mares. This trend is noted for several mares in this vaccination group, with the majority of ovulations occurring after antibody responses decrease (Appendix I).

Both GDF-9 and BMP-15 are known to inhibit premature luteinization of follicles by inhibiting FSH induced steroidogenesis in granulosa cells (Otsuka et al., 2000; Vitt et al., 2000). Therefore, inhibition of these growth factors could result in premature luteinization of follicles. This may explain the higher incidence of hemorrhagic and persistent anovulatory follicles in treated mares since steroidogenesis would not be inhibited, allowing for increase in estrogen concentrations and negative feedback on GnRH. Negative feedback on GnRH alters its pulsatility, which thereby favors LH secretion. Both BMP-15 and GDF-9 also inhibit LH receptor expression, which would be altered in treated mares (Orisaka et al., 2006; Otsuka et al., 2000). Lack of inhibition of LH receptors, coupled with an increase in LH concentrations would lead to earlier luteinization of follicles than normal. This would cause premature luteinization in follicles that had other factors preventing their ovulation, leading to hemorrhagic or persistent anovulatory follicles.

Both BMP-15 and GDF-9 promote proliferation of granulosa cells and inhibit luteinization by decreasing effects of gonadotropins on granulosa and theca cells (Otsuka et al., 2001; Vitt et al., 2000). This suggests that inhibition of BMP-15 and GDF-9 would lead to increased responsiveness of follicles to gonadotropins. Without influence of these proteins, FSH-R and LH-R expression would not be limited, which would allow the follicle to be more responsive to both

LH and FSH, resulting in early ovulation and luteinization. This may be the cause of the small pre-ovulation follicle sizes observed in mares in both the BMP-15 and GDF-9 treatments in this study.

Just prior to ovulation, GDF-9 and BMP-15 regulate expansion of the cumulus cells. GDF-9 does so by upregulating hyaluronan synthase 2 (HAS2) and cyclooxygenase 2 (PTGS2), while BMP-15 upregulates epidermal growth factor (EGF)- like growth factors and EGF receptors (Hussein, 2005; Su et al., 2007). Although these pathways are also influenced by other factors, such as the increase in LH, lack of BMP-15 or GDF-9 may lead to inadequate cumulus cell expansion, resulting in an oocyte trapped within the intact cumulus cell layer (Kawashima et al., 2012). This lack of cumulus cell expansion, coupled with early luteinization and ovulation of small follicles in BMP-15 and GDF-9 immunized mares may lead to infertile ovulations. Therefore, it is important to further investigate the fertility of mares that ovulated in the treatment groups in this study.

Decreased ovulation rate in BMP-15 treated mares is similar to effects observed in sheep and cattle studies (Juengel, 2002; Juengel et al., 2009; McNatty et al., 2007). It is hypothesized that this decrease in ovulations is as a result of failure of follicular growth past the primary stage. Follicles that progress past the primary stage in treated animals were abnormal, with few numbers of granulosa and theca cells surrounding the oocyte, abnormally large oocytes, and clusters of granulosa-like cells with no oocyte present. (Juengel, 2002; Juengel et al., 2009; McNatty et al., 2007). Given that BMP-15 supports the proliferation of granulosa cells, it can be deduced that the failure of follicles to mature past the primary stage in immunized animals may be due to lack of granulosa cells required to support follicular growth resulting from decreased proliferative signals from BMP-15 (Hayashi et al., 1999). It is possible that these abnormalities can be observed in histological analysis of the ovaries of treated mares in this study, and is a focus of future research.

Total days observed in estrus was decreased for both BMP-15 and GDF-9 treatment following the first booster vaccination, indicating an alteration in estrous cycle behavior. This would be expected since BMP-15 immunized mares also ovulated less frequently with a smaller average pre-ovulation follicle size than controls. Given that ovulating follicles are smaller and less frequent, there would be less estrogen available to the system, limiting estrogen-stimulated estrus behavior. In GDF-9 vaccinated mares, a higher incidence of abnormal follicles may alter the circulating levels of progesterone and estrogen, thus altering estrous behaviors. Part of the BLM's goal for a contraceptive for use in wild horses is minimal effects on natural behaviors (National Research Council, 2013). This is one of the negatives of immunocontraception with PZP, which decreases herd fidelity possibly as a result of increased stallion harassment of immunized mares exhibiting continuous estrous cyclicity (Madosky et al., 2010; Ransom et al., 2010). Uncontracepted wild horses normally become pregnant early in the season and subsequently do not exhibit continuous cyclicity. Therefore, vaccination against BMP-15 or GDF-9 as a contraceptive would likely have less of an effect on natural herd interactions given that it decreases total days that mares are in estrus and would simulate lack of estrus behavior expected during pregnancy.

## FUTURE PROJECTS

Based on results of this study, both immunization against GDF-9 and BMP-15 demonstrate the ability to alter ovarian function in horses; however, further research is needed to adapt these vaccines to induce long-term sterility in mares. It is important to determine if GDF-9 vaccinated mares demonstrate a decrease in ovulation rates in the second year of this project. This would be similar to the observed outcome of the Eckery et al. (2014) study in deer, which successfully induced sterility in the second and third years of the project. If the GDF-9 treatment fails to induce

sterility in mares in this study, there is potential for success with a different GDF-9 peptide sequence, which may elicit a better antibody response to GDF-9.

Efficacy of both BMP-15 and GDF-9 antibodies in binding their mature protein in the equine ovary can also be determined through analyzing binding specificity of these antibodies to ovarian samples. This would better confirm that antibodies being produced as a result of immunization are able to successfully target BMP-15 or GDF-9 protein within ovarian follicles, which is crucial for efficacy of the vaccination.

In order to truly investigate the ability of these immunizations to serve as a contraceptive, future research should include fertility studies to determine pregnancy rates in immunized mares. This would also determine if the oocytes from the abnormally small ovulations observed in both treatment groups are viable and capable of being fertilized. Additionally, future studies involving evaluation of ovarian histology, follicular morphology, and follicle counting would give a better representation of the effects immunization against these growth factors has on the equine ovary and its follicles. Additionally, it would help decipher if these treatments are indeed depleting the oocyte reserve as hypothesized.

