THESIS

IMMUNOREACTIVITY OF ANTI PZP ANTIBODIES FROM THE SERUM OF SPAYVAC VACCINATED MARES TO EQUINE ZONA PROTEIN

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ABSTRACT

IMMUNOREACTIVITY OF ANTI PZP ANTIBODIES FROM THE SERUM OF SPAYVAC VACCINATED MARES TO EQUINE ZONA PROTEIN

Immunocontraception with the porcine zona pellucida (pZP) antigen is a well-published means of wild horse contraception. There are three pZP vaccines currently proposed for use in horses, Zonastat-H®, PZP-22, and SpayVac®. Despite abundant research concerning the safety and contraceptive efficacy of pZP vaccines in numerous species, the contraceptive mechanisms of the pZP antigen remain unclear and have not been investigated thoroughly for SpayVac. We investigated the immunoreactivity of anti pZP antibodies from the serum of SpayVac vaccinated mares to equine zona protein using Western blot and immunohistochemical techniques. These techniques were first applied using a bovine model because bovine oocytes are more easily obtained in large quantities relative to equine oocytes. Once the procedure was validated, equine samples were utilized. Western blot analysis revealed immunoreactivity of anti pZP antibodies that were produced in response to SpayVac vaccination to protein isolated from mature equine oocytes, equine zona pellucidae, equine follicular tissue, and equine ovarian stromal tissue. Immunohistochemical analysis identified the location of binding of anti pZP antibodies to the zona pellucida of mature oocytes isolated from Graafian follicles as well as the zona pellucida of immature oocytes in ovarian tissue. Western blot and immunohistochemical analyses also indicate high specificity of anti pZP antibodies for equine zona protein and predominant affinity for zona protein 3. Collectively, results suggest a model where anti pZP antibodies produced in

response to SpayVac vaccination are immunoreactive to equine zona protein in vitro. If available in the follicular fluid and able to permeate the ovary following SpayVac vaccination, anti pZP antibodies may act on not only mature oocytes, but also oocytes of growing follicles in vivo. The results of this study lend insight into the infertility observed following SpayVac vaccination, and may also help explain the long-term ovarian effects following pZP vaccination reported by other studies.

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CHAPTER I: INTRODUCTION

In 1971, wild horses became federally protected under the Wild Free-Roaming Horses and Burros Act. The Bureau of Land Management (BLM) was tasked with the protection, management, and control of wild horses on BLM managed federal lands in accordance with this law (92 Public Law 195). Provided this protection, in combination with a lack of natural predators, many wild horse populations began to increase rapidly. Current annual growth rates are recognized to range from 10-25% (Eberhardt et al. 1982, Berger 1986, Wolfe 1986, Garrott et al. 1991, Greger and Romney 1999, Goodloe et al. 2000, Roelle et al. 2010) while habitats remain constant in size and limited in forage availability. These horse populations are therefore faced with a finite supply of resources that is unable to sustain increasing herd sizes. In response to this, BLM removes thousands of horses annually and places them into their National Adopt-A-Horse or Burro Program to avoid issues of overpopulation (GAO 2008). When adoption demand does not balance removals, the remaining horses are transferred to holding facilities where they are fed and cared for by the government (NRC 2013).

As of 2013, BLM manages approximately 34,000 free-roaming feral horses across 10 western states and an additional 47,800 captive feral horses in government funded holding facilities. In 2013, the costs to feed and care for horses in holding facilities accounted for more than half of the BLM's annual appropriations for the Wild Horse and Burro Program (NRC 2013). Unless management strategy adjustments are made, the costs of holding animals in long-term facilities will continue to overwhelm the program (GAO 2008, Garrott and Oli 2013). Since wild horse populations can double every four years (Garrott and Oli 2013), and the continued removal of horses will only perpetuate the issue (Garrott and Oli 2013, NRC 2013), relying on a

gather-removal program is no longer a viable option (NRC 2013). Researchers have thus investigated other methods of population growth suppression.

Research on population control techniques has largely focused on suppressing female fertility, particularly through the use of immunocontraceptive vaccines. While there are two antigens actively being studied, gonadotropin releasing hormone (GnRH) and porcine zona pellucida (pZP), the NRC declared in their 2013 report that immunocontraception with pZP may be the more favorable option (NRC 2013). Porcine zona pellucida is an antigen harvested from the zona pellucida of porcine oocytes. When incorporated into a vaccine, combined with an adjuvant, and injected intramuscularly into another species, the pZP antigen has been shown to stimulate an immune response resulting in infertility (Sacco 1977, Liu et al. 1989, Ransom et al. 2011, Bechert 2013). Despite numerous efficacy studies in multiple species, the mechanisms by which the pZP antigen acts to inhibit fertility are not thoroughly understood. When injected, the pZP antigen induces an immune reaction that can be measured by serum antibody titers. These antibodies are thought to bind zona proteins on ovulated oocytes, thereby inhibiting sperm binding and penetration (Sacco 1977, Liu et al. 1989, Kirkpatrick et al. 1990). Since the zona pellucida is thought to play a critical role in fertilization and early embryogenesis (Wolgemuth et al. 1984, Prasad et al. 2000), it is an optimal target for fertility control research.

The use and efficacy of pZP vaccines was first investigated in the early 1980s in mammals including dogs (Mahi-Brown et al. 1985), rabbits (Wood et al. 1981), and baboons (Dunbar et al. 1989). The promising contraceptive results from these studies prompted research on its use in horses. Liu et al. (1989) was the first to demonstrate that an aqueous pZP vaccine may be a viable contraceptive agent to control wild horse populations through a study in captive, feral mares. Kirkpatrick et al. (1990, 1992) further investigated the use of this vaccine, now

known as ZonaStat-H®, in a herd of free-roaming horses inhabiting Assateague Island, Maryland. In the two decades that followed, a succession of studies investigated delivery methods (Kirkpatrick et al. 1990, Willis et al. 1994), duration of effect (Turner et al. 2002, 2007, 2008, Ransom et al. 2011), effect on behavior (Nunez et al. 2009, Nunez et al. 2010, Ransom et al. 2010), and return to fertility (Kirkpatrick et al. 1992, Ransom et al. 2013). Researchers also began investigating new formulations of pZP vaccines that promised longer duration of effect than the original aqueous formulation. Turner et al. (2007) tested the efficacy of a presumptive 2-year pZP vaccine, known as PZP-22 (Turner et al. 2007), and SpayVac® (Immunovaccine Inc., Halifax, Nova Scotia, Canada) emerged as a long-lasting, single injection alternative to the traditional pZP vaccines in seals (Brown et al 1997) and deer (Hernandez et al. 2006, Locke et al. 2007). SpayVac has been recently reported for use in horses (Killian et al. 2004, Bechert et al. 2013), but the contraceptive mechanism of the vaccine is not well documented in published literature.

While pZP vaccines all incorporate the pZP antigen, they are not a homogenous set of compounds. Since each vaccine has a slightly different method of preparation and antigen isolation, levels of purity between the vaccines may differ. This could account for variability in efficacy, duration of effect, and return to fertility between the vaccines (Miller et al. 2009, NRC 2013). Likewise, individual differences in immunity between mares vaccinated with the pZP antigen leads to variations in antibody titer level and efficacy.

Despite many studies investigating the use of pZP vaccines, the contraceptive mechanisms remain unclear. While researchers have investigated serum anti pZP titer levels (Willis et al. 1994, Turner et al. 1996), estrogen and progesterone profiles (Kirkpatrick et al. 1992, Killian et al. 2008), and even ovarian function (Mahi-Brown et al. 1981, Skinner et al.

1984, Dunbar et al. 1989, Bechert 2013) as a result of pZP vaccination, few studies have focused on SpayVac. Likewise, varying levels of purity, and thus efficacy, among the vaccines may be indicative of slight differences in the mechanism of action between the compounds. We tested the hypothesis that anti pZP antibodies produced in response to SpayVac vaccination are immunoreactive to equine zona protein to better understand the contraceptive mechanisms resulting from SpayVac vaccination. The objectives of this study were to (1) develop and use a Western blot assay to determine whether vaccination of wild horse mares with SpayVac results in the production of antibodies immunoreactive to equine zona protein, and (2) identify location of binding of antibodies from the serum of SpayVac vaccinated mares to equine oocytes and ovarian tissues using immunohistochemistry techniques.

CHAPTER II: REVIEW OF THE LITERATURE

Population Growth Suppression Research

In 1971, the U.S. Congress enacted the Wild Free-Roaming Horses and Burros Act, which charged the Bureau of Land Management (BLM) with the protection, management and control of free-roaming horses and burros on BLM managed federal lands (92 Public Law 195). BLM currently manages approximately 34,000 free-roaming feral horses on public lands and an additional 47,800 horses in long- and short- term holding facilities. With holding facility capacity nearly at its limit, and wild horse populations capable of growing 10-25% annually (Eberhardt et al. 1982, Berger 1986, Wolfe 1986, Garrott et al. 1991, Greger and Romney 1999, Goodloe et al. 2000, Roelle et al. 2010), relying on a gather-removal program is no longer a viable option (GAO 2008). Researchers have thus investigated other methods of population control.

Such research has largely focused on suppressing female fertility, particularly through the use of surgical sterilization (NRC 2013), intrauterine implants (Daels and Hughes 1995, Killian et al. 2004) and immunocontraception (Kirkpatrick et al. 2011). While the first two methods are less favorable due to their invasive nature, the latter is associated with ease of administration and nominal discomfort to the animal during application. Ideal characteristics of a potential fertility control method are ease of delivery, high efficacy, prolonged duration of effect, and minimal physiological and behavioral side effects (NRC 2013). Immunocontraception with porcine zona pellucida (pZP) vaccines may be the most promising option at present as it fulfills these criteria (NRC 2013).

Surgical sterilization procedures, such as ovariectomy and ovariohysterectomy, are commonly practiced in domestic species and offer complete, irreversible infertility. These procedures, however, are seldom applied to free-ranging species since accessing the female reproductive tract in a field setting is difficult and often carries the risk of post-operative complications, such as septic peritonitis (Freeman et al. 2007). The possibility that these procedures may be followed by prolonged bleeding or peritonitis makes them inadvisable for field application (NRC 2013). Additionally, surgeries such as these typically require movement restriction and monitoring, making them logistically difficult to apply to a free-ranging species (NRC 2013).

Two studies of note investigated the use of intrauterine devices (IUDs) in horses as a method of fertility control. Daels and Hughes (1995) fitted 6 domestic mares with silastic O-ring-shaped IUDs to investigate the contraceptive efficacy. The IUDs were in place for just less than 1 year and 100% efficacy was achieved during this time period. After removal of the IUDs in early spring, each mare was able to return to fertility, conceive, and deliver a normal foal at term. Uterine cytology and histopathology indicated that the IUD caused a mild inflammation of the uterus sufficient to interfere with fertility while not permanently affecting reproductive health (Daels and Hughes 1995). Killian et al. (2004) evaluated three different IUDs in pony mares for ease of placement, retention and efficacy. The results of this preliminary study in ponies led the authors to implant 15 captive, feral mares with a copper containing IUD (Killian et al. 2004 and 2008). After four years of study, rates of contraception for years 1, 2, 3, and 4 respectively were 80%, 29%, 14%, and 0%. Ultrasound analysis confirmed that the pregnancies of IUD-implanted mares were due to a loss of the IUD itself rather than a failure of the device. Likewise, the

authors note IUD retention may be a function of uterine size, as pony mares implanted with IUDs had higher retention and contraception rates than larger, feral mares (Killian et al. 2008).

While IUDs are not as invasive as surgical sterilization procedures, implantation of the IUD requires capture of the animal, restraint, and transcervical insertion of the device. Killian et al. (2008) described this process as cumbersome and time consuming, even while using chemical restraint for IUD placement. The device itself may provoke undue uterine inflammation and further research concerning loss rates of the IUDs is needed before widespread use (NRC 2013).

One of the most encouraging areas of fertility control research focuses on immunocontraception. Immunocontraceptive vaccines are advantageous as they can be remotely delivered, are noninvasive, and offer high efficacy with few adverse side effects (Kirkpatrick et al. 2011, NRC 2013). These vaccines use the animal's own immune system to interfere with a critical reproductive event (Miller et al. 2008, Gray et al. 2010). Likewise, since the vaccine is specific to a chosen tissue or biochemical, it can target specific hormones or tissues within the animal while not causing harm to other tissues (Barber and Fayrer-Hosken 2000). The most studied immunocontraceptive vaccines are the gonadotropin-releasing hormone (GnRH) vaccine, GonaCon™, and the three formulations of porcine zona pellucida (pZP) incorporating vaccines: Zonastat-H®, pZP-22 and SpayVac® (NRC 2013).

Immunocontraception with Gonadotropin Releasing Hormone (GnRH) Vaccines

GnRH is a trophic peptide hormone that is secreted in a pulsatile form from neurons in the preoptic area of the hypothalamus. It stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland (Irvine and Alexander 2005). Briefly, LH and FSH act on cells of the developing follicle to cause cellular proliferation,

follicular growth, ovarian estrogen and progesterone production, and ovulation (Alexander and Irvine 2005). When a GnRH peptide is conjugated to a large foreign protein, combined with an adjuvant, and injected into an animal an immune response is developed which ultimately results in infertility (Miller et al. 2008). Antibodies produced in response to vaccination bind to endogenous GnRH, preventing it from diffusing into the anterior pituitary from the blood (Gray et al. 2010). Infertility is thus achieved by limiting the availability of endogenous GnRH to the anterior pituitary gland, thereby reducing the secretion of both FSH and LH, culminating in an inhibition of ovulation (Miller et al. 2008, Gray et al. 2010).

Two formulations of GonaCon have been reported with varying levels of efficacy. GonaCon-KLH, where the GnRH peptide has been conjugated to a keyhole limpet hemocyanin protein, is typically less effective than GonaCon-B, where a blue mollusk protein is used in the conjugation (Killian et al. 2008, Miller et al. 2008). While these GnRH vaccines have proven effective in many species, including deer (Miller et al. 2000, Gionfriddo et al. 2011), wild boar (Killian et al. 2003, Miller et al. 2004a), bison (Miller et al. 2004b), and feral cats (Levy et al. 2004), there are only a few published studies of the use of GonaCon as a contraceptive agent in the horse. Killian et al. (2008) evaluated the multiyear contraceptive efficacy of GonaCon-KLH in captive, feral mares. Rates of contraception for years 1, 2, 3, and 4 respectively were 94%, 60%, 60%, and 40% (Killian et al. 2008). Gray et al. (2010) observed slightly lower contraception rates in free-roaming feral horses following immunocontraception with GonaCon-B; 39%, 42% and 31% respectively for years 1, 2 and 3 post treatment. Baker et al. (2013) also investigated the effects of GonaCon-B on a herd of free roaming horses. The estimated foaling rate in the first year following treatment was 48% for treated mares compared to 75% for control mares (Baker et al. 2013). Although these results contradict the general opinion that GonaCon-B

is more effective than GonaCon-KLH, the authors attribute this discrepancy to their use of a more conservative method of fertility estimation as well as potential differences in body condition between captive and free-ranging mares (Gray et al. 2010). Gray et al. (2010) monitored mares at least weekly and supplemented visual observations with fecal steroid hormone analysis. The authors indicate that this intense monitoring allowed them to observe events which may otherwise be missed when only assessing fertility as a function of foal production, such as whether a mare aborted a fetus early or lost a foal shortly after birth (Gray et al. 2010).

Immunocontraception with Porcine Zona Pellucida (pZP) Vaccines

Although GnRH vaccines were found to be an effective means of immunocontraception for the horse (Miller et al. 2008, Gray et al. 2010), studies concerning their duration of effect and efficacy in horses are rare. Likewise, GnRH vaccines are generally considered to be less efficacious in the short-term than pZP vaccines (NRC 2013). Alternatively, immunocontraception with pZP has been extensively tested and documented in literature, offers high contraceptive efficacy, and is more predictable as a contraceptive agent (Kirkpatrick et al. 2011, NRC 2013).

