# Final report

## Development of a single shot immunocontraceptive vaccine for cattle


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# Abstract

Previous MLA funded research has demonstrated that spayed cattle experience significant pain and stress. The aim of this project was to develop an immunocontraceptive vaccine to replace spaying. The project focussed on development of a vaccine targeting the zona pellucida (ZP), the unique outer glycoprotein layer of the ovum that sperm must bind to, and penetrate, to initiate fertilisation. Three vaccinations against whole porcine ZP (pZP) 6 weeks apart induced long term infertility in typical cull heifers, and a similar rate of infertility was induced with a purified mix of pZP proteins 3&4. Further, it was shown that a single vaccination with either of these proteins suspended in Montanide™ ISA VG 61 resulted in a moderate rate of infertility lasting at least 12 months. Although vaccination against these porcine ZP proteins were shown to induce infertility, they cannot be used in any commercial vaccine. Unfortunately, none of the synthetic (recombinant) ZP proteins produced and tested in this project induced acceptable levels of infertility. Alternative methods for producing appropriately folded and glycosylated ZP proteins and incorporation of immune potentiators should be considered for any future work.

# Executive summary


## Background

Historically and continuing spaying of surplus heifers and cull cows is an effective means of managing stocking rates, reducing aged cow mortalities and improving business profitability in northern Australia. However, research has clearly shown that the procedure is painful and stressful for cattle undergoing the procedure. A practical effective alternative has been sort by industry and the public is seeking greater evidence of implementation of measures to improve beef cattle well-being. Different physical, chemical, hormonal and immunological approaches to long-term prevention of cattle becoming pregnant have been investigated. However, most have been rejected due to unacceptable side-effects, efficacy and cost. A novel approach, which has been routinely used to control population of wildlife such as elephants, white tail deer and grey seals, is vaccination against the zona pellucida (ZP). The ZP is the glycoprotein shell-like structure which surrounds the developing oocyte(female) in ovarian follicles. Antibodies induced by ZP vaccination bind to the target females' ZP blocking sperm attachment and hence fertilisation. The ZP of cattle comprise three ZP glycoproteins, ZP2, ZP3 and ZP4. Current understanding is that a combination of ZP3 and ZP4 sequences are primarily responsible for sperm binding. The proteins which make up the ZP are unique and thus vaccination targeting the ZP is safe for the animal, and in the case of cattle, for the consumer.

## Objectives

The primary aim was to develop an immunocontraceptive vaccine capable of preventing cattle from becoming pregnant for a period of approximately 12 months following a single vaccination. The objectives were to identify:

1) Which porcine (p) ZP proteins should be incorporated in a vaccine,
2) how should the vaccine be formulated to enable infertility to be induce following a single vaccination, and
3) which synthetic (recombinant) ZP protein construct/production system should be used to produce selected ZP protein(s) able to be incorporated in a commercial vaccine.

## Methodology

The first experiment involved purification of the individual proteins which comprise the pig zona pellucida and then vaccination of tropically adapted heifers against each of these using a standard vaccine formulation (Freund's complete and incomplete adjuvants). The second experiment assessed the magnitude and duration of infertility following administration of a single vaccination consisting of the selected purified pZP proteins suspended in Montanide™ ISA VG 61. The final three experiment assessed the magnitude and duration of infertility following administration of a single vaccination consisting of different recombinant ZP proteins suspended in Montanide™ ISA VG 61. The efficacy of each different vaccination formulation was assessed by assessing the immune response to vaccination, the outcome of mating to a fertile bull for 2-3 periods of 6-12 weeks duration, and monthly assessment of ovarian morphology via transrectal ultrasound.

## Results/key findings

A practical method of producing large amounts of pZP suitable for cattle vaccination studies was developed. Vaccination of cattle with a crude preparation of pZP induced infertility in most cattle for

at least 12 months (Exp.1 and 2). Administration of vaccines to cycling heifers containing either purified pZP2 or pZP3&4 suspended in Freund's adjuvant (Exp.1) induced prolonged infertility in a high proportion of heifers (71% and 100% first mating, 71% and 78% second mating, respectively), similar to that achieved with native pZP. The anti-ZP immune response was significantly higher in infertile compared to fertile heifers. Infertility persisted in spite of a decline in the anti-ZP immune response to close to prevaccination levels. At slaughter 419 days after the last vaccination there was histologic evidence of some to severe follicular atrophy in the ovarian cortex. The loss of developing follicles is likely to have contributed to the observed long term infertility. Although, most vaccinated heifers became infertile a small proportion remained fertile and became pregnant to one or both matings i.e these animals apparently failed to respond as expected to vaccination.