Another avenue of research would be the combined administration of vaccines against BMP-15 and GDF-9. A study in mice determined that ovarian activity was more abnormal in double knockout mice (BMP-15$^{-/-}$ GDF-9$^{-/-}$) than either single knockout (Yan et al., 2001). Also, combined immunization against both growth factors in cattle more intensely altered ovulation rates than either immunization alone (Juengel et al., 2009). This suggests that BMP-15 and GDF-9 may be compensatory in their expression, with the expression of one growth factor increasing in the absence of the other. Therefore, immunization against both proteins would remove this compensatory ability and increase sterilization effects.

Once efficacy of BMP-15 and GDF-9 as a contraceptive is determined, subsequent studies should focus on development of the immunizations with stronger, more immunogenic adjuvants in order to elicit a long-term response in treated mares. This would decrease or eliminate the number of booster injections required, improving the appeal of the vaccination to be used in BLM wild horse herds. The fewer number of booster vaccinations required limits the need for herd gathers or individual horse identification for re-administration. Additionally, a stronger adjuvant may increase the efficacy of the vaccine to induce sterility. Multiples studies determined immunization against these growth factors in sheep using a weak adjuvant increased ovulation rates, while immunization with a strong adjuvant decreased ovulation rates (Juengel, 2002; Juengel et al., 2004). Therefore, a stronger adjuvant may increase efficacy overall as well as increase the length of efficacy.

Another important aspect of research is determining the impact that these immunizations have on estrous cycle behavior and herd dynamics. Social hierarchies within wild horse herds are based on mares' sexual behaviors and their interactions with the harem stallion (Asa et al., 1979). Therefore, alterations in breeding behaviors resulting from immunocontraceptive use may have unintended consequences on herd dynamics. To be successful in wild horse herds, the contraceptive should have minimal effects on herd behavior.

## POTENTIAL APPLICATIONS

Results of this study show promise that immunization against either BMP-15 or GDF-9 may serve as a contraceptive in mares in the future. However, a multitude of follow-up studies are required to improve vaccination and prove that the effects observed in this study translate into decreased pregnancy rates. If successful, immunization against BMP-15 and/or GDF-9 would be a good option for the BLM to utilize in their efforts to control the growth of the population of wild

horses. In order for a vaccine to be effective for use in wild horse populations, the BLM requires it to be effective and reversible, safe for use in pregnant mares, minimally invasive to limit stress on the animal, have limited potential to affect unintended species and minimal effects on behavior (BLM, 2005). Vaccination against BMP-15 and GDF-9 could potentially offer a permanent immunocontraception option that is not available for use in horses today. Since we hypothesize immunization against these growth factors accelerates the depletion of the oocyte reserve, effects would be permanent. Given the specificity of BMP-15 and GDF-9 role in follicular growth, it is hypothesized that there would be minimal risk of affecting mares that are already pregnant as well as minimize other effects on the health of the animal. Also, since this immunocontraceptive would be administered via injection, it would have minimal chance of impacting unintended animals and would have the potential to be remotely delivered via darting, reducing the stress on the animal. Given that both vaccinations decreased estrus behavior, they are likely to have less of an effect on natural herd behaviors than other immunocontraception options that induce continuous estrous cyclicity. Considering these effects, vaccination against BMP-15 and/or GDF-9 shows promise in ability to meet the immunocontraception requirements outlined by the BLM.

## CONCLUSION

Immunizations against BMP-15 and GDF-9 successfully alter ovarian function in the mare, with BMP-15 vaccinations decreasing the ovulation rate in treated mares. Although further research is needed for confirmation of results, these treatments may also accelerate depletion of the oocyte reserve by increasing the number of abnormal anovulatory follicles. Therefore, both vaccinations show promise that immunization against these growth factors can induce sterility in mares and may be a successful contraceptive. This would be beneficial for controlling overpopulation of wild horses in the United States. Results of this study also aid in determining

the role that BMP-15 and GDF-9 play in folliculogenesis in mares through investigating ovarian function during altered expression of these growth factors in the horse.

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# APPENDICES

## APPENDIX I: INDIVIDUAL ANTIBODY RESPONSE VS CL OCCURRENCE

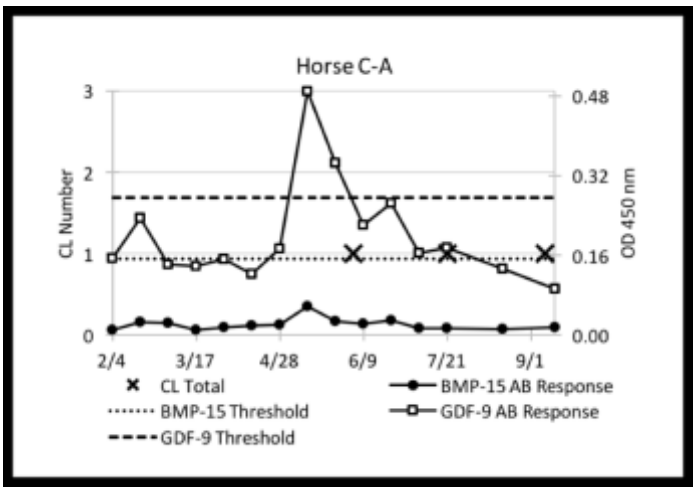


***Figure 8: Individual Antibody Response vs CL Occurrence- Horse C-A***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse A in the Control group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody thresholds for both BMP-15 and GDF-9 are indicated by horizontal dashed lines.

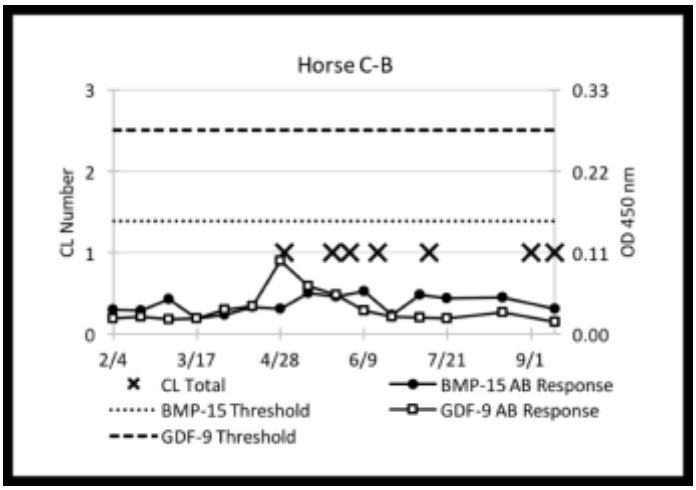


***Figure 9: Individual Antibody Response vs CL Occurrence- Horse C-B***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse B in the Control group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody thresholds for both BMP-15 and GDF-9 are indicated by horizontal dashed lines.