The zona pellucida is a unique glycoprotein matrix that surrounds the mammalian oocyte. It is thought that zona proteins play a critical role in folliculogenesis, fertilization, and early embryogenesis (Wolgemuth et al. 1984, Prasad et al. 2000). The zona pellucida's assumed role in fertilization makes it an expected target for fertility control research. Specifically, when zona proteins harvested in one species are used as an antigen in another, they have been shown to stimulate an immune response, measured by serum antibody titers, that results in infertility

(Sacco 1977, Liu et al. 1989). Despite efficacy studies in multiple species, the mechanism by which the pZP antigen acts to inhibit fertility is not thoroughly understood. When injected, pZP vaccines induce an immune reaction in which anti pZP antibodies are generated. These antibodies are thought to bind zona pellucida proteins on mature oocytes, thereby interfering with sperm binding and penetration (Sacco 1977, Liu et al. 1989, Kirkpatrick et al. 1990). Other studies demonstrated follicular dysgenesis in the rabbit (Skinner et al. 1984) and mare (Kirkpatrick et al. 1995) following vaccination, including diminished ovulatory ability (Kirkpatrick et al. 1995), absence of mature oocytes and follicles (Skinner et al. 1984), and a decline in ovarian estrogen secretion (Kirkpatrick et al. 1995). These results suggest an effect of pZP vaccination on ovarian function in addition to an interruption at the level of sperm binding sites. Paterson and Aitken (1990) agree with both of these hypotheses and suggest that anti pZP antibodies can cause infertility by disturbing both folliculogenesis and sperm-egg interactions. It is well documented that these effects occur following vaccination, regardless of which stage of the reproductive process pZP vaccines target.

The use and efficacy of pZP vaccines was first investigated in the early 1980s in mammals including dogs (Mahi-Brown et al. 1985), rabbits (Wood et al. 1981), and baboons (Dunbar et al. 1989). The promising contraceptive results from these studies prompted further research on its use in mares (Liu et al. 1989, Kirkpatrick et al. 1990, Willis et al. 1994). In recent decades, pZP vaccines have grown in popularity with wild horse managers due to their relative ease of application, lack of significant side-effects, and proven temporary efficacy (Ransom et al. 2011).

Liu et al. (1989) was the first to document the use of an aqueous pZP vaccine, later to become known as Zonastat-H, in mares. Fourteen fertile mares were immunized with a series of

four aqueous pZP intramuscular injections and their immune responses were measured by an enzyme-linked immunosorbent assay (ELISA). Inhibition of fertility occurred in 12 of the 14 mares (86%) and persisted for a minimum of seven months. While only a preliminary study, Liu et al. (1989) demonstrated that pZP vaccination may be a viable contraceptive option to control wild horse populations. The authors suggested the need for more studies to investigate the effects on long-term fertility and improve the method of administration (Liu et al. 1989).

Kirkpatrick et al. (1990) further explored this aqueous pZP vaccine, known as ZonaStat-H, by documenting its use in a free-roaming herd of feral horses inhabiting Assateague Island National Seashore, Maryland. In 1988, 26 mares were inoculated with a series of two aqueous pZP injections, the majority of which were delivered by dart from 25 to 30 meters distance. Eighteen of the original 26 mares received a third injection 2-4 weeks after the second. Of the original 26 treated mares, 14 were pregnant at the time of inoculation and all 14 produced normal healthy foals in the spring of 1988, verifying a lack of observed effect on existing pregnancies. By August 1989, one year after vaccination, only 1 of the 26 mares foaled. This study was the first to document successful fertility inhibition among free-roaming horses by means of remotely delivered immunocontraception. The authors concluded that remote inoculation of feral mares with this aqueous pZP vaccine was an effective means of fertility inhibition (Kirkpatrick et al. 1990).

Kirkpatrick et al. (1992) continued to monitor this herd of mares on Assateague Island in order to determine the long-term effects of ZonaStat-H on ovarian function and contraceptive efficacy. Ten free-roaming mares that were initially inoculated in 1988 with aqueous pZP received 2 additional annual boosters in 1989 and 1990. Following the third annual pZP inoculation, ovarian function was monitored for 51 days during the breeding season by means of

urinary estrone conjugates and nonspecific progesterone metabolites. One treated mare was pregnant as a result of conception in 1989 and none of the ten treated mares became pregnant in 1990. Four of the treated mares revealed no evidence of ovulation and displayed significantly depressed urinary estrogen concentrations. Observations indicate that a third consecutive annual pZP booster is more than 90% effective in inhibiting pregnancy, but that three consecutive years of treatment may interfere with normal ovarian function (Kirkpatrick et al. 1992).

In the years to follow, a succession of studies investigated new remote-delivery methods (Willis et al. 1994), duration of efficacy (Turner et al. 2002, 2007, 2008, Ransom et al. 2011), behavioral effects as a result of contraception (Nunez et al. 2009, Nunez et al. 2010, Ransom et al. 2010), and return to fertility (Kirkpatrick et al. 1992, Ransom et al. 2013). Likewise, researchers began investigating formulations of the pZP antigen that promised multiyear efficacy. Turner et al. (2007) tested the effectiveness of a targeted 2-year pZP vaccine that incorporated a controlled-release adjuvant component in feral mares. The vaccine, later to become known as PZP-22, consisted of two components: a primer dose of aqueous pZP and a booster dose in the form of controlled-release polymer pellets. The study by Turner et al. (2007) reported high efficacy for 2 years post treatment with some residual contraceptive effect in year 3. Similarly, SpayVac (Immunovaccine Inc., Halifax, Nova Scotia, Canada) emerged as a long-lasting, single injection pZP vaccine in seals (Brown et al. 1997) and deer (Hernandez et al. 2006, Locke et al. 2007). SpayVac has been recently reported for use in horses (Killian et al. 2004, Bechert et al. 2013), but the contraceptive mechanism of the vaccine is not well documented in published literature.

Efficacy studies have demonstrated that heteroimmunization with the pZP antigen, that is, using porcine zona proteins as a vaccine for another species, not only effectively induces

infertility, but also results in follicular dysgenesis in some cases (Mahi-Brown et al. 1985, Dunbar et al. 1989, Paterson and Aitken 1990, Killian et al. 2008). These results raise the question of how the pZP antigen, or the antibodies produced in response to vaccination, elicits these responses. Previous studies have determined the equine zona pellucida to be composed of the zona protein families ZP1, ZP2, ZP3 and ZP4 that have antigenic properties similar to those found in other species, such as the pig (Liu and Shivers 1982, Miller et al. 1992, Goudet et al. 2008). When pZP is prepared, the entire porcine zona pellucida is homogenized; resulting in a vaccine composed of all zona protein families. Wassarman (1988) indicated ZP3 to be the most prominent component of the porcine zona pellucida, representing as much as 70-80% of total protein content. It therefore follows that pZP vaccines are likely composed of a larger proportion of ZP3 than the other zona proteins. Since ZP3 is primarily implicated in fertilization events, pZP's possible effect on sperm binding to and penetration of the equine zona pellucida is of particular importance. However, with the vaccine being a compilation of all zona protein families, a possible effect on zona pellucida structure and follicular development is also of interest.

Porcine Zona Pellucida (pZP) Vaccine Preparations

While ZonaStat-H, PZP-22 and SpayVac incorporate the pZP antigen, the three vaccines are not a homogenous set of compounds. Rather, differences in antigen preparation result in variations in purity and protein content (NRC 2013). It is likely that the SpayVac formulation contains more non-zona pellucida proteins than the method described by Liu et al. (1989). Miller et al. (2009) suggests that these differences in antigen preparation may account for the variability in efficacy levels observed among vaccines. Likewise, the methods of pZP antigen isolation

described in published literature simply cite that porcine ovaries were obtained from local slaughter houses for antigen isolation and never mention the age or reproductive status of the sows the ovaries were harvested from (Yurewicz et al. 1983, Liu et al. 1989). These are important confounding factors that can contribute to the complex nature of the pZP antigen among vaccine preparations.

ZonaStat-H is a two-injection, aqueous formulation of pZP that is registered with the Environmental Protection Agency (EPA) as a mammalian contraceptive. ZonaStat-H includes a primer of aqueous pZP emulsified in Freund's Complete Adjuvant, Modified and a booster of aqueous pZP in Freund's Incomplete Adjuvant, Modified given 2 to 4 weeks later (Kirkpatrick et al. 1990). The primer is prepared by emulsifying 0.5cc aqueous pZP (65µg pZP suspended in 0.5cc phosphate buffered saline; PBS) with 0.5cc Freund's Complete Adjuvant (Kirkpatrick et al. 1990). The booster injection also contains 0.5cc aqueous pZP, but it is emulsified in Freund's Incomplete Adjuvant (Kirkpatrick et al. 1990). The preparation of aqueous pZP for this vaccine is outlined by Liu et al (1989) and details slicing porcine ovaries to release oocytes from surrounding tissues prior to a series of passages through filters of decreasing pore size. Trapped oocytes are further homogenized and the resulting zona pellucidae are trapped on a 48µm screen. The zona pellucidae are washed in PBS and heat solubilized at 70° Celsius for 30 minutes in PBS. The supernatant is finally concentrated through ultrafiltration and stored as an aqueous solution. ZonaStat-H targets one year of contraceptive efficacy (Liu et al. 1989).

PZP-22 also involves two injections, a liquid primer followed by a booster in the form of three heat-extruded pellets. The liquid primer is made by emulsifying 65µg aqueous pZP in PBS, prepared as detailed above for ZonaStat-H, with 0.5 cc Freund's Complete Adjuvant. The booster pellets incorporate lyophilized pZP and QS-21, a water-soluble saponin adjuvant,

matrixed in homogenous lactide and glycolide polymers (Turner et al. 2007). The pellets are designed to act in a time-release fashion, where the contents of the first pellet are released 1 month, the second released 3 months, and the final released 12 months, after initial inoculation. The first pellet incorporates 70μg pZP with 175μg QS-21, the second pellet incorporates 90μg pZP with 200μg QS-21, and the third pellet incorporates 250μg pZP with 500μg QS-22 (Turner et al. 2007). This time-release delivery method targets 22 months of efficacy (Turner et al 2007).

Finally, SpayVac is a single injection formulation in which the pZP antigen is encapsulated in liposomes for targeted multi-year contraceptive efficacy. Alternative to the preparation of aqueous pZP detailed above by Lui et al. (1989), Yurewicz et al. (1983) details a preparation where frozen-thawed whole porcine ovaries are homogenized in Tris-zona buffer and zona-encased oocytes are recovered by a succession of passages of the homogenate through nylon screens of decreasing pore sizes. Oocytes are trapped on a 100μm screen, collected by washing into Tris-zona buffer, and homogenized further. The resulting zona pellucidae are trapped on a 50μm screen, heat solubilized at 73° Celsius for 20 minutes, and centrifuged at 27,000 x g for 15 minutes. The supernatant is finally concentrated through ultrafiltration and stored as an aqueous solution. An aliquot of 100μg aqueous pZP is encapsulated in liposomes and mixed with Freund's Complete Adjuvant, Modified (Yurewicz et al. 1983, Brown et al 1997).

Immune Response to Vaccination

The immune system has evolved as a critical defense mechanism against the risks imposed by infectious microorganisms. While immunity has many facets, two main immunological branches are required for proper immune response. These are the innate

immunity and adaptive immunity (Hoebe et al. 2004). The innate immune response is responsible for the rapid release of inflammatory cytokines and activation of antigen-presenting cells as the first line of defense against a foreign microbe. In contrast, adaptive immunity is much slower in nature and utilizes a very specific mechanism of selection and clonal expansion to recognize antigens and provide long-lasting immunological memory (Pashine et al. 2005). These two branches of the immune system perform very distinct functions and their interaction and coexistence is critical for proper immune system function (Hoebe et al. 2004).

The innate immune response incorporates a wide variety of pattern-recognition receptors to recognize pathogen associated molecular patterns (PAMPs) and activate the release of inflammatory mediators (Pashine et al. 2005). Pattern-recognition receptors are differentially expressed on a variety of immune cells, including neutrophils, B cells, natural killer cells and macrophages (Pashine et al. 2005). When the receptor recognizes a PAMP, it activates the secretion of cytokines and chemokines from the immune cells it is expressed on, recruits additional immune cells to the site of infliction, and induces phagocytosis of the antigen by antigen-presenting cells (Hoebe et al. 2004). This culminates in an inflammatory response that will influence the establishment of the adaptive immune response.

Once immune cells have phagocytized the antigen, rendering them activated, immune cells migrate to the lymph nodes where T-cells of the adaptive immune system recognize the antigen and undergo clonal expansion (Pashine et al. 2005). T cells interact with and activate B cells, which differentiate into plasma and memory cells (Pashine et al. 2005). Plasma cells exit the lymph nodes and secrete antigen specific antibodies, while memory cells are reserved for a secondary encounter with the specific antigen (Pashine et al. 2005). In this way, the inflammatory environment established by the innate immune response serves to condition the

site of infliction for adaptive immunity initiation. Hoebe et al. (2004) further indicates a dependency of the adaptive immune response on antigen presentation by the innate immune response.

This is the manner by which an immune response ensues when an animal is vaccinated with an antigen. Innate immunity stimulates an inflammatory response, antigen-presenting cells condition adaptive immunity, and antigen-specific antibodies are produced (Pashine et al. 2005). When an antigen is presented to the immune system for the first time, no memory cells exist to guide the immune response. In this case, stimulation of the innate immune system is critical to elicit an initial immune response to the antigen. Inclusion of immunological agents or potentiators, such as adjuvants, in the vaccine is thus commonly used to facilitate this process.

Adjuvants are immunological agents that improve vaccine efficacy. When incorporated into a vaccine, adjuvants have been shown to enhance and modulate the immune response in such a way as to reduce the amount of antigen and number of immunizations required to achieve a high rate of efficacy (Aguilar and Rodriguez 2007). Adjuvants therefore work to generate long-lasting antigen depots in order to prolong the presentation of the antigen to the immune system (Reed et al. 2008). As indicated earlier, the immune response is triggered by the recognition of PAMPs by pattern-recognition receptors on immune cells. For this reason, PAMPs are often used as the basis for many adjuvants (Reed et al. 2008). Adjuvants also typically incorporate cytokines, bacterial toxins and glycolipids to augment the immune response (Reed et al. 2008).

Water-in-oil emulsions, liposomes, and saponins are three adjuvants that are commonly used in conjunction with immunocontraceptive antigens. Water-in-oil emulsions, such as those formed with Freund's Complete and Incomplete Adjuvants, are microdroplets of water, stabilized by surfactant in a continuous oil phase (Cox and Coulter 1997). These adjuvants are

characterized by their ability to induce an antibody response when emulsified with an antigen while remaining immunologically inert in the absence of local irritant (Cox and Coulter 1997). Liposomes are single or multilamellar bilayer membrane vesicles that provide a safe means for immunomodulators to be incorporated into a vaccine (Cox and Coulter 1997). Saponins, including Quil A and QS21, are triterpene glycosides isolated from plants that are directly targeted to and efficiently taken up by antigen presenting cells (Reed et al. 2008).