Single administration of vaccines containing either pZP or pZP3&4 suspended in an emulsion of Montanide$^{TM}$ ISA VG 61 to cycling heifers, resulted in prolonged infertility in 70% of vaccinated heifers. Again the anti-ZP immune response was significantly higher in infertile compared to fertile heifers.

However, single administration of vaccines containing recombinant pZP3 or a 1;1 mixture of recombinant pZP3 and pZP4 suspended in an emulsion of Montanide$^{TM}$ ISA VG 61 to cycling heifers, failed to induce infertility. This was despite the fact that the anti-ZP immune response was similar to that achieved following vaccination with native pZP or purified pZP proteins. This suggests that although the recombinant pZP proteins incorporated in each vaccine were immunogenic the antibodies produced failed to effectively bind to the ZP in the follicles of vaccinated heifers. Differences in the glycosylation and conformation between the native and recombinant proteins, which has been reported before with other ruminant recombinant vaccines, is the most likely explanation.

Single administration of a vaccine containing a recombinant heterocomplex of bovine (b) ZP3&4 suspended in an emulsion of Montanide$^{TM}$ ISA VG 61 to cycling heifers induced infertility in some heifers initially. The outcome of two further mating periods is required to confirm and better understand this finding.

## Benefits to industry

This project has demonstrated that an immunocontraceptive vaccine targeting the zona pellucida could be developed for use in cattle, but more work needs to be done to create better quality recombinant ZP proteins. Vaccination of cattle with either pig derived or recombinant ZP/ZP proteins was shown to have little or no impact on their health, well-being, growth and carcass quality, however the latter failed to induce infertility in most vaccinated cattle. There was evidence that some vaccinated cattle could recover fertility after approximately 12 months, but this was not examined in detail. An outline for implementation of a single shot immunocontraceptive vaccine was developed in consultation with a senior cattle veterinarian servicing beef cattle businesses in northern Australia.

## Future research and recommendations

The failure of the administered recombinant pZP protein vaccines to induce infertility in a high proportion of heifers represents the major challenge for future research. It seems likely that the failure of the recombinant ZP protein vaccine to induce infertility was due to significant differences in glycosylation and conformation between the native pZP and the recombinant ZP. Glycosylation is a complex and critical process playing an important role in the biological activity, folding, stability and solubility of proteins. Failure of a recombinant protein to fold into the correct three-dimensional

structure typically results in the production of inactive proteins. These are some of the well-recognised challenges in producing recombinant protein vaccines. In addition, as there was evidence that vaccination against purified pZP2 protein induced infertility in a significant proportion of heifers development of a method of producing recombinant ZP2 should be investigated.

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# 1. Background

## 1.1 Role of spaying in the management of cull female cattle in northern Australia

In the extensive beef cattle production systems of northern Australia, where bull control is problematic, cull cows and heifers are commonly spayed (ovariectomy via a flank or vaginal incision), or in some cases 'webbed' (surgical removal of a section of each oviduct), to render them sterile (Jubb and Letchford, 1997). Spayed cattle can continue to be managed within their mob of origin and can then be subsequently either slaughtered or exported. Spaying of surplus cattle is an important means of managing stocking rates, and hence sustainability of pastures and has been shown to reduce the prevalence of cow mortality and improve business profitability (Niethe and Holmes, 2008). The majority of non-pregnant cull heifers and cows are spayed using the dropped ovary technique (DOT), but due to the risk of severe haemorrhage from the severed ovarian artery, pregnant cattle are often 'webbed' via a flank laparotomy. A major concern with spaying of cattle is the adverse impact on animal welfare. The MLA funded project 'Evaluation of the impact on animal welfare of various manipulative and surgical procedures performed on the reproductive tract of female cattle in the northern beef industry' (B.AHW.0143) demonstrated very clearly that spaying causes significant pain and stress, with typically 2 to 4 % of spayed cattle subsequently dying of surgical haemorrhage or other sequelae (McCosker *et al.* 2010; Petherick *et al*. 2013). Although now many cattle undergoing spaying receive pain relief treatment, a key question arising from completion of B.AHW.0143 was, could spaying be replaced with some form of practical and cost effective chemical or immunological treatment. Further, Petherick (2005) has highlighted the growing public concern about the welfare of livestock undergoing surgical procedures.