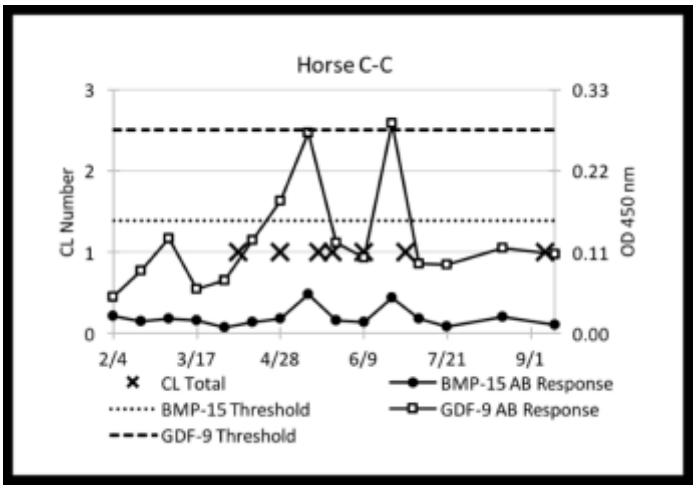


***Figure 10: Individual Antibody Response vs CL Occurrence- Horse C-C***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse C in the Control group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody thresholds for both BMP-15 and GDF-9 are indicated by horizontal dashed lines.

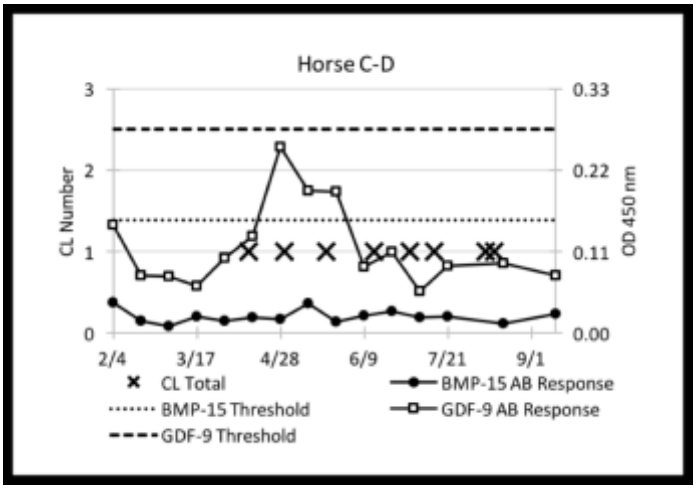


***Figure 11: Individual Antibody Response vs CL Occurrence- Horse C-D***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse D in the Control group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody thresholds for both BMP-15 and GDF-9 are indicated by horizontal dashed lines.

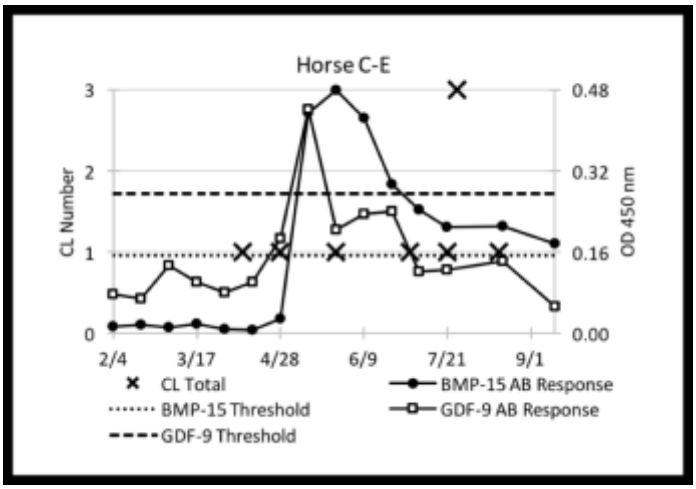


***Figure 12: Individual Antibody Response vs CL Occurrence- Horse C-E***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse E in the Control group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody thresholds for both BMP-15 and GDF-9 are indicated by horizontal dashed lines.

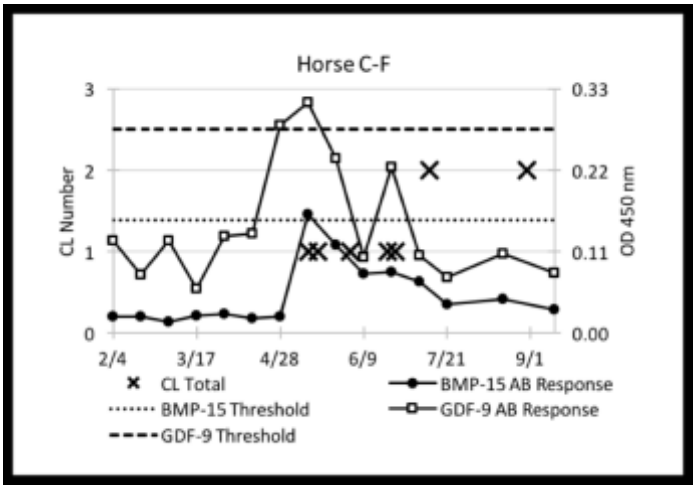


***Figure 13: Individual Antibody Response vs CL Occurrence- Horse C-F***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse F in the Control group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody thresholds for both BMP-15 and GDF-9 are indicated by horizontal dashed lines.