Folliculogenesis and Zona Pellucida Biosynthesis

Folliculogenesis is an intricate, highly regulated process that is inextricably coupled with oogenesis. At birth, the female ovary contains a lifetime's supply of primordial follicles. Oocytes in primordial follicles are arrested in meiotic prophase I and surrounded by a single layer of squamous granulosa cells (Pierson 2005). Commencing primarily at puberty in response to hormones, and continuing in a cyclic fashion throughout reproductive life, primordial follicles are recruited to enter the maturation and differentiation process known as folliculogenesis. Primordial follicles first develop into primary follicles. This transition involves differentiation of the squamous layer of granulosa cells into a cuboidal epithelium, formation of the zona pellucida, and oocyte growth (Richards 1980). A cohort of these primary follicles is further selected to develop into antral follicles, characterized by a follicular fluid-filled antrum and the differentiation of theca cells. Finally, one dominant follicle matures into a Graafian follicle, marked by a dramatic increase in follicular fluid volume, and awaits ovulation (Pierson 2005). The oocyte remains in meiotic arrest throughout this process, with resumption of meiosis only occurring as a result of the LH surge at ovulation (Richards 1980).

During the early stages of folliculogenesis, the zona pellucida is formed between the plasma membrane of the mammalian oocyte and surrounding granulosa cells (Pierson 2005). The zona pellucida is a unique extracellular matrix composing three or four glycoproteins, depending on the species, that are synthesized as an integrated action of both the oocyte and the granulosa cells (Wolgemuth et al. 1984, Leveille et al. 1987, Rankin et al. 1996, Blackmore et al. 2004). The different glycoproteins, known as zona proteins, share an overall similar architecture around a conserved C-terminal zona protein domain, while sequences downstream from this domain are much more variable and contribute to the functional differences between each of the proteins (Prasad et al. 2000). Zona proteins are generally categorized into four protein families based on structure and function similarity. Specifically, zona protein families ZP1, ZP2, and ZP4 are thought to be involved in zona pellucida structure whereas ZP3 has a known role in sperm binding and penetration (Prasad et al. 2000).

Furthermore, while zona proteins within a protein family share an overall similar structure, they also exhibit heterogeneity due to extensive post-translational modifications. Heavy glycosylation and sulfation patterns lend to variances in electrophoretic mobilities and carbohydrate moeities among species and between proteins (Dunbar et al. 1994, Prasad et al. 2000). Likewise, proteolytic cleavage and deglycosylation events have been shown to process these large zona glycoproteins into polypeptides of smaller molecular weights. Zona proteins are also known to exist as heterodimers, and thus appear to be of increased molecular weight. (Wassarman 1988, Prasad et al. 2000) These post-translational modifications complicate the ability to accurately identify zona proteins by electrophoresis (Wassarman 1988). For instance, Dunbar et al. (1985) published a 2D PAGE gel illustrating the zona protein families of a porcine zona pellucida ranging from 6 to 93 kDa. The authors suggest that the protein family appearing

in the 6-30 kDa range during 2D PAGE analysis is formed due to proteolytic processing of the ZP2 protein family, which would typically appear in the 60-65 kDa range (Dunbar et al. 1995). Wassarman (1988) confirmed these results by suggesting that the ZP4 porcine glycoprotein appears to have a molecular weight of 15-25 kDA, particularly under reducing conditions. Wasserman (1988) continues to define this protein family as being derived from proteolytic events and identifies molecular weight differences between glycoproteins and polypeptides of the same zona protein family. Additionally, Wasserman (1988) found the porcine zona pellucida to be composed of zona protein families in the 68-90, 52-99, 32-55, and 15-25 kDa ranges, indicating a broad spectrum of protein weights within and among each zona protein family. Meanwhile, Miller et al. (1992) found 3 major zona protein glycoprotein families in the horse varying in sizes of 93-120, 73-90, and 45-80 kDa. Collectively, these experiments define and explain the broad range of molecular weights observed within zona protein families and among species when the zona pellucida is analyzed by gel electrophoresis.

The zona pellucida ultimately develops into a selectively permeable barrier in the primary follicle that is thought to be involved in folliculogenesis, fertilization, and early embryogenesis (Wolgemuth et al. 1984, Prasad et al. 2000). Concurrent with the development of the zona pellucida, the establishment of gap junctions between neighboring granulosa cells and the oocyte permit communication between these cell types. The presence of gap junctions is particularly important after the formation of the zona pellucida, which would otherwise serve as a barrier limiting diffusion of materials needed for oocyte growth. Oocyte growth is thus thought to be mediated primarily by granulosa cells by way of gap junctions (Prasaad et al. 2000, Eppig et al. 2002). Eppig et al. (2002) proposes that this communication is bidirectional, where oocytes promote granulosa cell proliferation, differentiation and function while granulosa cells support

the growth, development and progression through meiosis of the oocyte. The functional role of the zona pellucida in folliculogenesis is less clear. Due to the location of the zona pellucida between the oocyte and surrounding granulosa cells, some argue that it plays a direct role in this cell-cell communication necessary for folliculogenesis and oocyte maturation (Wolgemuth et al. 1984, Prasaad et al. 2000). Conversely, others have shown that follicular maturation proceeds as normal in the absence of zona matrix (Rankin et al. 1996).

Role of the Zona Pellucida in Fertilization

Fertilization is the process by which one spermatozoon interacts with and fuses to an ovulated oocyte to form a zygote. At this time, somatic chromosome number is restored and development of a new individual is initiated. Fertilization is classically thought to be the cumulative result of a number of highly regulated events (Wassarman 1999). These events include ovulation, spermatozoa migration into the oviduct, spermatozoa capacitation, binding of spermatozoa to the zona pellucida matrix, the acrosome reaction, sperm egg fusion, and finally oocyte activation (Wassarman 1999, Okabe 2013).

At the time of ovulation, an oocyte completes meiosis I and is deposited into the oviduct to await fertilization (Richards 1980). Meanwhile, spermatozoa migrate into the female reproductive tract and undergo the physiological change known as capacitation (Okabe 2013). Capacitation results in the hyperactivation of spermatozoa, which is characterized by a vigorous mobility thought to be responsible for the thrusting motion necessary to penetrate the zona pellucida (Okabe 2013). In a sequence of events that is unclear, spermatozoa undergo the acrosome reaction and bind ZP3 on the zona pellucida of the oocyte (Wassarman 1999, Okabe 2013). During the acrosome reaction, lytic enzymes are released and zona binding proteins are

exposed providing spermatozoa with the ability to penetrate and bind the zona pellucida (Okabe 2013). Once spermatozoa have bound to and penetrated the zona pellucida, they reach the perivitelline space between the oocyte plasma membrane and zona pellucida (Wassarman 1999). One spermatozoon fuses with the plasma membrane of the oocyte and releases PLC-zeta, which is implicated in the activation of the oocyte and subsequent calcium oscillations (Okabe 2013). The oocyte completes the second meiotic cell division and releases cortical granules. Finally, ovastacin, which is an enzyme accumulated in the cortical granules, is released, cleaves ZP2 and effectively prevents polyspermy (Okabe 2013).

The classical models of fertilization focus on the ability of sperm to bind the zona pellucida and the involvement of the zona pellucida in the acrosome reaction (Okabe 2013). Researchers postulate that these two events are both dependent on one another and required for fertilization to occur (Okabe 2013). However, recent research involving gene disruption and knock-out experiments has questioned the importance of these events in fertilization. Spermatozoa from mice with impaired zona binding ability due to either a deficiency in the testis specific molecular chaperone PDILT (Tokuhiro et al. 2012) or a knockout in the Adam 1a gene (Nishimura et al. 2004) retained their ability to fertilize cumulus-covered oocytes. These studies suggest that the ability of spermatozoa to bind the zona pellucida may be dispensable for fertilization (Okabe 2013). Likewise, many researchers hypothesize that the acrosome reaction is initiated upon contact with the zona pellucida and spermatozoa that have prematurely undergone the acrosome reaction prior to contact are unable to fertilize the egg (Bleil and Wassarman 1983). Nevertheless, recent studies suggest that the action of penetration through the zona matrix, rather than surface binding, induces the acrosome reaction as the researchers were unable to observe the acrosome reaction of GFP transgenic spermatozoa upon binding to the zona

pellucida (Nakanishi et al. 1999, Baibakov et al. 2007). Jin et al. (2011) agrees with this and suggests that most of the fertilizing spermatozoa are acrosome reacted prior to arriving at the zona pellucida in vitro, indicating the acrosome reaction is zona-independent. These studies question the importance of the zona-induced acrosome reaction and zona-binding ability in the success of fertilization (Okabe 2013).

Conversely, Rankin et al. (1996) found mice with a genetic defect in the ZP3 gene to be infertile. While homozygous mutant $ZP3^{-/-}$ mice had oocyte containing follicles that underwent folliculogenesis and ovulation, the oocytes were marked by an absence of the zona pellucida and disrupted cumulus-oocyte complex upon ovulation (Rankin et al. 1996). When mated with normal males, these mice did not become visibly pregnant and produced no litters (Rankin et al. 1996). This study suggests a necessity of the zona pellucida for successful in vivo fertilization. This is in contrast to the known ability to fertilize zona-free oocytes in vitro (Fulka et al. 1982, Rankin et al. 1996). The contradictory nature of these studies implies that the zona pellucida's role in fertilization is not thoroughly understood and the molecular mechanisms underlying fertilization remain largely unknown.

Contraceptive Mechanism

The contraceptive mechanisms of pZP vaccines remain unclear. Many studies have investigated serum anti pZP titer levels (Willis et al. 1994, Turner et al. 1996), estrogen and progesterone levels (Kirkpatrick et al. 1992, Killian et al. 2008), and even ovarian function (Mahi-Brown et al. 1981, Skinner et al 1984, Dunbar et al. 1989, Bechert et al. 2013) following vaccination. However the ability of anti pZP antibodies produced in response to SpayVac vaccination to recognize equine zona protein has not been thoroughly investigated. One notable

exception is a study that used immunohistochemical staining of an equine oocyte to demonstrate that antibodies raised in the mare in response to PZP-22 vaccination are able to bind the zona proteins of the zona pellucida (Liu et al. 2005). While the study by Liu et al. (2005) lends valuable insight into the mechanism of action behind PZP-22 vaccination, variations in purity and antigen content among vaccines prevent these results from being directly applicable to SpayVac vaccination. As noted by the National Research Council (NRC 2013), it is likely that the SpayVac vaccine contains more non-zona pellucida proteins than the other pZP vaccines. This may account for antigenic differences between SpayVac and PZP-22 (Miller et al. 2009). Since SpayVac is emerging as a longer-lasting alternative to PZP-22, we investigated the mechanism of action of SpayVac vaccination by demonstrating the immunoreactivity of antibodies produced in response to SpayVac vaccination to equine zona protein.

CHAPTER III: DEVELOPMENT OF AN ASSAY TO TEST THE IMMUNOREACTIVITY OF ANTI PZP ANTIBODIES RAISED IN THE MARE IN RESPONSE TO SPAYVAC VACCINATION TO ZONA PROTEIN

Summary

We developed and verified an assay to test the immunoreactivity of anti porcine zona pellucida antibodies raised in the mare in response to SpayVac® vaccination (anti pZP antibodies). Initially, bovine oocytes were used as they are more easily obtained in large quantities than horse oocytes, and bovine zona proteins share a strong homology to equine zona proteins, as verified by the National Library of Medicine Basic Alignment Search Tool (BLAST®). BLAST analysis confirmed greater than 75% homology between all sequences of zona proteins across the two species. The presence of zona protein 3 (ZP3) and zona protein 4 (ZP4) was then confirmed in bovine oocytes and zona pellucidae through Western blot analysis. Immunoreactivity of serum from a SpayVac vaccinated mare to zona protein was tested by a modified Western blot approach in which serum from SpayVac vaccinated and non-vaccinated mares was used as the primary antibody. A horseradish peroxidase (HRP) conjugated goat anti horse IgG polyclonal antibody was used as the secondary antibody to determine whether anti pZP antibodies present in the serum samples of vaccinated mares are capable of binding a protein of interest. Similarly, we modified an immunohistochemistry (IHC) protocol to use serum as the primary antibody and a HRP conjugated goat anti horse IgG polyclonal antibody as the secondary antibody in order to identify the location of binding of anti pZP antibodies to whole oocytes. Serum from non-vaccinated mares was used as a control to ensure that the antibodies binding the protein samples were a result of SpayVac vaccination. These experiments

led to the development of a binding assay that tests whether antibodies present in serum can bind to a specific protein of interest. Results suggest that anti pZP antibodies in the serum of SpayVac vaccinated mares are capable of binding protein isolated from whole bovine oocytes and bovine zona pellucida.

Materials and Methods

Samples

Bovine ovaries were donated by a local slaughterhouse (JBS Swift & Co, Greeley, CO) to the Colorado State University Animal Reproduction and Biotechnology Laboratory (ARBL, 3107 Rampart Road, Fort Collins, CO 80523) for ongoing research at the facility. Follicular fluid was aspirated from follicles on these ovaries using a syringe fitted with an 18-gauge needle. The fluid was separated into Petri dishes and oocytes were visualized under a dissecting microscope. Oocytes were transferred to a 15mL conical tube containing 3% bovine serum albumin (BSA) in 1% phosphate buffered saline (PBS) using an embryo pipette and stored at 4°C until needed.

Serum from vaccinated mares of known titer levels was obtained from stored samples previously collected as part of a study on SpayVac immunocontraception (Roelle, USGS, Pauls Valley, OK). Serum samples from 8 different mares of high titer levels were chosen and used throughout the study (Appendix I). Serum of two non-vaccinated mares was obtained from samples previously collected and stored as part of ongoing research at the ARBL. All serum samples were stored at -80°C until needed. Non-vaccinated mares were of known reproductive history and proven fertility.

Sample preparation and protein isolation

Isolation of total protein from oocytes was performed using radioimmunoprecipitation assay (RIPA) lysis buffer (Appendix II). Whole bovine oocytes were removed from storage and washed in PBS. Cumulus cells were mechanically stripped from the oocytes by a series of passages through an embryo pipette while the oocytes were suspended in PBS. The oocytes were then placed in a 1.7mL conical tube containing RIPA lysis buffer. The samples were homogenized twice using a homogenizing pestle and centrifuged for 10 minutes at 10,000 revolutions per minute at 4°C. The supernatant containing the protein was transferred to a separate 1.7mL conical tube and the pellet was discarded. Protein was isolated just prior to use and never frozen.

To isolate protein from the zona pellucida and ooplasm fractions of oocytes, the zona pellucidae were first stripped from the oocytes by removing them from storage, washing in PBS and placing them in a Petri dish containing a droplet of 100µL PBS. Ultra-sharp splitting blades (Bioniche, Pullman, WA, catalog #ESE020) were used to make an incision in the zona pellucida, allowing the ooplasm to be released and separated from the zona pellucida. The zona pellucida was then washed once more in PBS to free it of extra cells and placed in a 1.7mL tube. Likewise, once the oocytes are lysed, the ooplasm was recovered using an embryo pipette and transferred to a 1.7mL conical tube. Protein was then isolated from zona pellucidae and ooplasm as described above for whole oocytes.

BLAST analysis

The protein sequences of ZP3 and ZP4 were compared among species using the National Library of Medicine BLAST. The protein sequence for each zona protein (Appendix V) was

entered into the tool and queried against all of the public sequence databases (Appendix V). Percent identity for each sequence to other protein sequences in the database is reported. Percent identity represents the extent to which the two protein sequences have the same residues at the same positions in an alignment. The higher the percentage, the more similar the protein sequences are. The percent identity between bovine, porcine, and equine ZP3 and ZP4 are reported (Table 1).