## 1.2 Non-surgical methods of preventing pregnancy in cattle

Various chemical contraception treatments, intrauterine devices and contraceptive vaccines have been developed and evaluated in a large number of domestic and wild animal species (Herbert and Trigg, 2005; Hardy and Braid, 2007). The MLA funded project 'Review of the alternatives to castration and spaying of ruminants B.AWW.0225) evaluated how effective and practical non-surgical alternatives to spaying were. D'Occhio *et al.* (2002) reported that treatment of Brahman heifers and cows with subcutaneously administered slow-release implants containing the gonadotrophin releasing hormone (GnRH) agonist, deslorelin (8 or 12 mg), was effective in preventing pregnancy in a high proportion of cattle. Twelve months after treatment, 60 to 90% and 80 to 100% of untreated heifers and cows, respectively, were pregnant compared to 10 to 28% and 6 to 24% of deslorelin treated heifers and cows, respectively. Unfortunately, use of deslorelin implants to control reproduction and prevent pregnancy in dogs, horses and many species of wildlife has become very common and thus the cost of the implants has become too high for routine use in cattle e.g. a 9.4 mg implant (Suprelorin, Virbac) costs $76.50. Lower dose implants with different deslorelin release patterns have been investigated, however their ability to prevent pregnancy has been lower than is required.

Insertion of an intrauterine device developed in Argentina was evaluated in Brahman heifers and cows in north Queensland (Fordyce *et al*. 2001). Transcervical insertion was not possible in 25% of heifers and 8% of cows. In cattle in which the device was able to be inserted, perforation of the uterus occurred in 35% of heifers and 45% of cows, and 21% of heifers and 33% of cows became pregnant within a year of treatment. Although there has been some reported success with chemical sterilization (intraovarian injection of zinc- or calcium chloride solutions) of animals (Cavalieri, 2017)

a study in Brahman heifers found cattle experienced pain and or failed to become infertile (Cavalieri ,2016).

Herbert and Trigg (2005) have reviewed vaccination of females against GnRH as a means of controlling reproduction. Vaccination of cattle against GnRH induces antibodies which bind to GnRH, thus preventing it from binding to receptors in the pituitary, resulting in failure of ovulation. In the last 30 years two commercially available vaccines have been developed and evaluated in Australian feedlot and grazing cattle, Vaxstrate® and Bopriva®. Both require a priming vaccination followed by one or more booster vaccinations. The interval between primary and secondary vaccination varies but typically is 2-4 months. Schatz et al (2016) reported that vaccination of cull cows with Bopriva (twice 12 weeks apart) resulted in 98% of cows being non-pregnant 4 months after the second vaccination but this reduced to 69% by 5.5 months. Herbert and Trigg (2005) stated that the primary problem with vaccination against GnRH was the highly variable response of individual animals.

## 1.3 Vaccination against the zona pellucida to prevent pregnancy

Since 1980 immunocontraception through vaccination has gained traction as a potential way to limit wild animal populations, to control breeding of zoo animals, to limit feral pest impacts by reducing pest numbers, and to provide an alternative to surgically neutering domestic pets and farm animals (Naz and Saver, 2016). Development of vaccines targeting the porcine Zona Pellucida (pZP) have been shown to induce an effective immunocontraceptive response in over 80 animal species (Kirkpatrick et al. 2009). The ZP of cattle and pigs comprises three ZP glycoproteins, ZP2, ZP3 and ZP4. The amino acid sequences of porcine and bovine ZP2, ZP3, and ZP4 are 77%, 85% and 75% identical, respectively (Kanai *et al*. 2007). Although the ZP proteins of the pig ZP are similar to those of cattle, they are sufficiently different that if included in a vaccine administered to cattle they should induce a significant immune response. Chains of carbohydrate (mainly mannose type) bind to the ZP protein amino acids forming a glycoprotein complex. The pattern of binding of these carbohydrate chains (glycosylation) plays an important role in the species-specificity of sperm-zona binding (Yonezawa *et al*. (2005).

Interestingly, although pZP vaccines have been shown to be effective in a number of large ruminant species (bison, water buffalo, yak, banteng), little or no work has been done on domestic cattle. Bison and domestic cattle are genetically similar, and their reproductive physiology of bison is similar. Duncan *et al*. (2013) reported the that the pregnancy rate in a herd of bison cows vaccinated against pZP (two initial vaccinations prior to the breeding season and then an annual booster prior to subsequent breeding seasons) was reduced from 67% to 11% after approximately 12 months and to 3% after 24 months. Longitudinal monitoring of this herd revealed that following repeated annual pZP vaccination for 3-4 years treatment with PZP (3 or 4 years), vaccinated bison cows remained infertile for at least 4 or 5 years (Duncan *et al.* 2017).