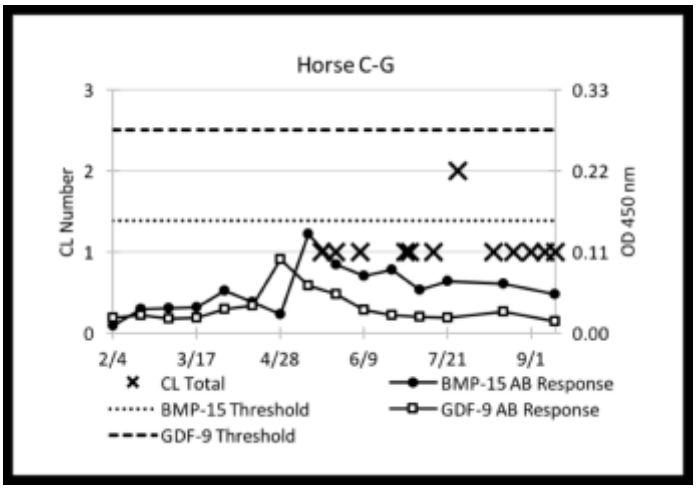


***Figure 14: Individual Antibody Response vs CL Occurrence- Horse C-G***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse G in the Control group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody thresholds for both BMP-15 and GDF-9 are indicated by horizontal dashed lines.

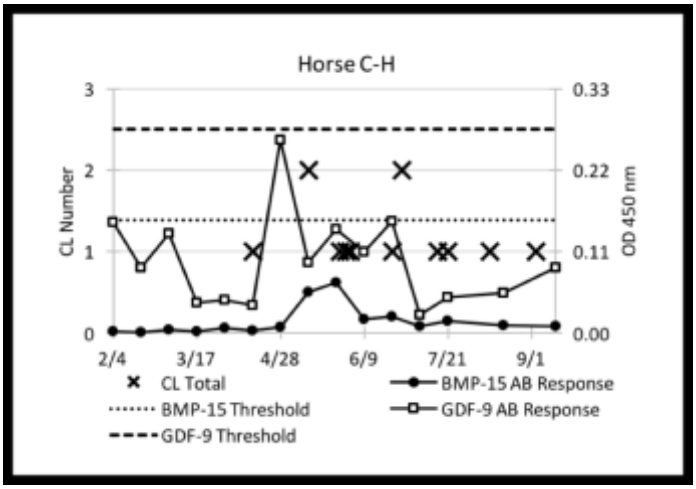


***Figure 15: Individual Antibody Response vs CL Occurrence- Horse C-H***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse H in the Control group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody thresholds for both BMP-15 and GDF-9 are indicated by horizontal dashed lines.

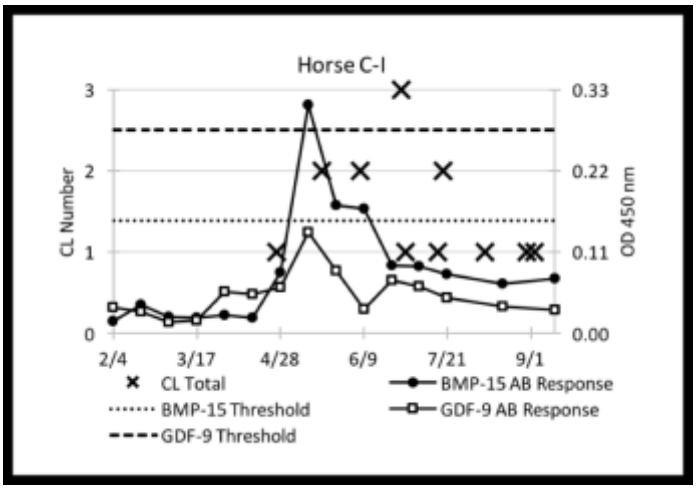


***Figure 16: Individual Antibody Response vs CL Occurrence- Horse C-I***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse I in the Control group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody thresholds for both BMP-15 and GDF-9 are indicated by horizontal dashed lines.

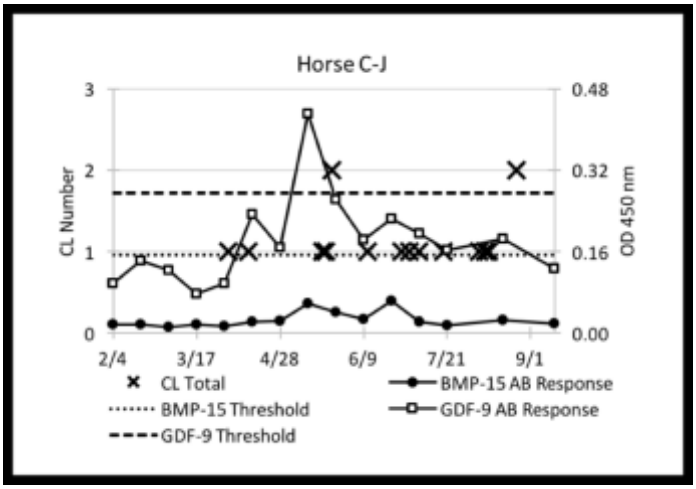


***Figure 17: Individual Antibody Response vs CL Occurrence- Horse C-J***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse J in the Control group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody thresholds for both BMP-15 and GDF-9 are indicated by horizontal dashed lines.

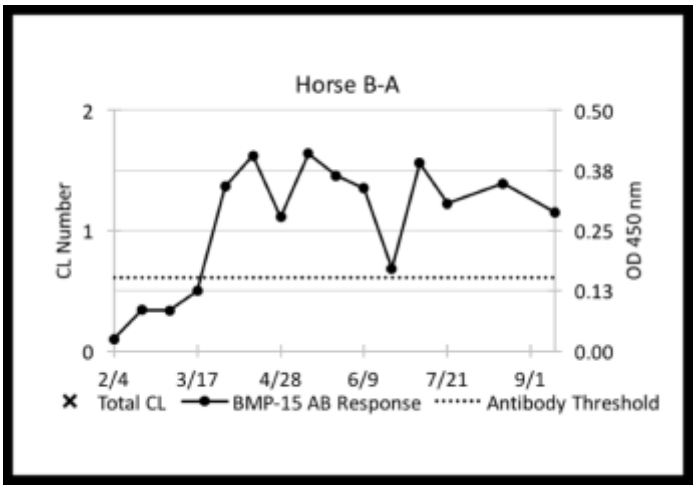


***Figure 18: Individual Antibody Response vs CL Occurrence- Horse B-A***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse A in the BMP-15 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for BMP-15 is indicated by horizontal dashed line.