Western blot analysis

The presence of ZP3 and ZP4 in bovine oocytes was determined by Western blot analysis. Protein samples were incubated with 6x sample buffer containing dithiothreitol (DTT; Appendix IV) for 5 minutes at 95°C prior to loading into 12% 1.5mm SDS-page polyacrylamide gels (Appendix III). Electrophoresis was conducted at 30mA for 1 hour at room temperature and protein was transferred at 100V for 1 hour at 4°C to a Protran nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA, Catalog #10402468). Membranes were blocked in 5% blocking buffer (5g non-fat dried milk in 100mL 1X TBST) for 1 hour at room temperature and washed with 1X TBST (three 5 minute washes). Membranes were then incubated in either rabbit anti ZP4 polyclonal antibody or rabbit anti ZP3 polyclonal antibody (Abgent, San Diego, CA, ap12724b and ap17235b respectively) as the primary antibody overnight at 4°C. Primary antibodies were diluted 1:100 in 1% blocking buffer (1g non-fat dried milk in 100mL 1X TBST). The following day, membranes were washed in 1X TBST and incubated with HRP conjugated goat anti rabbit IgG polyclonal secondary antibody (1:1000 in 1% blocking buffer; BioRad, Hercules, CA, #170-6515) for 1 hour at room temperature. Membranes were again washed in 1X TBST, exposed to Super Signal® West Dura Extended Duration Substrate (Thermo Scientific,

Rockford, IL, #34075) and imaged immediately on a Molecular Imager ChemiDoc XRS+ System (Bio-Rad, Hercules, CA). The absence of non-specific binding was confirmed by adding 100μg of ZP3 or ZP4 synthetic blocking peptide (Abgent, San Diego, CA, bp1723b and bp12724b respectively) to each primary antibody dilution prior to incubation. The westerns blots were then carried out as described above.

The immunoreactivity of anti pZP antibodies in the serum of SpayVac vaccinated mares to oocyte and zona protein was determined by modifying the Western blot protocol detailed above. Protein isolated from whole oocytes or zona pellucidae were loaded into a polyacrylamide gel in duplicate, separated by electrophoresis, transferred to a nitrocellulose membrane, and blocked for 1 hour at room temperature in 5% blocking buffer. Membranes were washed with 1X TBST and cut down the middle, such that each half contained one set of the duplicate protein samples, and kept separate for the remainder of the protocol. One half of each membrane was incubated in a 1:50 dilution of serum from a SpayVac vaccinated mare in 1% blocking buffer, while the other half was incubated in a 1:50 dilution of serum from a non-vaccinated mare in 1% blocking buffer as a negative control. Membranes were incubated in these serum dilutions overnight at 4°C. The following day, the membranes were washed in 1X TBST and incubated with HRP conjugated goat polyclonal secondary antibody to horse IgG (1:10,000 in 1% blocking buffer; Abcam, San Francisco, CA, ab102396) for 1 hour at room temperature. Membranes were again washed in 1X TBST, exposed to Super Signal® West Dura Extended Duration Substrate, and imaged immediately on a Molecular Imager ChemiDoc XRS+ System.

As a loading control, after the above procedure was completed, the membrane that was incubated with non-vaccinated serum was reanalyzed using rabbit anti ZP3 polyclonal antibody. Specifically, after imaging, the membrane was re-blocked in 5% blocking buffer for 1 hour at

room temperature and incubated with rabbit anti ZP3 polyclonal antibody overnight at 4°C. The following day, the membrane was incubated with HRP conjugated goat anti rabbit IgG polyclonal antibody, exposed to the signal substrate, and imaged once more.

Oocyte immunohistochemistry

Three oocytes, each in 10μL sterile PBS, were placed on slides individually and allowed to dry at room temperature. Once dry, the oocytes were fixed in 4% paraformaldehyde (PFA) for 30 minutes. PFA was rinsed off with 1X PBS and the oocytes were then blocked in 5% normal goat serum (NGS) in 1% BSA (.1g bovine serum albumin in 10mL 1X PBS) for 2 hours at room temperature in a moist chamber. After washing the blocking solution off with 1X PBS, each oocyte was then incubated overnight in a moist chamber at 4°C with one of the following solutions: a dilution of serum from a SpayVac vaccinated mare, a dilution of serum from a non-vaccinated mare, or 1% BSA as a negative control. The serum dilutions were all 1:50 in 1% BSA. The following day, the oocytes were washed in 1X PBS and incubated with HRP conjugated goat anti horse IgG polyclonal secondary antibody (1:1000 in 1% BSA) for 1 hour at room temperature in a moist chamber. The secondary antibody was washed off with 1X PBS. Immpact DAB Peroxidase Substrate Solution (Vector Laboratories, Burlingame, CA, Catalog #SK-4105) was applied to each oocyte for 10 minutes, rinsed off with water to stop the reaction, and allowed to dry. Once dry, slides were mounted with coverslips using cytoseal (Thermo Scientific, Catalog #8310-4) and imaged with light microscopy.

Results

BLAST analysis of ZP3 and ZP4 protein sequences

The protein sequences for bovine ZP3 and ZP4 were compared to those of the horse using BLAST analysis. BLAST finds regions of local similarity between protein sequences and reports the percent identity between matches (Table 1). According to these results, bovine and equine ZP3 and ZP4 are ≥ to 75% homologous for all sequences. Likewise, comparing equine and bovine sequences to those of the pig resulted in at least 75% homology among the species (Table 1).

Table 1: BLAST analysis of ZP3 and ZP4 protein sequences

	Accession	Amino Acids	Sus scrofa ZP3	Bos taurus ZP3	Equus caballus ZP3	Sus scrofa ZP4	Bos taurus ZP4	Equus caballus ZP4
Sus scrofa ZP3	NP_999058	421	-	84%	77%			
Bos taurus ZP3	AAI46036	421	84%	-	75%			
Equus caballus ZP3	XP_001493094	426	77%	75%	-			
Sus scrofa ZP4	Q07287	536				-	76%	75%
Bos taurus ZP4	XP_005226261	534				76%	-	76%
Equus caballus ZP4	XP_001490803	538				75%	76%	-

Presence of ZP3 and ZP4 in bovine oocyte and zona protein

Western blot analysis confirmed the expression of ZP3 and ZP4 proteins in protein isolated from whole bovine oocytes (Figure 1). Bovine ZP3 has a predicted weight of 46kDa and bovine ZP4 is predicted at 59kDa. The Western blots for these respective proteins show protein bands at the correct molecular weights according to a molecular weight standard loaded into the left-most lanes of each gel (Figure 1). Western blots were repeated twice for each antibody with newly isolated protein samples to validate consistency.

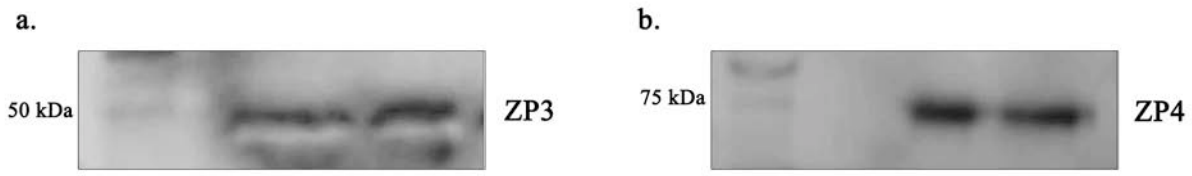

Figure 1: Western blot analysis of bovine oocytes for ZP3 and ZP4

Western blot analysis of protein isolated from whole bovine oocytes (7.5 oocytes per lane) using (**a**) rabbit anti ZP3 polyclonal antibody or (**b**) rabbit anti ZP4 polyclonal antibody. Molecular weight standards appear in the left-most lanes of each gel.

Protein isolated from whole bovine oocytes, bovine oocyte ooplasm, and bovine zona pellucidae was assessed individually to determine whether all three fractions express ZP3. Protein was loaded into a polyacrylamide gel in both 1 and 5 oocyte-per-lane quantities. Western blot analysis revealed the presence of ZP3 in all three fractions and verified that protein isolated from 1 zona pellucida is sufficient to run the assay (Figure 2).

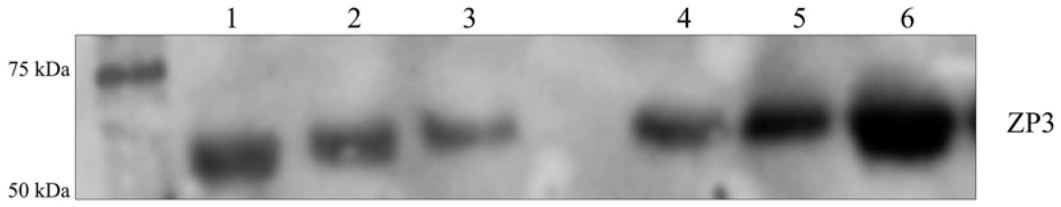

Figure 2: Western blot analysis for ZP3 of protein isolated from whole bovine oocytes, bovine oocyte ooplasm and bovine zona pellucidae

Western blot analysis of protein isolated from whole bovine oocytes (lanes 1 and 4), bovine oocyte ooplasm (lanes 2 and 5), and bovine zona pellucida (lanes 3 and 6) using rabbit anti ZP3 polyclonal antibody. Lanes 1-3 contain protein isolated from 1 oocyte per lane. Lanes 4-6 contain protein isolated from 5 oocytes per lane. Molecular weight standard appears in the left-most lane.

Immunoreactivity of serum from SpayVac vaccinated mares to bovine oocyte protein

A modified Western blot approach was used to assess the immunoreactivity of serum from SpayVac vaccinated mares to bovine oocyte protein. Antibodies in the serum from a SpayVac vaccinated mare bound bovine oocyte protein (Figure 3b). Antibodies in the serum from a non-vaccinated mare did not bind the protein (Figure 3a), confirming that the immunoreactivity of serum from a vaccinated mare to oocyte protein is a result of antibodies produced in response to SpayVac vaccination. The protein bands appeared at the correct molecular weight range for ZP3. This experiment was repeated four times to verify consistency. Each time the experiment was repeated, serum from different mares and newly isolated protein samples were used.

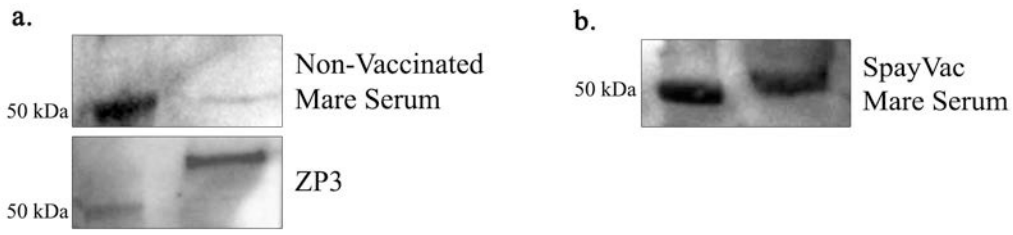

Figure 3: Western blot analysis of protein isolated from whole bovine oocytes using serum from a SpayVac vaccinated mare

Western blot analysis of protein isolated from whole bovine oocytes (2 per lane) using (**a**) serum from a non-vaccinated mare and rabbit anti ZP3 polyclonal antibody as a loading control, or (**b**) serum from a SpayVac vaccinated mare. Molecular weight standards appear in the left-most lanes of each gel.

Localization of anti pZP antibody binding to the zona pellucida of bovine oocytes

Whole bovine oocytes were mounted on slides to locate the binding of anti pZP antibodies. Immunohistochemical staining using serum from a SpayVac vaccinated mare

revealed localization of anti pZP antibodies to the zona pellucida of the bovine oocyte (Figure 4a). Immunohistochemical staining of oocytes using serum from a non-vaccinated mare was comparable to that of the negative control (Figures 4b and 4c). IHC experiments were repeated three times with different serum samples and oocytes.

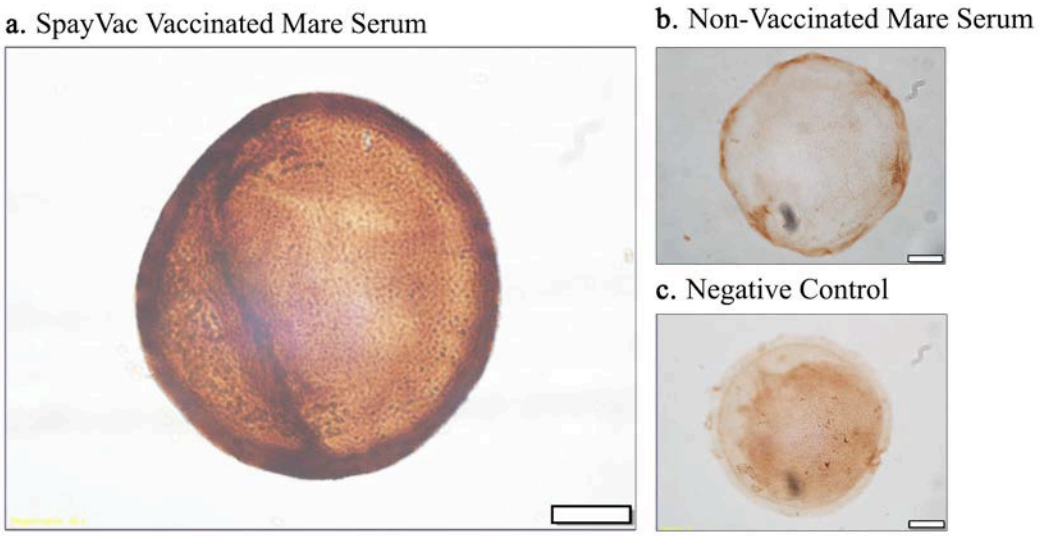

Figure 4: Localization of anti pZP antibodies to the zona pellucida of the bovine oocyte

Immunhistochemical staining of whole bovine oocytes using (**a**) serum from a SpayVac vaccinated mare diluted 1:50 in 1% BSA, (b) serum from a non-vaccinated mare diluted 1:50 in 1% BSA, or (c) 1% BSA as a negative control. (bars = 20μm)

Discussion of Results

These experiments were performed to develop an assay to investigate the immunoreactivity of serum from a SpayVac vaccinated mare to zona protein. Bovine and equine ZP3 and ZP4 are ≥ 75% homologous for all sequences according to BLAST analysis (Table 1). Likewise, comparing equine and bovine ZP3 and ZP4 sequences to those of the pig resulted in at least 75% homology among species (Table 1). Western blot analysis further confirmed the

presence of ZP3 and ZP4 in bovine oocytes (Figures 1 and 2). Importantly, these experiments determined that the protein content in the zona pellucida from one oocyte is sufficient to run the assay.

While all four zona proteins are of interest, some studies argue whether the pig has ZP1 and recombinant antibodies to ZP2 are not commercially available. ZP3 was chosen for use in this experiment due to its critical role in sperm binding and fertilization, along with its assumed participation in the mechanism of action of SpayVac. ZP4 was also of interest due to its role as a structural protein. Western blot and BLAST results therefore confirm the adequacy of using bovine oocytes to develop the Western blot approach.

We then investigated the immunoreactivity of antibodies in serum from SpayVac vaccinated mares to zona protein using a modified Western blot approach. Western blot analysis of bovine oocyte protein using serum dilutions from SpayVac vaccinated and non-vaccinated mares revealed that antibodies in the serum from SpayVac vaccinated mares are immunoreactive to bovine oocyte protein samples (Figure 3). Serum from non-vaccinated mares did not have antibodies immunoreactive to the protein. This confirms that the antibodies capable of binding zona protein present in the serum of vaccinated mares are not endogenously present in the serum of non-vaccinated mares. These antibodies are likely horse polyclonal antibodies to porcine zona pellucida, or horse anti pZP antibodies. As the name implies, these antibodies are raised in the horse in response to vaccination with porcine zona protein. Likewise, these anti pZP antibodies in the serum of SpayVac vaccinated mares recognized protein at the correct molecular weight range for ZP3, suggesting that antibodies in the serum of vaccinated mares are capable of binding the zona pellucida of mature bovine oocytes, perhaps primarily at ZP3 binding sites.

Immunohistochemistry (IHC) techniques were also adapted to use serum as the primary antibody. This modification demonstrates that anti pZP antibodies in the serum of SpayVac vaccinated mares bind to the zona pellucida of mature bovine oocytes (Figure 6). While high background staining was observed from immunohistochemical labeling of bovine oocytes using serum from a non-vaccinated mare, the background staining was comparable to that of the negative control.