pZP is most commonly used in vaccines because of its homology with the ZP proteins of many mammalian species, and also the ready availability pf pig ovaries from abattoirs. pZP is isolated from the oocytes, yielding a relatively homogenous preparation (Hendrick and Wardrip,1087). Techniques to isolate the 4 individual glycoproteins which compose pZP using high-performance liquid chromatography (HPLC) are well established. However, because of their substantial sugar content (about 80%) only a few studies have reported vaccination of animals with recombinant ZP proteins. There is significant homology between porcine ZP proteins and those of other mammalian species (e.g. porcine ZP3 protein has 65.6% amino acid homology with mouse, 75.7% with dog, and 83.6% with cow ZP3). Hetero-immunisation with pZP provokes a much stronger immune response than

alloimmunisation. The antibodies produced induce infertility by interfering with sperm binding to the ZP, which is the initial step leading to fertilization. pZP antibodies do not bind to any other cell or tissue in the body, indicating very strong specificity of the induced immune response. pZP has been formulated with a number of different adjuvants before immunisation. The most common and the most effective in inducing a large immune response are Freund's adjuvants and AdjuVac, which is a lipid-based adjuvant containing heat-killed *Mycobacterium avium*. Neither at this point would receive Australian Pesticides and Veterinary Medicines Authority (APVMA) approval for use in a commercial cattle vaccine.

Vaccination against pZP has been shown to induce minimal local (transient swelling at site of vaccination) or systemic changes (Kirkpatrick *et al.* 2009) in a range of species, and no undesired tissue pathology, which collectively indicates a lack of any serious side effects. Further, pregnant animals from multiple species have been immunised with pZP without adverse consequences (Naz and Saver, 2016). However, there is evidence that vaccination can induce a cell-mediated immune response in some animals resulting in significant transient or permanent destruction of developing ovarian follicles (Kirkpatrick, *et al*. 2009; Joone *et al.* 2017). The infertility effect is closely related to the titre of the antibody induced, which appears to be species-specific. Animals generally become fertile again when the antibody titre significantly decreases. Re-exposure to pZP rapidly induces a significant increase in antibody titre to levels above which they become infertile again. Thus, while there are important gaps in our knowledge of how to best produce pZP proteins for immunisation and especially which adjuvant and which antigen-adjuvant formulation works most effectively, there is sufficient knowledge indicating the value of pZP in inducing an immunocontraceptive response.

In 2010 as part of a PhD research programme (King, 2014) funded by the CSIRO – Flagship Collaboration Fund and The University of Queensland, we demonstrated that vaccination of 2-year-old Brahman heifers against semi-purified porcine zona pellucida (100 µg pZP in Freund's complete adjuvant followed in 1 month and again at 6 months with 100 µg pZP in Freund's incomplete adjuvant) induced infertility in 89% of animals for 46 weeks and in 78% of animals for at least 80 weeks. A third of the vaccinated animals in this experiment continued to cycle normally, another third cycled but not regularly, and the remaining third became anovulatory. Although this immunisation regime induced a strong immunocontraceptive response, it is neither practical in requiring three injections over a 6-month period when access to extensively managed cattle is typically biannual, nor is it likely to meet APVMA registration requirements because it uses proteins derived from pig ovaries.

Therefore, the aim of the research conducted in this project was to identify the key ZP protein to target, to produce a synthetic version of this protein, and to formulate a vaccine which would induce infertility in a high proportion of heifers/cows for at least 12 months following a single vaccination. The major applications of such a vaccine would be to prevent cull heifers from becoming pregnant, thus enabling them to be exported or finished in a feedlot, and to prevent cull cows from reconceiving, thus enabling them to be fattened and sent to slaughter. Producers are familiar with the use of vaccination to ensure the health and well-being of their cattle, and thus it was considered that where spaying was routinely conducted, an effective immunocontraceptive vaccine could be readily adopted to replace surgical spaying as part of annual herd management. Another consideration in developing this non-surgical alternative was cost per animal, with the starting point being that the cost would need to be similar to the cost of spaying i.e. approximately $20-25 per animal. This estimate is based on 2009 calculations provided by Ian Braithwaite (cattle veterinarian, Mt Isa) on the true cost of DOT spaying per animal - stock camp cost + total spay cost (includes veterinarian/technician travel cost) + cost of spay related mortalities (Appendix 8.1). More recent

costing provided by Peter Letchford (cattle veterinarian, Kununurra) was very similar. Finally, there is no evidence that use of an immunocontraceptive vaccine would impact meat quality or result in any unacceptable residues, and it is likely that development of a non-surgical alternative to spaying would be favourably viewed by consumers.