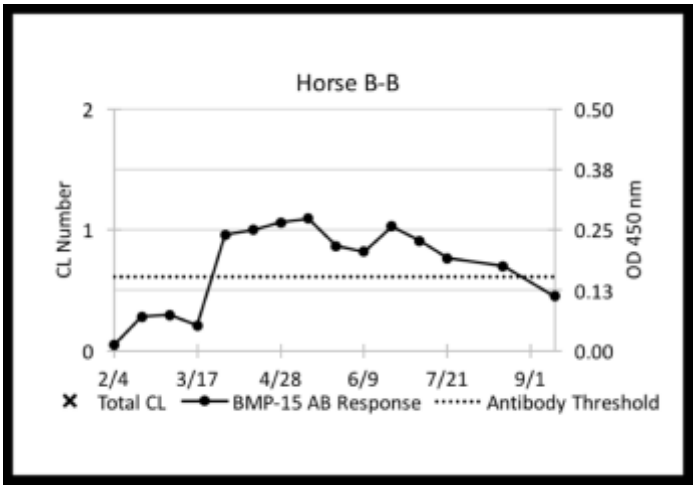


***Figure 19: Individual Antibody Response vs CL Occurrence- Horse B-B***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse B in the BMP-15 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for BMP-15 is indicated by horizontal dashed line.

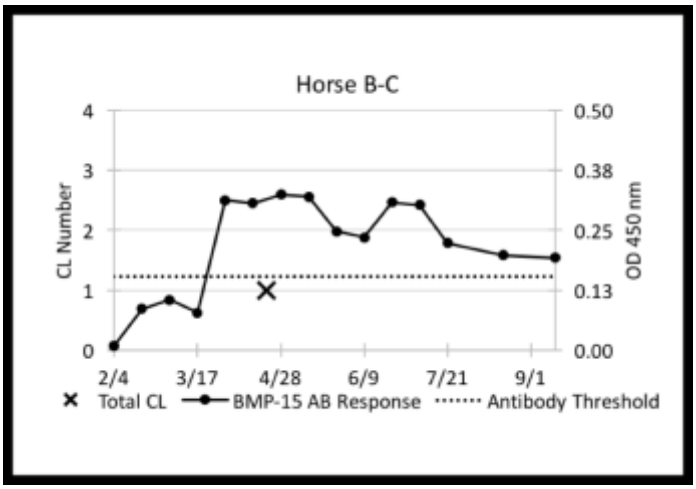


***Figure 20: Individual Antibody Response vs CL Occurrence- Horse B-C***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse C in the BMP-15 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for BMP-15 is indicated by horizontal dashed line.

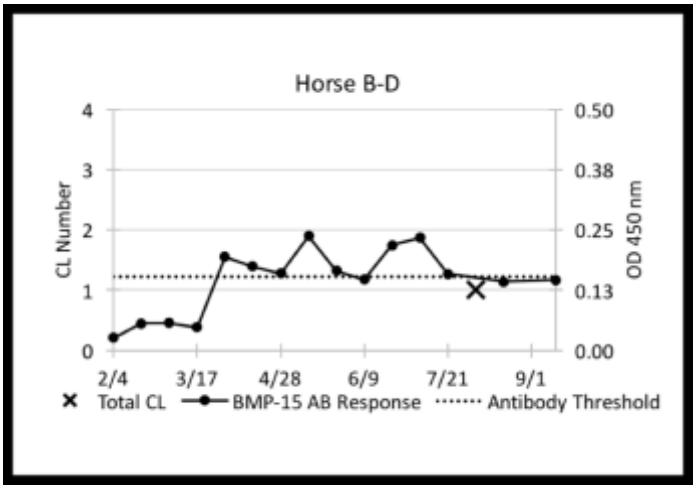


***Figure 21: Individual Antibody Response vs CL Occurrence- Horse B-D***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse D in the BMP-15 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for BMP-15 is indicated by horizontal dashed line.

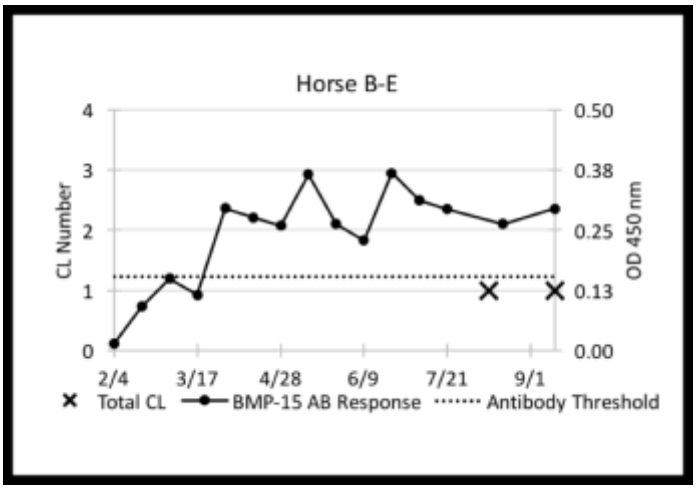


***Figure 22: Individual Antibody Response vs CL Occurrence- Horse B-E***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse E in the BMP-15 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for BMP-15 is indicated by horizontal dashed line.

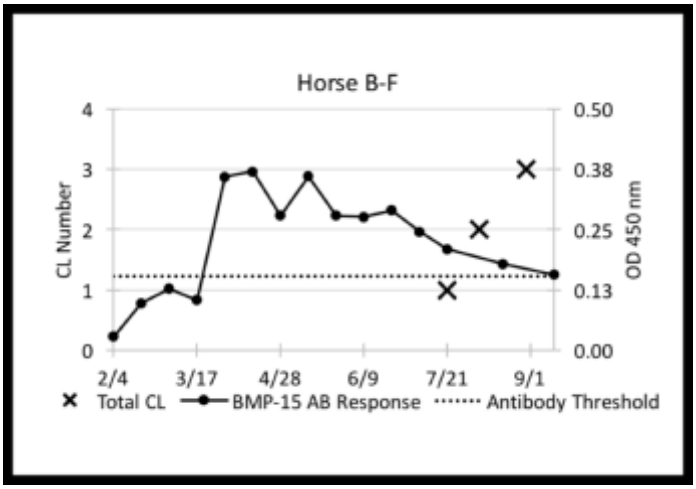


***Figure 23: Individual Antibody Response vs CL Occurrence- Horse B-F***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse F in the BMP-15 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for BMP-15 is indicated by horizontal dashed line.