These experiments led to the development of a repeatable assay that can be used to investigate the immunoreactivity of serum to a protein of interest. In the process, Western blot and immunohistochemical analysis also confirmed the presence and specificity of antibodies in the serum of SpayVac vaccinated mares for bovine oocyte protein. Since antibodies in the serum of non-vaccinated mares fail to recognize and bind the protein samples, anti pZP antibodies raised in the vaccinated horse against the SpayVac vaccine are most likely responsible for the binding of protein observed.

CHAPTER IV: IMMUNOREACTIVITY OF ANTI PZP ANTIBODIES FROM THE SERUM OF SPAYVAC VACCINATED MARES TO EQUINE ZONA PROTEIN

Summary

We investigated the immunoreactivity of serum from SpayVac vaccinated mares to equine zona protein using a modified Western blot approach. Western blot analysis for anti-porcine zona pellucida (anti pZP) antibodies from SpayVac vaccinated mares was first conducted using protein isolated from whole equine oocytes and equine zona pellucidae, as the zona pellucida is of primary interest in investigating the mechanism of action of the vaccine. Previous studies have reported follicular dysgenesis following vaccination with the pZP antigen (Skinner et al. 1984, Kirkpatrick et al. 1995), suggesting a mechanism of action beyond the scope of simply binding to the zona pellucida of ovulated oocytes. We therefore conducted Western blot analysis of equine ovarian and follicular tissues as well. Immunohistochemical analysis of consecutive slices of equine ovarian and follicular tissues, along with whole equine oocytes, was used to determine the site of binding of anti pZP antibodies to these tissues. To verify the specificity of anti pZP antibodies for zona protein, ZP3 and ZP4 blocking peptides were used to pre-saturate the serum from a SpayVac vaccinated mare prior to the serum being used as the primary antibody for immunohistochemical analysis. Likewise, Western blot membranes were pre-incubated with polyclonal antibodies to ZP3 and ZP4 before incubation with serum from a SpayVac mare. Finally, serum samples from mares with known low- and high- titer levels were analyzed side by side in a Western blot analysis to test their relative immunoreactivity to the same equine zona protein samples. Results indicate immunoreactivity of anti pZP antibodies

from the serum of SpayVac vaccinated mares, with both high- and low- antibody titers, to isolated equine zona protein.

Materials and Methods

Samples

We obtained equine ovaries, renal tissue, and oocytes from necropsy samples previously collected and stored at the Animal Reproduction Biotechnology Laboratory (ARBL, 3107 Rampart Road, Fort Collins, CO 80523) at Colorado State University as part of ongoing research. Follicular fluid was aspirated from large follicles visible on the ovaries and oocytes were recovered as described for the bovine model. The remainder of the follicles were lysed and washed with phosphate buffered saline (PBS). The washing fluid was then searched using a dissecting microscope for additional oocytes released from smaller follicles. Oocytes were stored in PBS at 4°C. Tissue was excised from the ovarian stroma and follicular walls of equine ovaries. Tissues were fixed in 4% paraformaldehyde (PFA) overnight. The following day, the fixative was replaced with 70% ethanol for storage and samples were sent to the Colorado State Veterinary Diagnostics Laboratory (Fort Collins, CO) to be embedded in paraffin blocks. The rest of the tissues were frozen and stored at -80°C until thawed for protein isolation.

Serum from vaccinated mares of known titer levels was obtained from stored samples previously collected as part of a study on SpayVac immunocontraception (Roelle, USGS, Pauls Valley, OK). Serum samples from eight mares of high titer levels and one mare of low titer level were chosen and used throughout the study (Appendix I). Serum from two non-vaccinated mares was obtained from samples previously collected and stored as part of ongoing research at the ARBL. All serum samples were stored at -80°C until needed. Non-vaccinated mares were of known reproductive history and proven fertility.

Protein isolation and quantification

Protein was isolated from equine oocytes and zona pellucidae using radioimmunoprecipitation assay (RIPA) lysis buffer (Appendix II) as described for the bovine model. Protein from ovarian, follicular, and renal tissue was also isolated utilizing RIPA lysis buffer. Tissue samples were homogenized in RIPA lysis buffer, sonicated on ice to disrupt cellular membranes, and centrifuged at 10,000 revolutions per minute at 4°C for 10 minutes. The resulting supernatant containing the protein was separated into a 1.7mL conical tube and used immediately. Protein content was quantified following the manufacturer's protocol for the Pierce BCA Protein Assay Kit (Thermo Scientific, catalog #23227). Standards containing 2.0mg/mL bovine serum albumin and working reagents were prepared. A microplate was loaded with 12.5µL of each standard or unknown sample and 100µL of working reagent per well. The microplate was then incubated for 30 minutes at 37°C. Protein content was quantified using spectrophotometry with the Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT). Unknown sample concentrations were determined by comparison to the standard curve of the known standards.

Western blot analysis

The immunoreactivity of anti pZP antibodies in the serum of SpayVac vaccinated mares to protein isolated from whole equine oocytes, equine zona pellucidae, ovarian tissue, and follicular tissue was determined using the Western blot techniques developed in the bovine model. Protein was isolated from these tissues and incubated with 6x sample buffer (Appendix IV) containing dithiothreitol (DTT) for 5 minutes at 95°C prior to loading into 12.0% 1.5mm

SDS-page polyacrylamide gels in duplicate (Appendix III). Electrophoresis was conducted for 1 hour at 30mA and protein was transferred to a Protran nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA, Catalog #10402468) for 1 hour at 100V at 4°C. Membranes were blocked in 5% blocking buffer (5g dried milk in 100mL 1X TBST) for 1 hour at room temperature and washed with 1X TBST (three, five minute washes). Membranes were then cut down the middle, such that each half contained one set of the duplicate protein samples, and kept separate for the remainder of the protocol. One half of each membrane was incubated in a 1:50 dilution of serum from a SpayVac vaccinated mare in 1% blocking buffer (1g dried milk in 100mL 1X TBST), while the other half was incubated in a 1:50 dilution of serum from a non-vaccinated mare in 1% blocking buffer. Membranes were incubated overnight at 4°C. The following day, the membranes were washed in 1X TBST and incubated with horseradish peroxidase (HRP) conjugated goat polyclonal secondary antibody to horse IgG (1:10,000 in 1% blocking buffer; Abcam, San Francisco, CA, ab102396) for 1 hour at room temperature. Membranes were washed in 1X TBST, exposed to Super Signal® West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, #34075) and imaged immediately on a Molecular Imager ChemiDoc XRS+ System (Bio-Rad, Hercules, CA).

Mouse anti beta actin monoclonal antibody (1:100 in 1% BSA; Abcam, San Francisco, CA, ab8226) was used for protein isolated from follicular and ovarian tissues, and rabbit anti ZP3 polyclonal antibody (1:100 in 1% blocking buffer; Abgent, San Diego, CA, ap17235) was used for protein isolated from oocytes and zona pellucidae, as loading controls. After initial imaging, the membranes were blocked in 5% BSA (.5g bovine serum albumin in 10mL 1X TBST) or 5% blocking buffer, respectively, for 1 hour at room temperature. Membranes were then incubated with their respective antibodies overnight at 4°C. The following day, the

membranes were washed in 1X TBST and incubated with either HRP conjugated donkey anti mouse IgG polyclonal antibody (1:1000 in 1% BSA; Abcam, San Francisco, CA, ab 97030) or HRP conjugated goat anti rabbit IgG polyclonal antibody (1:1000 in 1% blocking buffer; BioRad, Hercules, CA, 170-6515), depending on the primary antibody used, for one hour at room temperature. The membranes were finally exposed to substrate and imaged.

Binding specificity was determined by pre-incubation of the Western blot membranes with either rabbit anti ZP3 polyclonal antibody or rabbit anti ZP4 polyclonal antibody (Abgent, San Deigo, CA, ap17235b and ap12724b respectively). After protein loading, separation, transfer to membrane, and blocking, membranes were cut in half. One half of each membrane was pre-incubated in rabbit anti ZP3 polyclonal antibody (1:100 in 1% blocking buffer) while the other half was pre-incubated in rabbit anti ZP4 polyclonal antibody (1:100 in 1% blocking buffer) overnight at 4°C. The following day, the membranes were washed in 1X TBST and incubated with serum from a SpayVac vaccinated mare (1:50 in 1% blocking buffer) overnight at 4°C. On the third day, the membranes were washed in 1X TBST, incubated with HRP conjugated goat anti horse IgG polyclonal secondary antibody (1:10,000 in 1% blocking buffer), exposed to Super Signal West Dura Extended Duration Substrate, and imaged. As a loading control, after imaging the membranes were blocked in 5% BSA and incubated with mouse monoclonal antibody to beta actin (1:1000 in 1% BSA in 1X TBST) overnight at 4°C. On the fourth day, the membranes were washed in 1X TBST and incubated in HRP conjugated donkey anti mouse IgG polyclonal antibody for 1 hour at room temperature. The membranes were then exposed to developing substrate and imaged one more time.

Relative immunoreactivity of high- and low- titer serum samples was investigated using the same Western blot procedures detailed above. The difference being that one half of each

membrane was incubated in the serum from a SpayVac vaccinated mare with high antibody titer, while the other half was incubated in a dilution of serum from a SpayVac vaccinated mare with low antibody titer overnight at 4°C as the primary antibody (Appendix I). All serum dilutions were 1:50 in 1% blocking buffer. HRP conjugated goat anti horse IgG polyclonal antibody (1:10,000 in 1% blocking buffer) was used as the secondary antibody. Anti ZP3 and anti beta actin polyclonal antibodies were used as loading controls for protein isolated from oocytes and tissues, respectively.

Western blot quantification

Densitometry was performed using a Molecular Imager ChemiDoc XRS+ System with ImageLab™ software (Bio Rad, Hercules CA). Bands were detected by the software and values of integrated optical density were exported to an Excel spreadsheet. Each protein band of interest was normalized to total protein content in the lane using its respective loading control. Graphs were then produced by Excel to compare the normalized densities of protein bands between high- and low- titer serum samples. The y-axis of each graph is read as a ratio of protein of interest to the loading control.

Immunohistochemistry and Hematoxylin and Eosin (H&E) staining of tissues

Four consecutive 5μm sections of tissue were placed on two glass slides, such that each slide had two slices of tissue, and incubated overnight at 37°C. The following day, the slides were incubated at 55°C for 30 minutes. Citrisolve (Thermo Scientific, Catalog# 22-143975) along with an alcohol gradient was then used to dewax and rehydrate the tissue slices.

Following rehydration, one of the two slides was subjected to a H&E staining protocol. The slide was incubated in hematoxylin for 1 minute, rinsed under running tap water for 5 minutes, incubated in eosin for 5 minutes, rinsed once more under running tap water, and finally dehydrated through an alcohol and citrisolve gradient. The slide was then left to air dry at room temperature prior to being mounted with a coverslip using cytoseal and imaged with light microscopy.

Meanwhile, the other slide was boiled in 10mM sodium citrate (pH 6.0) for 20 minutes for antigen retrieval. Afterward, the slide was washed in 1X PBS for 10 minutes and blocked in 5% natural goat serum (NGS) in 1% BSA (0.1g bovine serum albumin in 10mL 1X PBS) at room temperature for 2 hours in a wet chamber. After blocking, the slide was drained and blotted dry. A dilution of serum from a SpayVac vaccinated mare was applied to the first tissue slice while a dilution of serum from a non-vaccinated mare was applied to the second tissue slice on the slide. The slide was then incubated overnight in a wet chamber at 4°C. Serum dilutions were all 1:50 in 1% BSA. The following day, the slide was washed in 1X PBS and incubated at room temperature in a wet chamber for 1 hour with HRP conjugated goat polyclonal secondary antibody to horse IgG (1:1000 in 1% BSA, Abcam, ab102396). The slide was washed again in 1X PBS and Immpact DAB Peroxidase Substrate Solution (Vector Laboratories, Burlingame, CA, Catalog #SK-4108) was applied for 10 minutes. To stop the reaction, the slide was rinsed in running water for 5 minutes, taken back through the alcohol gradient for dehydration, and allowed to dry. The slide was finally mounted with cytoseal (Thermo Scientific, Rockford, IL, Catalog #8310-4) and imaged with light microscopy.

Binding specificity of anti pZP antibodies to zona protein was determined by pre-saturating a sample of serum from a SpayVac vaccinated mare with ZP3 and ZP4 synthetic

blocking peptides (Abgent, San Diego, CA, bp1723b and bp12724b respectively) prior to use as the primary antibody for immunohistochemical analysis. 30µg of each blocking peptide were added to a dilution of serum from a SpayVac vaccinated mare (10µL serum in 500µL 1% BSA). The immunohistochemistry protocol was then followed out as described above. HRP conjugated goat anti horse IgG polyclonal antibody was used as the secondary antibody (1:1000 in 1% BSA).

Oocyte immunohistochemistry

Immunohistochemical staining of the equine oocyte was performed as described for the bovine oocytes. A whole equine oocyte was mounted on a slide, fixed in 4% paraformaldehyde, and blocked in 7.5% NGS in 1% BSA. The oocyte was then incubated in a 1:50 dilution of serum from a vaccinated mare in 1% BSA overnight at 4°C in a wet chamber. The following day, the oocyte was incubated in HRP conjugated goat anti horse IgG polyclonal antibody(1:1000 in 1% BSA), developed using Immpact DAB Peroxidase Substrate, mounted with a coverslip, and imaged using light microscopy.

Results

Immunoreactivity of serum from SpayVac vaccinated mares to equine zona protein

Modified Western blot techniques were used to assess the immunoreactivity of serum from SpayVac vaccinated mares to protein isolated from whole equine oocytes and equine zona pellucidae. The binding of antibodies in the serum from a SpayVac vaccinated mare to equine zona protein was demonstrated (Figure 5b). Serum from a non-vaccinated mare did not have antibodies capable of binding the protein, confirming that the immunoreactivity of serum from a

vaccinated mare is a result of antibodies produced in response to SpayVac vaccination (Figure 5). This experiment was repeated twice with different serum and protein samples.

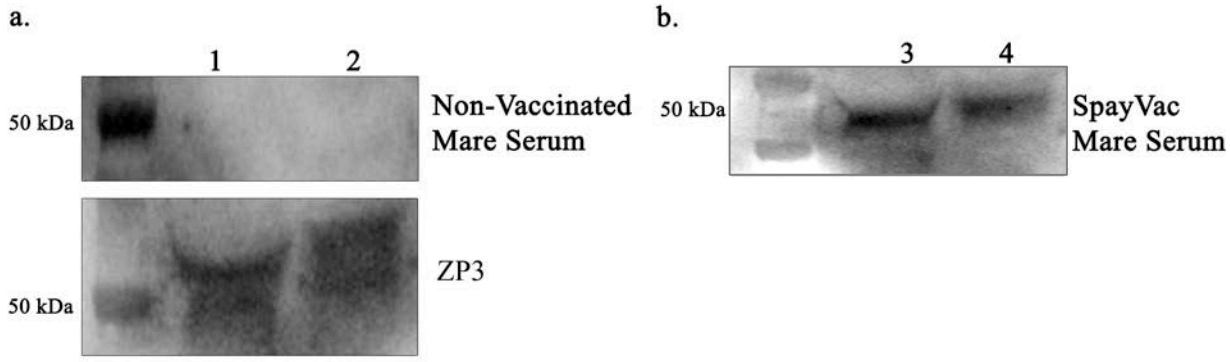

Figure 5: Western blot analysis of protein isolated from equine oocytes and zona pellucidae using serum from a SpayVac vaccinated mare

Western blot analysis of protein isolated from equine zona pellucidae (2 per lane; lanes 1 and 4) and whole equine oocytes (2 per lane; lanes 2 and 3) using (**a**) serum from a non-vaccinated mare and anti ZP3 polyclonal antibody as a loading control, or (**b**) serum from a SpayVac vaccinated mare. Molecular weight standards appear in the left-most lanes of each gel.

Immunoreactivity of serum from SpayVac vaccinated mares to equine ovarian and follicular tissues

Western blot analysis revealed the immunoreactivity of serum from a SpayVac vaccinated mare to protein isolated from equine ovarian and follicular tissues. Anti pZP antibodies in the serum of a SpayVac vaccinated mare bound the protein samples (Figure 6b) while antibodies in the serum from a non-vaccinated mare did not bind the protein (Figure 6a). Renal tissue was used as a negative control in these experiments. Western blot analysis of equine tissues using serum from SpayVac and non-vaccinated mares was performed four times to verify repeatability.

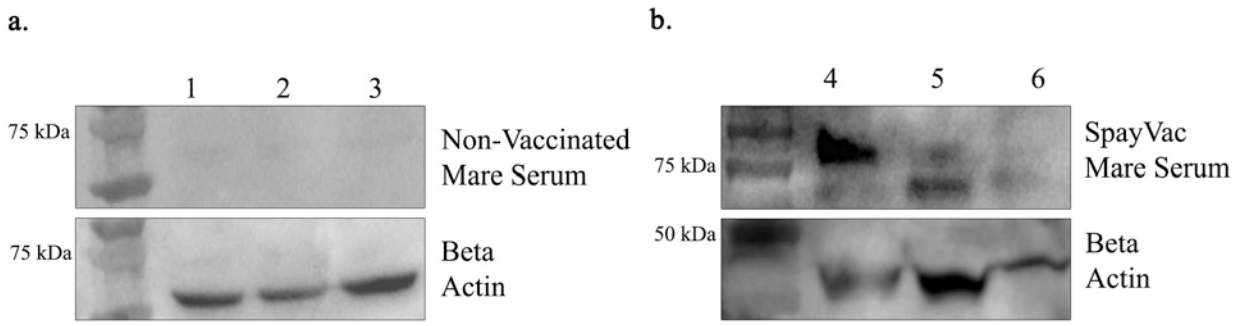

Figure 6: Western blot analysis of protein isolated from equine ovarian and follicular tissues using serum from a SpayVac vaccinated mare

Western blot analysis of protein isolated from equine ovarian tissue (lanes 1 and 4), equine follicular tissue (lanes 2 and 5), and equine renal tissue (lanes 3 and 6) using (**a**) serum from a non-vaccinated mare and (**b**) serum from a SpayVac vaccinated mare. 40µg of each protein were loaded per lane. Molecular weight standards appear in the left-most lane of each gel. Western blot analysis for beta actin of each membrane was performed as a loading control.

Localization of anti pZP antibodies from the serum of SpayVac vaccinated mares to the zona pellucida of mature and immature equine oocytes

Immunohistochemical staining of equine ovarian tissue using serum from a SpayVac vaccinated mare located the binding of anti pZP antibodies to the zona pellucidae of immature oocytes of primary follicles embedded in the tissue (Figure 7e). Immunohistochemical analysis did not locate the binding of anti pZP antibodies to oocytes of primordial follicles, where a zona pellucida is not yet formed (Figure 7b). Antibodies in the serum from a non-vaccinated mare did not bind the oocytes; implying antibodies present in the serum of vaccinated mares are a result of SpayVac vaccination (Figures 7c and 7f). H&E staining enhances visualization of structures (Figures 7a and 7d). Similarly, immunohistochemical staining of a whole equine oocyte using serum from a SpayVac vaccinated mare located the binding of anti pZP antibodies to the zona pellucida (Figure 8).

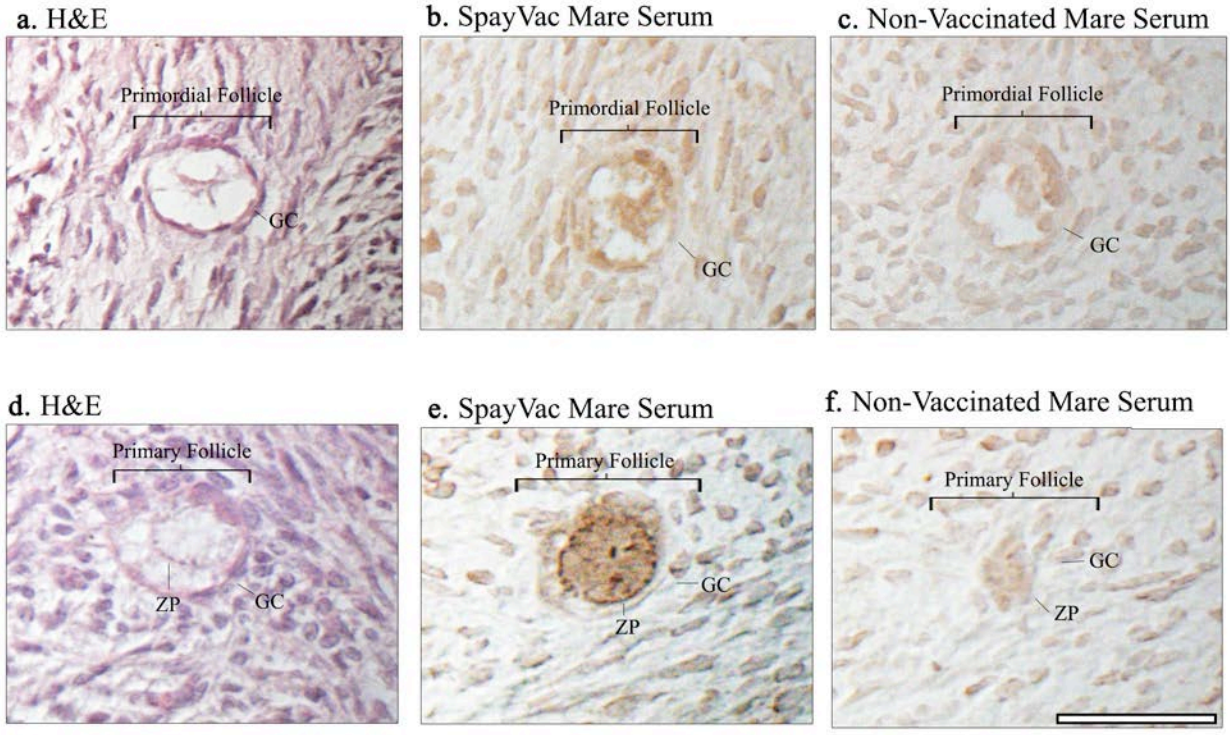

Figure 7: Anti pZP antibody binding to the zona pellucida of oocytes in primary follicles embedded in ovarian tissue

Immunohistochemical staining of equine ovarian tissue using (**b** and **e**) serum from a SpayVac vaccinated mare and (**c** and **f**) serum from a non-vaccinated mare. Anti pZP antibodies bind the zona pellucida of oocytes in primary follicles (**e**) but did not bind primordial follicles where the zona pellucida has yet to be formed (**b**). Immunohistochemical staining using serum from non-vaccinated mares (**c** and **f**) is not observed. (ZP: zona pellucida; GC: granulosa cells; bar = 20μm)

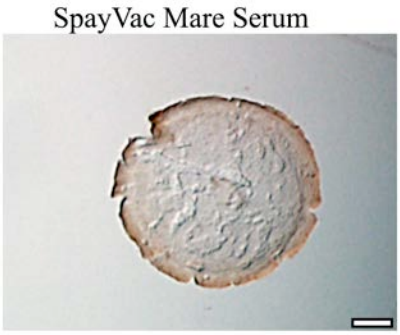

Figure 8: Anti pZP antibody binding to the zona pellucida of the equine oocyte

Immunohistochemical staining using serum from a SpayVac vaccinated mare reveals localization of anti pZP antibodies to the zona pellucida of the mature, pre-ovulatory equine oocyte. (bar = 20μm)

Specificity of anti pZP antibodies in the serum of SpayVac vaccinated mares for equine zona protein

Pre-incubation of the Western blot membrane with rabbit anti ZP3 polyclonal antibody prior to incubation with serum from a SpayVac vaccinated mare completely blocked the ability of anti pZP antibodies to bind to the protein sample. Anti pZP antibodies maintained their ability to bind the protein sample following pre-incubation with rabbit anti ZP4 polyclonal antibody. This indicates that anti pZP antibodies in the serum of a SpayVac vaccinated mare may be more specific for ZP3 than ZP4 (Figure 9).

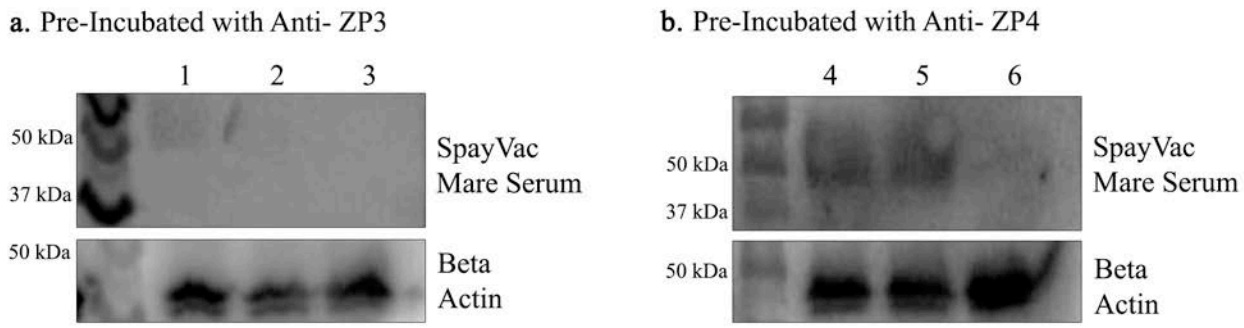

Figure 9: Western blot analysis of equine ovarian and follicular tissues using serum from a SpayVac vaccinated mare following pre-incubation with anti ZP3 and anti ZP4 polyclonal antibodies

Western blot analysis of protein isolated from equine ovarian (lanes 1 and 4), equine follicular (lanes 2 and 5), and equine renal (lanes 3 and 6) tissues using serum from a SpayVac vaccinated mare following pre-incubation with (**a**) rabbit anti ZP3 polyclonal antibody and (**b**) rabbit anti ZP4 polyclonal antibody. Western blot analysis using anti-beta actin antibody was performed as a loading control. Renal tissue was used as a negative control. Molecular weight standards appear in the left-most lanes of each gel. 40μg of protein were loaded per lane.

Similarly, immunohistochemical techniques were modified to further investigate the specificity of anti pZP antibodies to zona protein. Pre-incubation of serum from a SpayVac vaccinated mare with ZP3 and ZP4 blocking peptides prior to immunohistochemical staining of equine ovarian tissue using the serum inhibited the binding of anti pZP antibodies to the oocyte (Figure 10b). When the serum from the same SpayVac vaccinated mare was used in the absence of blocking peptides for immunohistochemical analysis, anti pZP antibodies in the serum localized to the zona pellucida of an oocyte embedded in the tissue (Figure 10c). H&E staining of a consecutive tissue slice enhances visualization of the cellular structures. Collectively, these two experiments confirm the specificity of anti pZP antibodies for equine zona protein.

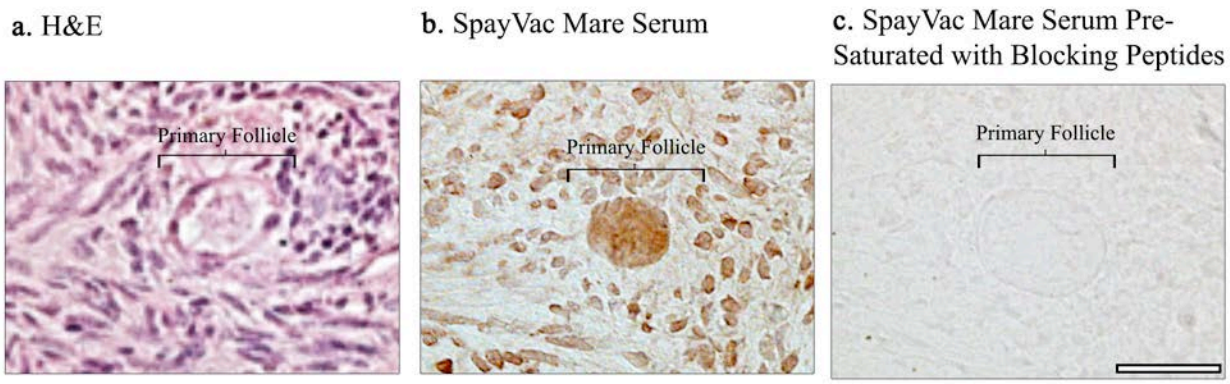

Figure 10: Immunohistochemical analysis of equine ovarian tissue using serum from a SpayVac vaccinated mare following pre-saturation with ZP3 and ZP4 blocking peptides

Immunohistochemical staining of equine ovarian tissue using (**b**) serum from a SpayVac vaccinated mare demonstrates localization of anti pZP antibodies to the zona pellucida of an oocyte in a primary follicle embedded in the tissue. Pre-saturation of serum from the same mare with ZP3 and ZP4 blocking peptides prior to immunohistochemical staining of equine ovarian tissue blocked the ability of anti pZP antibodies to bind the oocyte (**c**). (bar = 20μm)

Relative immunoreactivity of anti pZP antibodies from the serum of SpayVac vaccinated mares with high and low antibody titers to equine zona protein

Western blot analysis was used to compare the immunoreactivity of anti pZP antibodies in the serum of SpayVac vaccinated mares with high and low antibody titer levels to equine zona protein. The first serum sample had a titer level of 178.33 while the latter was 39.66, representing a 4x difference in titer levels between the samples. The binding of anti pZP antibodies from the serum a SpayVac vaccinated mare with high antibody titers to protein isolated from equine oocytes (Figure 11a) and equine ovarian and follicular tissues (Figure 11c) was verified by Western blot analysis. Likewise, the binding of anti pZP antibodies from the serum of a SpayVac vaccinated mare with low antibody titers to the same protein samples was demonstrated (Figures 11b and 11d). Antibodies to ZP3 and beta-actin were used as loading controls and as normalization factors in the quantitative analysis of relative protein expression. A Molecular Imager ChemiDoc XRS+ System was used to perform densitometry to detect the

relative immunoreactivity between high and low titer serum samples. Integrated optical density was detected and recorded for each protein band, and then normalized against the respective loading control for each lane. Finally, the ratio of protein to loading control was expressed as a function of high and low titer serum samples (Figures 11e and 11f). While it appears that anti pZP antibodies in the serum of SpayVac vaccinated mares bind the protein samples without regard to titer level, this experiment was merely a demonstration of concept and no statistical evidence can be offered.

Discussion of Results

Western blot analysis of equine oocyte and zona protein using serum from a SpayVac vaccinated mare revealed immunoreactivity of antibodies in the serum to the protein samples (Figure 5). Antibodies in the serum from a non-vaccinated mare did not bind the protein samples. This indicates that the antibodies immunoreactive to the protein samples found in serum from SpayVac vaccinated mares are a result of SpayVac vaccination, and are not endogenously present in the non-vaccinated mare. These antibodies are likely horse polyclonal antibodies to porcine zona pellucida (anti pZP). As the name implies, they are raised in the vaccinated horse against porcine zona protein. Western blot analyses confirmed these antibodies to be immunoreactive to equine ZP3. Immunohistochemical analysis located the binding of anti pZP antibodies to the zona pellucida of a mature, pre-ovulatory oocyte aspirated from a Graafian follicle (Figure 8).