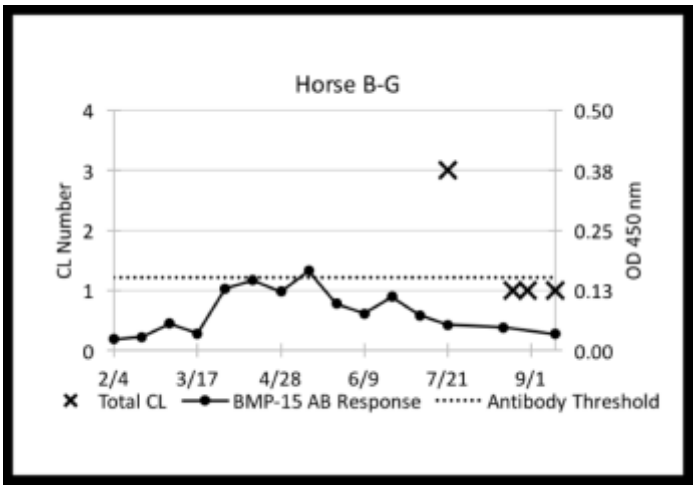


***Figure 24: Individual Antibody Response vs CL Occurrence- Horse B-G***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse G in the BMP-15 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for BMP-15 is indicated by horizontal dashed line.

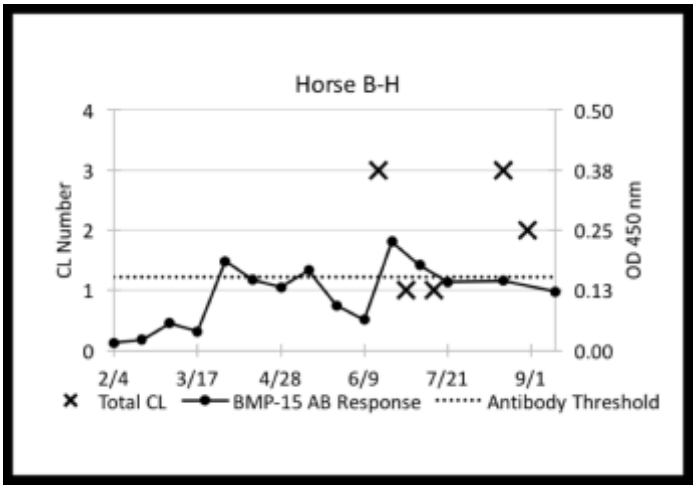


***Figure 25: Individual Antibody Response vs CL Occurrence- Horse B-H***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse H in the BMP-15 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for BMP-15 is indicated by horizontal dashed line.

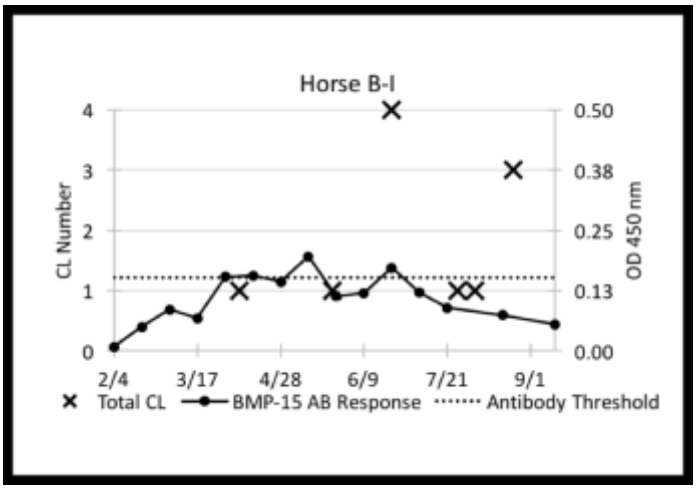


***Figure 26: Individual Antibody Response vs CL Occurrence- Horse B-I***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse I in the BMP-15 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for BMP-15 is indicated by horizontal dashed line.

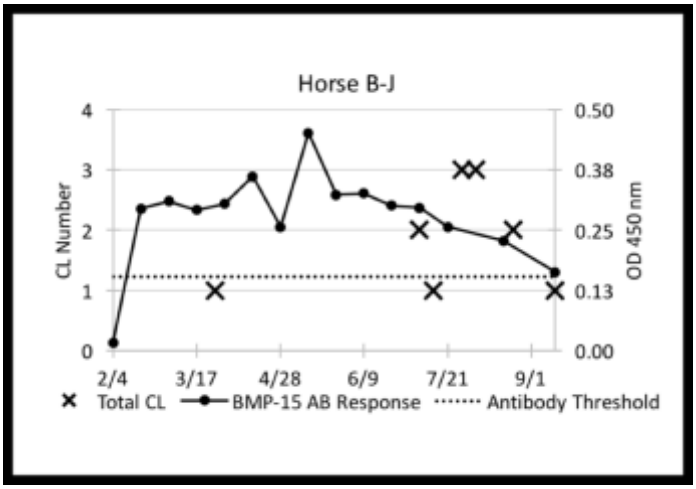


***Figure 27: Individual Antibody Response vs CL Occurrence- Horse B-J***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse J in the BMP-15 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for BMP-15 is indicated by horizontal dashed line.

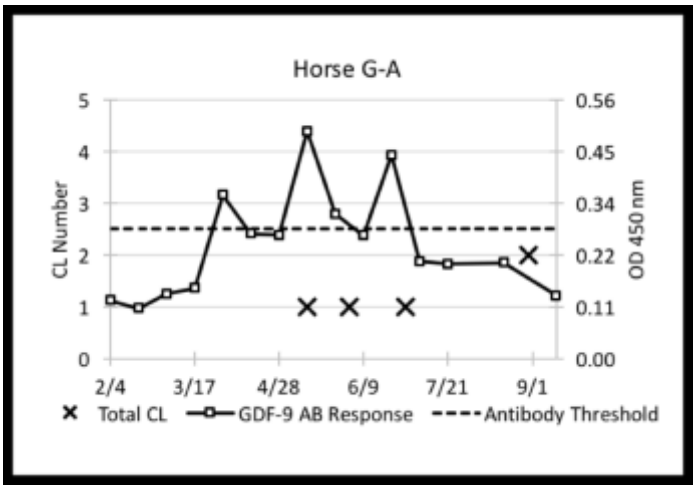


***Figure 28: Individual Antibody Response vs CL Occurrence- Horse G-A***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse A in the GDF-9 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for GDF-9 is indicated by horizontal dashed line.