Since previous studies have suggested a mechanism of action beyond anti pZP antibodies only binding mature oocytes (Skinner et al. 1984, Kirkpatrick et al. 1995), anti pZP reactivity to equine ovarian and follicular tissues was also tested. Western blot analysis revealed the binding

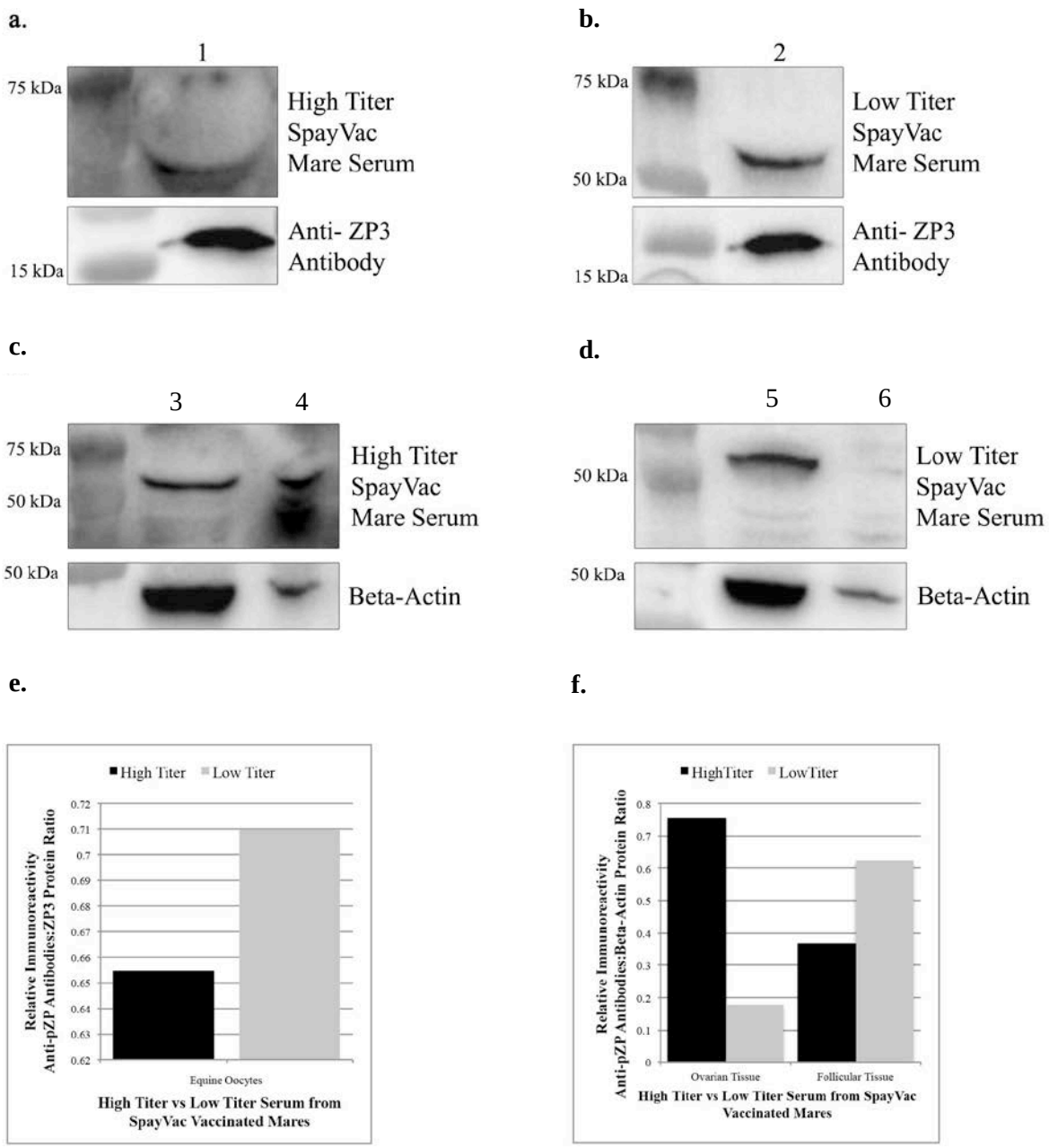

Figure 11: Immunoreactivity of anti pZP antibodies from the serum of SpayVac vaccinated mares with high and low antibody titers

Western blot analysis using (**a.** and **c.**) serum from a SpayVac vaccinated mare with high antibody titers or (**b.** and **d.**) serum from a SpayVac vaccinated mare with low antibody titers. Lanes 1 and 2 each contain protein isolated from 2 equine oocytes, lanes 3 and 5 each contain 40µg of protein isolated from equine follicular tissue, and lanes 4 and 6 each contain 40µg of protein isolated from equine ovarian tissue. Anti ZP3 antibody (**a.** and **b.**) and anti beta-actin antibody (**c.** and **d.**) were used as loading controls. Molecular

weight standards appear in the left-most lane of each gel. Densitometry was performed using a Molecular Imager ChemiDoc XRS+ System, with values expressed as integrated optical density. Immunoreactivity values are normalized to total protein content using ZP3 (**e.**) or beta-actin (**f.**). Relative immunoreactivity of anti pZP antibodies from high and low titer serum to protein isolated from equine oocytes (**e**.) and equine ovarian and follicular tissues (**f**.) is illustrated.

of anti pZP antibodies to protein isolated from these tissues (Figure 6). Immunohistochemical analysis of equine ovarian tissue localized the binding of anti pZP antibodies to the zona pellucidae of immature oocytes of primary follicles embedded in the tissue (Figure 7). Interestingly, immunohistochemical analysis of the equine follicular wall could not replicate the immunoreactivity described in the Western blot analysis to follicular tissue. The synthesis of the zona pellucida is thought to be an integrated process where both the oocyte and surrounding granulosa cells contribute to the overall creation of the structure (Sinowatz et al. 2001, Blackmore et al. 2004). It is likely that the granulosa cells express zona protein precursors that are recognized by anti pZP antibodies during Western blot analysis. While Western blot analysis may have detected these zona protein precursors in the granulosa cells of follicular tissue, immunohistochemical analysis did not confirm this. When protein is isolated for Western blot analysis, tissue is homogenized, sonicated and subjected to a cell lysis buffer. This disrupts cellular membranes and releases intracellular proteins, which are used in the analysis. Alternatively, preparation of tissue for immunohistochemical analysis does not involve these protein isolation methods. Similar to Western blot results, antibodies in the serum from non-vaccinated mares were unable to bind the tissues.

Anti pZP antibodies from the serum of SpayVac vaccinated mares were found to be specific for zona protein 3. Pre-saturating a serum sample from a SpayVac vaccinated mare with ZP3 and ZP4 blocking peptides successfully blocked the ability of anti pZP antibodies to bind

the tissues during immunohistochemical analysis (Figure 10). Similarly, pre-incubating the Western blot membrane with anti ZP3 antibodies prior to incubation with serum from a SpayVac vaccinated mare inhibited the ability of antibodies in the serum to bind the protein samples (Figure 9). Pre-incubation of the Western blot membrane with anti ZP4 antibodies attenuated the ability of anti pZP antibodies to bind, but did not remove it completely (Figure 9). These experiments are indicative of both a specificity of anti pZP antibodies for equine zona protein 3 as well as a lack of non-specific binding. The antibodies are, in fact, binding zona protein in these tissues and protein samples.

Following SpayVac vaccination in late March 2011, serum samples were taken from treated mares in early May 2011 and analyzed for titer level. Although each mare was vaccinated with the same dose of SpayVac, individual differences in immune system response may result in variable titer levels and efficacy among mares. Accordingly, 2 of the 9 serum samples used in this study were from SpayVac treated mares that were still able to conceive in 2011 despite vaccination. Nonetheless, experiments demonstrated that all 9 of the serum samples contained antibodies produced in response to SpayVac vaccination that are immunoreactive to equine zona protein. To further investigate whether the serum from a mare of high titer level is more immunoreactive to zona protein than serum from a mare with a low titer level, who also conceived despite vaccination, the Western blot protocol developed above was conducted with both serum samples for zona protein side-by-side. While the high titer serum sample had greater than four times the titer level of the low titer serum sample, both serum samples appeared to be immunoreactive to the protein sample (Figure 11). Western blot analysis revealed that both serum samples were able to bind zona protein, with no clear trend of one sample being more

effective than the other. With that said, this is purely a demonstration of concept as sufficient sample size to determine statistical power for the observation was not pursued.

As anticipated, results reveal immunoreactivity of anti pZP antibodies raised in the horse in response to SpayVac vaccination to zona protein. The zona pellucida emerges early on in folliculogenesis, and is abundantly apparent by the primary follicle stage of development. Anti pZP antibodies appear immunoreactive to not only the zona pellucidae of mature, pre-ovulatory oocytes, but also immature oocytes embedded in ovarian tissue. While Western blot analyses revealed immunoreactivity between anti pZP antibodies and protein isolated from equine follicular tissue, immunohistochemical analysis did not demonstrate this immunoreactivity, indicating that this topic should be investigated further. Collectively, results illustrate that anti pZP antibodies are capable of binding equine zona protein of both mature, pre-ovulatory oocytes and oocytes of immature, developing follicles. It is important to note, however, that while these experiments demonstrate an immunoreactivity of anti pZP antibodies to zona protein, it has yet to be demonstrated whether anti pZP antibodies are able to permeate the ovary or available in the follicular fluid following vaccination and thus able to act at these levels in vivo. These experiments merely illustrate a model by which anti pZP antibodies in the serum of SpayVac vaccinated mares may behave under the right conditions.

CHAPTER V: DISCUSSION

Immunocontraception with the porcine zona pellucida (pZP) antigen can be a highly effective means of wild horse contraception. Porcine zona pellucida vaccines have proven to be safe and predictable, with minimal side effects following injection and exceptional ease of administration. (Ransom et al. 2011, NRC 2013) Nonetheless, the contraceptive mechanisms of pZP vaccines remain unclear. This study thus investigated the fundamental question of whether anti pZP antibodies produced in response to SpayVac® vaccination are immunoreactive to equine zona protein. When used as a vaccine in another species, pZP stimulates an immune response that can be measured by antibody titer levels and results in infertility. Infertility is thought to be achieved by the binding of anti pZP antibodies to the zona pellucida proteins on mature oocytes, thereby inhibiting sperm binding and penetration (Sacco 1977, Liu et al. 1989, Kirkpatrick et al. 1990). Other studies have demonstrated follicular dysgenesis following vaccination; including diminished ovulatory ability, absence of mature oocytes and follicles, and a decline in ovarian estrogen secretion (Skinner et al. 1984, Kirkpatrick et al. 1995). Collectively, these studies suggest that anti pZP antibodies act on not only ovulated oocytes in the oviduct, but also on ovarian tissue, follicular tissue, and immature oocytes. However, the central question underlying these mechanisms that remains unclear is whether anti pZP antibodies are capable of recognizing equine zona protein.

Through Western blot and immunohistochemical analysis, this study demonstrated an immunoreactivity of anti pZP antibodies produced in response to SpayVac vaccination to equine zona protein when made artificially available to one another in vitro. A bovine model was used to develop an assay to test the immunoreactivity of antibodies present in the serum of a

vaccinated animal to a protein of interest. Bovine samples were chosen due to the relative ease of which they are obtained in large quantities and the strong homology of bovine zona proteins to equine. Once the assay was consistent and repeatable, equine samples were used in place of the bovine samples. Western blot analysis revealed immunoreactivity of anti pZP antibodies produced in response to SpayVac vaccination to protein isolated from equine oocytes, equine zona pellucidae, equine follicular tissue, and equine ovarian tissue (Figures 5 and 6). Immunohistochemical analysis then verified the location of binding of anti pZP antibodies to the zona pellucidae of both immature oocytes embedded in ovarian tissue and mature, pre-ovulatory oocytes isolated from Graafian follicles (Figures 7 and 8). These results lend insight into not only the mechanism underlying the interference of the sperm-egg interaction detailed by Kirkpatrick et al. (1995), but also the follicular dysgenesis reported in other studies.

The immunoreactivity of anti pZP antibodies to protein isolated from equine ovarian tissues is explained by the localization of antibodies to the zona pellucidae of immature oocytes embedded in the tissue. Likewise, the immunoreactivity to equine follicular tissue demonstrated by Western blot analysis is thought to be due to the presence of zona protein precursors in the granulosa cells of the follicle. However, multiple attempts to analyze follicular tissue by immunohistochemistry failed to demonstrate localization to these cells. The synthesis of the zona pellucida is thought to be an integrated process where both the oocyte and surrounding granulosa cells contribute to the overall creation of the structure (Sinowatz et al. 2001, Blackmore et al. 2004). It is therefore likely that both the granulosa cells and oocyte express, at least in small quantities, zona protein precursors. While Western blot analysis may have detected these zona protein precursors due to its high level of sensitivity (in the femtogram level) and stringent cell lysis protocol to release intracellular proteins, high background staining observed in

immunohistochemical analysis may have masked any localization to these cells. Ramos-Vara et al. (2008) indicates that high background staining is known to occur when the primary antibody used is from the same species as the tissue being analyzed. This is the case with figures 7, 8, and 10 where both the tissue and antibody used are of equine origin. Likewise, when protein is isolated for Western blot analysis, tissue is homogenized, sonicated and subjected to a cell lysis buffer. This disrupts cellular membranes and releases intracellular proteins, which are used in the analysis. Alternatively, preparation of tissue for immunohistochemical analysis does not involve these protein isolation methods. Since zona protein precursors in the granulosa cells are not integral membrane proteins, and therefore not expressed on the cell surface, it is likely that immunohistochemical analysis cannot detect these proteins as they were not exposed.

Although results indicate an immunoreactivity of anti pZP antibodies raised in the mare in response to SpayVac vaccination to equine zona protein, there are many limitations to this study. First, while there are three pZP vaccines currently being reported for use in horses, Zonastat-H®, PZP-22, and SpayVac, this study focused on SpayVac as it is less documented in published literature and emerged as a longer-lasting alternative to the other two vaccines. Although all three vaccines incorporate the same pZP antigen, they are not a homogenous set of compounds. Rather, differences in antigen preparation result in variations in purity and protein content among the vaccines (NRC 2013). Miller et al. (2009) suggests that these differences in antigen preparation may account for the variability in efficacy levels observed among vaccines. In addition to focusing on only one vaccine, serum samples from vaccinated mares were selectively chosen for their high antibody titer levels, with the exception of figure 11. Differences in antibody titer and quality depend on the animal immunized and contribute to variability in efficacy among individuals (Ramos-Vara 2005, NRC 2013). Therefore, not only

are the results of this study specific for anti pZP antibodies produced in response to SpayVac, but they may also not be indicative of antibodies produced in animals of all titer levels. Despite the attempt to compare high- and low- titer serum samples in figure 11, this experiment was simply illustrative of proof of concept and statistical evidence cannot be offered.

Similarly, the use of serum from a SpayVac vaccinated mare as a primary antibody in Western blot and immunohistochemical analysis is analogous to the use of a polyclonal antibody. As detailed by Ramos-Vara (2005), polyclonal antibodies are produced by immunizing an animal with purified antigen. The animal's immune response produces antibodies against the antigen. These antibodies migrate into the blood where serum containing the antibodies can be harvested and used as antisera against the antigen during immunological protocols (Ramos-Vara 2005). In the case of SpayVac vaccination, a horse is immunized with purified pZP antigen, resulting in the production of anti pZP antibodies. Serum harvested from the vaccinated horse contains anti pZP antibodies, and the serum can be used as a primary, polyclonal antibody during Western blot and immunohistochemical analyses. However, since whole serum is being used, polyclonal antisera harvested in this way will include not only antibodies to the antigen of interest, but also irrelevant antibodies present as part of normal immune function. For this reason, polyclonal antibodies are known to have higher affinity and wider reactivity, yet lower specificity when compared with monoclonal antibodies (Ramos-Vara 2005). Polyclonal antibodies are also more likely to identify multiple isoforms of the target antigen, as well as irrelevant proteins that other antibodies present in the serum may be reactive to. (Ramos-Vara 2005) The ability of polyclonal antibodies to recognize multiple epitopes of the same protein partly explains the variability seen during Western blot analysis for zona protein molecular weight. For instance, figure 5 shows antibodies in the serum of SpayVac vaccinated mares (5b)

and rabbit anti ZP3 polyclonal antibody (5a) binding 2 side-by-side protein samples at slightly different molecular weights.

Furthermore, zona proteins are large glycoproteins with extensive post-translational modifications. Heavy glycosylation and sulfation patterns lend to variances in electrophoretic mobilities and carbohydrate moieties among and within the protein families and species. This results in a range of molecular weights that can be observed for the same protein family (Dunbar et al. 1994, Prasad et al. 2000). Adding to the complexity, proteolytic cleavage and deglycosylation events have been shown to process the larger zona glycoproteins into smaller polypeptides; while zona proteins are also characterized by the tendency to self-assemble into matrices and thus exist as heterodimers. (Wassarman 1988, Prasad et al. 2000) These post translational modifications complicate the ability to accurately identify zona proteins by electrophoresis. Dunbar et al. (1985) and Wassarman (1988) confirm this by identifying a zona protein family in the 6-30 kDA range formed by proteolytic processing of the ZP2 protein. Similarly, multiple authors note molecular weight ranges for each zona protein, rather than cite a specific and consistent size (Dunbar et al. 1985, Wasserman 1988, Miller et al. 1992). The authors attribute this variation in molecular weight to differences in post-translational modification patterns. Variability in glycosylation level, proteolytic cleavage events, and species lends insight into why the same anti ZP3 polyclonal antibody bound in the 15-20 kDA range in figure 11 while binding in the 50-75 kDa range in figure 5. Similarly, Western blot analysis revealed the binding of antibodies in the serum of SpayVac vaccinated mares to zona protein anywhere from 50 kDa (Figure 5b) to 100 kDa (Figure 6b).

Figures 9 and 10 indicate a specificity of anti pZP antibodies from the serum of SpayVac vaccinated mares for equine zona proteins 3 and 4. Likewise, Western blot analyses revealed the

binding of anti pZP antibodies to zona proteins in the 37-75 kDa range; indicating an immunoreactivity of anti pZP antibodies to the zona proteins that typically reside in this molecular range. Nonetheless, it is difficult to conclude that anti pZP antibodies are specific for these two zona proteins knowing their tendency to form heterodimers and express varying levels of glycosylation. For instance, a polypeptide proteolytically cleaved from ZP2 could form a dimer with ZP3 and show up in this 50-75 kDa range. ZP3 itself is also thought to be composed of two components which, when exposed to proteases, yields two smaller polypeptides. (Wassarman 1988)

Alternatively, Wasserman (1988) declared ZP3 to be the major component of the porcine zona pellucida, representing as much as 70-80% of total protein content. When the pZP antigen is isolated, the entire zona pellucida is homogenized, resulting in a compilation of all zona protein families. It follows that 70-80% of the pZP antigen is thus accounted for by ZP3, with the remaining 20-30% representing the other zona and matrix proteins. When injected with pZP, the mare would produce substantially more antibodies against ZP3 than the other proteins. Antisera harvested from the mare therefore expresses higher specificity for ZP3 than the other zona proteins, and consequently bind ZP3 in Western blot and immunohistochemical analyses more prominently. This explains how pre-incubating the Western blot membrane with anti ZP3 antibodies blocked the ability of anti pZP antibodies to bind the protein, whereas pre-incubating the membrane with anti ZP4 antibodies only attenuated the binding of anti pZP antibodies to the protein.

Finally, due to the difficulty in obtaining equine samples, the sample size for these experiments was limited. When possible, each experiment was performed at least twice to verify results and repeatability. While this study was intended as a demonstration of a concept, an

increased sample size would have been preferable. Also, individual differences in immune response between mares may lead to variations in the quantity and biological ability of antibodies produced. Serum samples were selectively chosen from mares of high antibody titer level, and while it is generally assumed that titer level has a direct correlation with vaccine efficacy, this has yet to be scientifically proven. Similarly, although all nine serum samples used contained antibodies immunoreactive to equine zona protein, two of the nine serum samples were from SpayVac treated mares that were still able to conceive despite vaccination. A valuable control in this study would have been to have serum samples from each mare prior to and following vaccination, such that each mare could serve as her own negative control.

Collectively, results suggest a model where anti pZP antibodies produced in response to SpayVac vaccination are immunoreactive to equine zona proteins under the correct conditions. When zona proteins are isolated from equine tissues and made available to serum samples from SpayVac vaccinated mares, antibodies in the serum are consistently able to recognize the protein. When subjected to the same protocol, antibodies in the serum of non-vaccinated mares did not express this immunoreactivity to zona protein. This suggests that the antibodies immunoreactive to zona protein in the serum samples of SpayVac vaccinated mares are a result of SpayVac vaccination. Furthermore, these antibodies appear to be immunoreactive to zona protein isolated from both mature, pre-ovulatory oocytes isolated from graafian follicles as well as immature oocytes embedded in ovarian tissue. With that said, this study merely illustrates a pathway by which anti pZP antibodies could act if they are biologically available to these tissues under the correct conditions. It has yet to be investigated whether anti pZP antibodies are present in the follicular fluid of follicles or whether they are able to permeate ovarian tissue and access primary follicles following SpayVac vaccination. Similarly, the protein presented to anti pZP antibodies

during Western blot and immunohistochemical analysis has been subjected to various processing steps, including antigen retrieval, denaturing buffers, and lysis buffers to name a few. These steps aid in the unfolding of protein such that binding sites become more available to antibodies. In vitro, these proteins will be folded in 3D structures and thus may not be as available for antibody binding. This study therefore demonstrates a model by which anti pZP antibodies may bind zona proteins, thereby interfering with a critical reproductive event, and ultimately resulting in the inhibition of sperm-egg interaction and follicular dysgenesis.

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APPENDICES

Appendix I: Serum Samples from SpayVac Vaccinated Mares

Assay-HorseID	**Avg. OD**	**Std. Dev.**	**% of Std. (Titer)**
27-4131	0.672	0.009	117.28
24-1729	0.950	0.028	163.79
13-4133	0.633	0.020	109.08
28-3799[+]	0.721	0.007	125.83
79-6527	0.586	0.020	97.83
4-2816	0.826	0.021	142.47
43-6498	0.771	0.117	134.50
19-361*	1.034	0.014	178.33
15-1688**[+]	0.230	0.006	39.66

*Sample with the highest antibody titer
**Sample with the lowest antibody titer
[+] Mares conceived in 2011 after vaccination and foaled in 2012

List of serum samples used from SpayVac vaccinated mares along with relative antibody titer levels. Mares were vaccinated on March 31, 2011 in Pauls Valley, Oklahoma with SpayVac and serum samples were collected on May 3, 2011. Anti pZP antibody titers from these samples were determined by enzyme-linked immunosorbent assay (ELISA) by Immunovaccine Inc. (Halifax, Nova Scotia). Titers were read as average optical density (Avg. OD) and standard deviation (Std. Dev.) across 3 readings of the same sample. The samples were then reported as a percent of standard (% of Std.), where the standard was a serum sample thought to be of good response to the vaccination. The larger the percent of standard column value is, the higher the titer level of the individual mare is thought to be. Assay-HorseID simply refers to the assay number assigned to the serum sample followed by the horse number. Of the top 15 samples collected on May 3, 2011 with the highest titer levels, the above 8 were chosen at random for use. The last sample was used as a low titer comparison.

Appendix II: RIPA Lysis Buffer

RIPA Lysis Buffer is prepared as follows:

1. Combine:
    - 150mL ddH_2O
    - 0.484g Tris (pH 8.0)
    - 1.6 NaCl
    - 20mL Glycerol
    - 2mL Nonidet P-40 (NP-40)
    - 0.2g Sodium Dodecyl Sulfate (SDS)
    - 1.0g Deoxychlorate
    - 0.117g Ethylenediamine tetraacetic acid (EDTA)
2. Allow solvents to dissolve and adjust pH to 8.0 with HCL
3. Bring volume up to 200mL with ddH_2O

Appendix III: Western blot Polyacrylamide Gels Preparation

12% running gel

- 1.8mL H_2O
- 4.0mL 1M Tris (pH 8.8)
- 4.0mL Acrylamide 30:0.8
- 100µL 10% SDS
- 5µL TEMED
- 100µL 10% APS

Stacking gel

- 4.2mL H_2O
- 0.99mL Acrylamide
- 0.75mL 1M Tris (pH 6.8)
- 60µL 10% SDS
- 15µL TEMED
- 75µL 10% APS

Appendix IV: 6x Sample Buffer Recipe

50 mL 6x Sample Buffer

- 2.363g Tris-HCL (pH 6.8)
- 10mL glycerol
- 3g SDS
- 0.1g Bromo Blue
- Dilute 4:1 with 1M dithiolthreitol (DTT)

1M Diothiothreitol (DTT)

- 309mg DTT
- 2mL H2O

Appendix V: Zona Protein Sequences

Equus caballus ZP3

MGLSYGLFVCLLLWGGTELCYPQPIQQAGTHHPHPMPSKLPVVVECLEAQLVVTVSKDLF

GTGKLIQSADLTLGPKNCEPLVSMDTEDVVRFEVALHECGNSVQVTEDALVYSTFLLHNP

RPVGNLSILRTNRAEVPIECRYPRQGNVSSQAILPTWVPFQTTMFSEEKLVFSLLLMEED

WGAEKRSPTFQLGEIAHLQAEVHTGSHVPLRLFVDHCVATLTPDRNASPYHTIVDFHGCL

VDGLSDASSAFKAPRPGPETLQFMVAVFHFANDSRNMVYITCHLKVTLADHVPDQLNKAC

SFSKSSNSWSPVEGPADICQCCGNGSCGTPGHSGTQSRTVRQWHKSASRNRRHVTEEADV

TVGPLIFLGKASDRAEEGSTSSPASVMLGLGLAAVGSLTLATVGLGLARRRRAASHPVLC

PVSAPQ

Equus caballus ZP4 sequence

LPAEASTGTMWLLQSVLLCFSLSLALSGQRELEAPAYPGVLHCGLRSFQFTVNLSQETAT

PPALIAWDNRGMPRRLQNDSDCGTWVREDPGSSVVLEASYSSCYVTQWDSHYIMPVGVER

TDAAGHRTVTKMKLLECPMDFLALNAPSADLCDSVPVWDRLTCAPSPVTQGDCKERGCCY

NSEVNSCYYGNTVTSRCTQDGHFSIAVSRNVTSPPLLLSSVHLAFRNDSECNPVMATHAF

ALFRFPFSSCGTTRRVTGDQAVYENELVATRDVRTWSHGSITRDSIFRFQVSCSYSVSSN

AFPVNVQVFTLPPPLPETQPGPLTLELRIAKDKHYISYYNVSDYPVVKLLQDPIYVEVSI

LHRTDPYLGLMLHQCWATPSTNPLHQPQWPLLVKGCPYTGDSYQTQLIPVQKALDLPFPS

HYQRFSIFTFSFVDSVAKRAFKGLVYLHCSASVCQPAGTPPCMITCPVTRRRRSSDIRFQ

NSTASISSKGPMILLQATKDSSEKLHKYSTSPVDSQSLWVAGLSGTFIVGASLVSYLAIR

KQM

Sus scrofa ZP3 sequence

MAPSWRFFVCFLLWGGTELCSPQPVWQDEGQRLRPSKPPTVMVECQEAQLVVIVSKDLFG

TGKLIRPADLSLGPAKCEPLVSQDTDAVVRFEVGLHECGSSLQVTDDALVYSTFLRHDPR

PAGNLSILRTNRAEVPIECHYPRQGNVSSWAILPTWVPFRTTVFSEEKLVFSLRLMEENW

SAEKMTPTFQLGDRAHLQAQVHTGSHVPLRLFVDHCVATLTPDWNTSPSHTIVDFHGCLV

DGLTEASSAFKAPRPGPETLQFTVDVFHFANDSRNTIYITCHLKVTPADRVPDQLNKACS

FSKSSNRWSPVEGPAVICRCCHKGQCGTPSLSRKLSMPKRQSAPRSRRHVTDEADVTVGP

LIFLGKTSDHGVEGSTSSPTSVMVGLGLATVVTLTLATIVLGVARRRRAAAHLVCPVSAS

Q

Sus scrofa ZP4 sequence

MWLRPSIWLCFPLCLALPGQSQPKAADDLGGLYCGPSSFHFSINLLSQDTATPPALVVWD

RRGRLHKLQNDSGCGTWVHKGPGSSMGVEASYRGCYVTEWDSHYLMPIGLEEADAGGHRT

VTETKLFKCPVDFLALDVPTIGLCDAVPVWDRLPCAPPPITQGECKQLGCCYNSEEVPSC

YYGNTVTSRCTQDGHFSIAVSRNVTSPPLLWDSVHLAFRNDSECKPVMETHTFVLFRFPF

SSCGTAKRVTGNQAVYENELVAARDVRTWSHGSITRDSIFRLRVSCIYSVSSSALPVNIQ

VFTLPPPLPETHPGPLTLELQIAKDERYGSYYNASDYPVVKLLREPIYVEVSIRHRTDPS

LGLHLHQCWATPGMSPLLQPQWPMLVNGCPYTGDNYQTKLIPVQKASNLLFPSHYQRFSV

STFSFVDSVAKQALKGPVYLHCTASVCKPAGAPICVTTCPAARRRRSSDIHFQNGTASIS

SKGPMILLQATRDSSERLHKYSRPPVDSHALWVAGLLGSLIIGALLVSYLVFRKWR

Bos taurus ZP3 sequence

MGPCSRLFVCFLLWGSTELCSPQPFWDDETERFRPSKPPAVMVECQEAQLVVTVDKDLFG

TGKLIRPADLTLGPDNCEPLASADTDGVVRFAVGLHECGNILQVTDNALVYSTFLLHNPR

PAGNLSILRTNRAEVPIECHYPRQGNVSSWAIQPTWVPFRTTVFSEEKLVFSLRLMEENW

SAEKMTPTFQLGDRAHLQAQVHTGSHVPLRLFVDHCVATLTPDWSTSPYHTIVDFHGCLV

DGLTDASSAFKAPRPRPEILQFTVDVFRFANDSRNMIYITCHLKVTPVDRVPDQLNKACS

FSKSSNRWSPVEGPTDICRCCSKGRCGISGRSMRLSHREGRPVPRSRRHVTEEADVTVGP

LIFLRKMNDRGVEGPTSSPPLVMLGLGLATVMTLTLAAIVLGLTGRLRAASHPVCPVSAS

Q

Bos taurus ZP4 sequence

MWLLLQLVWLCFLLSLGLNSWHQSKVPEYPDELRCGLRSFQFTINPLSQETETPPVLVAW

DNHGLPHSLQNDSDCGTWVSEGPGSSLVGEASYSGCYVTEWESYYIMTVGIERAGVSGSG

AFIETKLFKCPVNLPDVPNAGLCDSVPVWDRLPCAPSPITQGDCKQLGCCYNSEEVISCY

YGNTVTSHCTQDGHFSIAVSRNVTSPPLLLNSVHLAFRNDSECKPVMATHTFVLFRFPFT

TCGTTKQITGKQAVYENELVAARDVRTWSRGSITRDSTFRLQVSCSYSASSSALPVNVQV

LTLPPPLPETQPGNLTLELKIAKDKRYRSYYTASDYPVVKLLRDPIYVEVSIHQRTDPSL

ELRLDQCWATPGADALLQPQWPLLVNGCPYTGDNYQTKLIPVWEASDLPFPSHYQRFSIS

TFSFVDSVAKRALKGPVYLHCSASVCQPAGTPSCVTLCPARRRRSSDIHFQNNTASISSK

GPLILLQAIQDSSEKLHKYSRSPVDSQALWVAGLSGILIVGALFMSYLAIRKWR