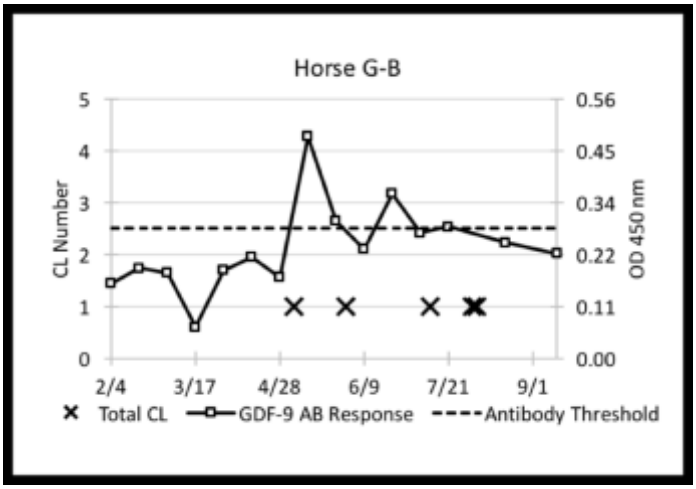


***Figure 29: Individual Antibody Response vs CL Occurrence- Horse G-B***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse B in the GDF-9 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for GDF-9 is indicated by horizontal dashed line.

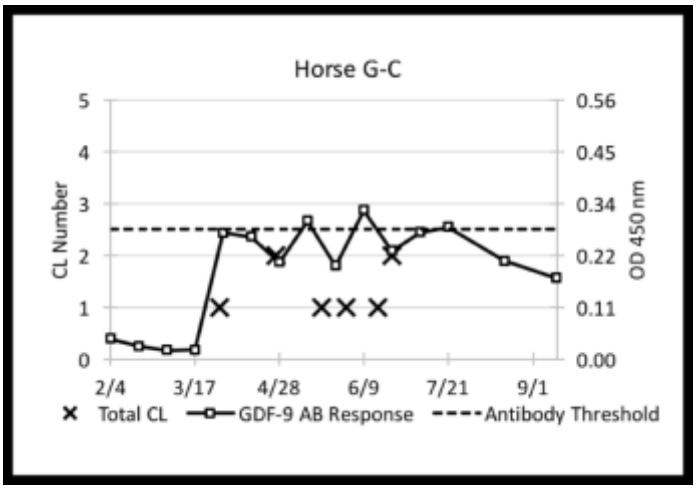


***Figure 30: Individual Antibody Response vs CL Occurrence- Horse G-C***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse C in the GDF-9 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for GDF-9 is indicated by horizontal dashed line.

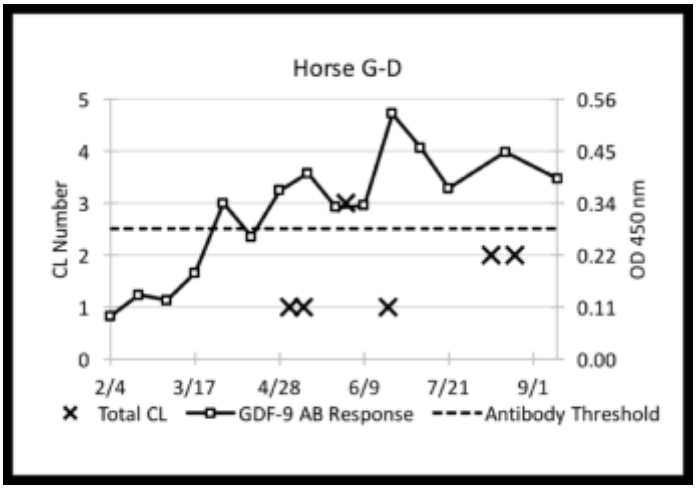


***Figure 31: Individual Antibody Response vs CL Occurrence- Horse G-D***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse D in the GDF-9 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for GDF-9 is indicated by horizontal dashed line.

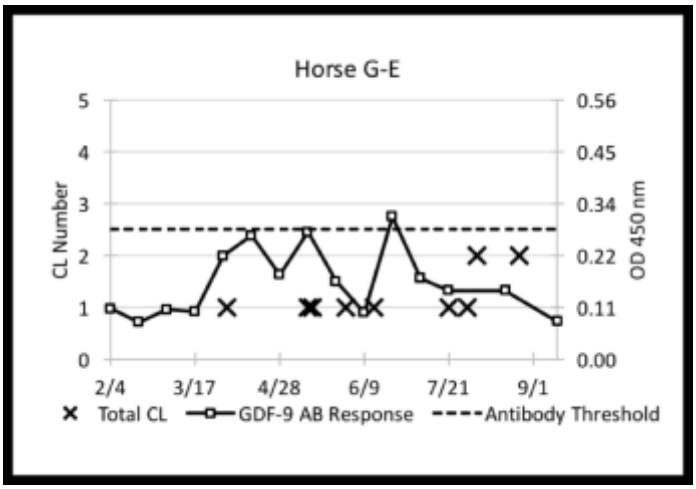


***Figure 32: Individual Antibody Response vs CL Occurrence- Horse G-E***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse E in the GDF-9 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for GDF-9 is indicated by horizontal dashed line.

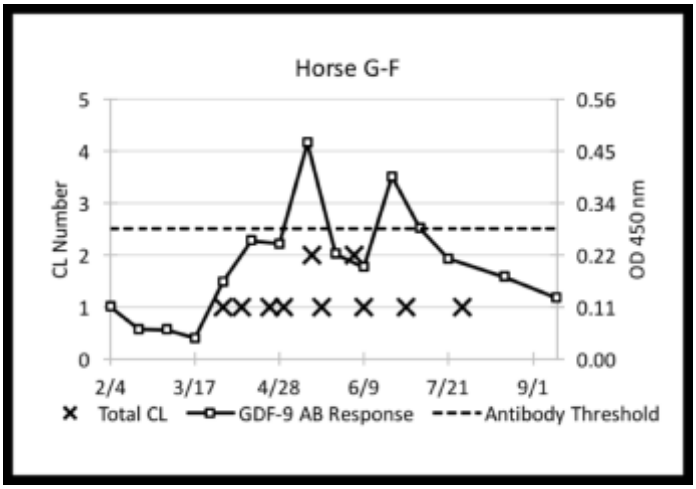


***Figure 33: Individual Antibody Response vs CL Occurrence- Horse G-F***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse F in the GDF-9 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for GDF-9 is indicated by horizontal dashed line.

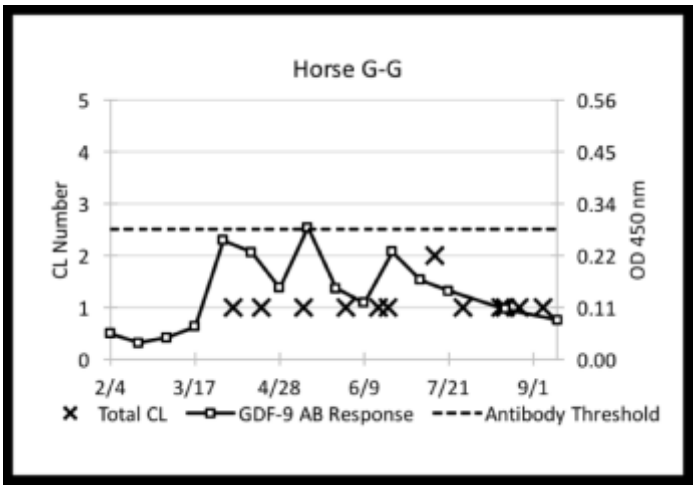


***Figure 34: Individual Antibody Response vs CL Occurrence- Horse G-G***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse G in the GDF-9 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for GDF-9 is indicated by horizontal dashed line.

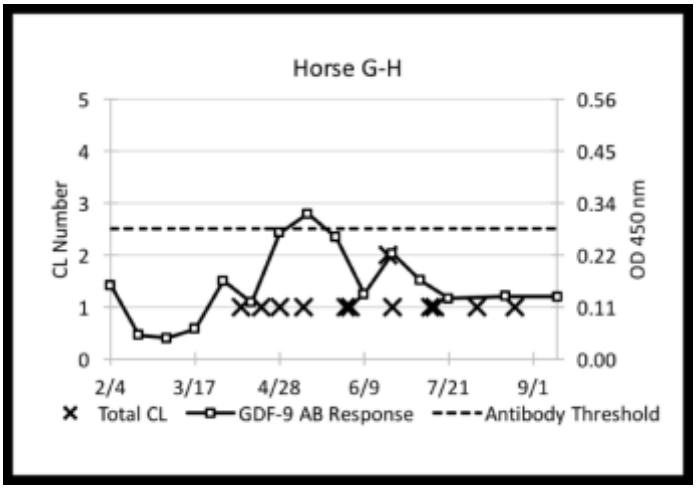


***Figure 35: Individual Antibody Response vs CL Occurrence- Horse G-H***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse H in the GDF-9 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for GDF-9 is indicated by horizontal dashed line.

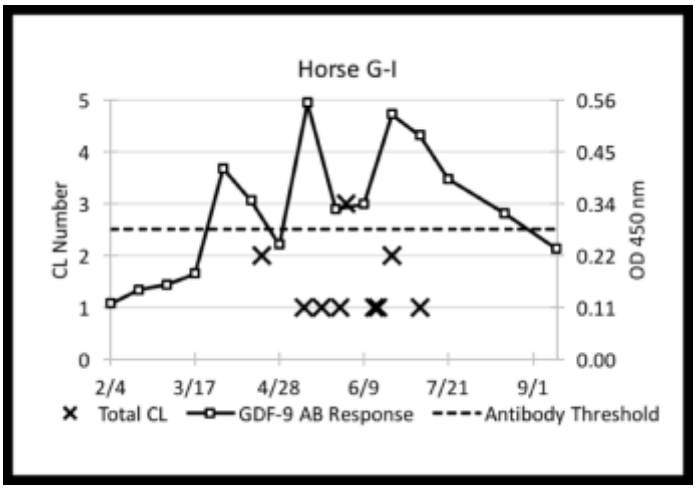


***Figure 36: Individual Antibody Response vs CL Occurrence- Horse G-I***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse I in the GDF-9 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for GDF-9 is indicated by horizontal dashed line.

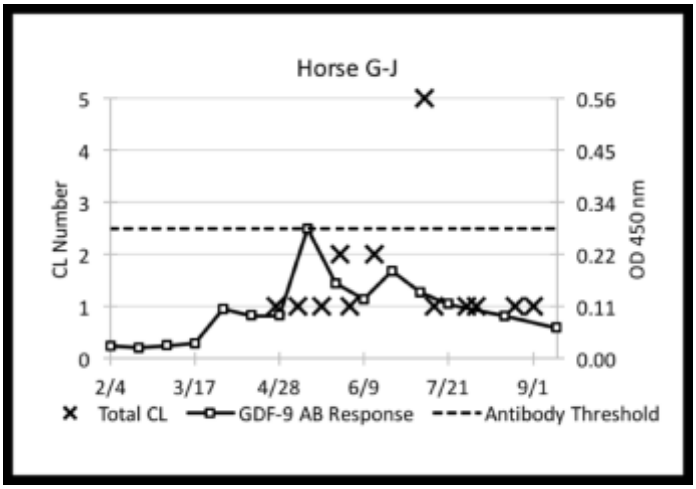


***Figure 37: Individual Antibody Response vs CL Occurrence- Horse G-J***

Individual antibody response in relation to the date of occurrence of corpus lutea for Horse J in the GDF-9 group. A black X indicates total number of corpora lutea observed during ultrasound observation on that date. Antibody threshold for GDF-9 is indicated by horizontal dashed line.