# 2. Objectives

The project aims to develop a vaccine against the zona pellucida (ZP), the outermost layer of the egg, of cattle which would provoke an immune response and in turn prevent fertilisation for 12 months.

By the end of this project, the researcher will have:

- **a.** Produced recombinant Zone Pellucida protein(s) based on selection from porcine ZP protein candidates. Successfully achieved
- **b.** Proved effective contraception with the recombinant ZP protein(s) in female cattle. Unsuccessful.
- **c.** Formulated the candidate protein(s) in a slow-release delivery system. Successfully achieved

# 3. Methodology

## 3.1 Production of native and recombinant porcine zona pellucida proteins

### 3.1.1 Isolation of whole porcine zona pellucidae

The method used to isolate and purify pZP was based on the method described by Dunbar et al (1978) but was modified to significantly increase the yield and relative purity of pZP. Instead of homogenising the ovaries, a standard food processor was used to finely slice the ovaries. This new process enabled more efficient separation of the oocytes, resulted in far less damage to the zona pellucidae, and significantly reduced the level of tissue protein contamination. The sliced ovarian tissue was rinsed through a series of progressively finer filters. The filtrate from the final filter was then microscopically examined to confirm the presence of either intact oocytes or free zona pellucidae. This final filtrate was then heat solubilised and a sample analysed to determine the yield of pZP. Typically, from 3 kg of trimmed pig ovary, 3.7 mg of pZP could be isolated per day. Assuming that 200ug of pZP is required per dose of vaccine this would enable 18 cattle to be vaccinated. The crude pZP was stored frozen at -20˚C (full description of method is provided in Appendix 8.2).

### 3.1.2 Purification of porcine zona pellucida proteins

Purification of pZP was conducted by The University of Queensland's Protein Expression Facility (PEF). The crude ZP protein preparation was thawed from -20˚C and concentrated using the Amicon Ultra-4 30 kDa MWCO filtration device (Millipore). This material was buffer exchanged into running buffer (50 mM Sodium Phosphate, 100 mM $Na_2SO_4$, 35 mM SDS, pH 7.0 (25˚C)) using a PD SpinTrapTM G-25 (GE Healthcare). The buffer exchanged sample was heated at 100˚C for 3 min, passed through a 0.22 µm filter and purified using a size exclusion chromatography (SEC) column. Fractions corresponding to the target protein were pooled. The SEC pooled samples were buffer exchanged into Phosphate Buffered Saline (PBS) using a PD midiTrap G25 from GE Healthcare. The purified protein was concentrated using an AmiconUltra-15 Centrifugal Filter with a 10 kDa molecular weight cut-off (Merk Millipore). The purified proteins were stored at 4°C. Using the SEC profiles, samples from each peak were run on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels under non reducing conditions.

### 3.1.3 Development of candidate recombinant zona pellucida proteins

Injecting proteins derived from one livestock species into another is, with good reason, very unlikely to be accepted by APVMA in any application for registration of a commercially available vaccine for cattle. Recombinant proteins produced synthetically in a Good Laboratory Practice standard laboratory is what is required. This is a safety issue but also a cost issue. Recombinant proteins typically can be produced consistently and cost effectively, lowering the final cost of the vaccine. A number of recombinant vaccines to prevent infectious diseases in livestock have already been developed (Pech-Cervantes *et al.* 2020).

The first step in development of recombinant ZP proteins was to examine the amino acid sequence for porcine ZP3 and ZP4. PZP3 is 421 amino acids in length with the first 22 containing the signal peptide and the final 89 (residues 333-421) being the transmembrane domain. Both the signal peptide and transmembrane domain can be cleaved enzymatically and removed, leaving a mature secreted protein of 310 amino acids. Porcine ZP4 is 442 amino acids in length, 132 amino acids or 42.6% longer than pZP3. It was recognised that it may be too difficult to produce as a single

recombinant protein. Preliminary analysis suggested that the first part of the molecule (residues 21-231) could be expressed independent of the second part (residues 231-460).

Initial development work focused on producing recombinant versions of pZP3 and pZP4 in mammalian expression cell lines because this would ensure the proteins were glycosylated. Porcine ZP proteins are heavily glycosylated, and it is widely believed that this contributes to the biological function of these proteins. Numerous constructs were developed, produced, and evaluated (Appendix 8.3). Attempted production of some constructs yielded only small quantities of recombinant protein insufficient to evaluate in animal experiments, while others produced proteins which were not secreted into the medium and the recombinant protein could not be readily recovered from the complex cellular debris without damage. In addition, this form of expression was very costly per mg of protein synthesized. These factors taken together forced a strategic rethink. The decision was made to produce the recombinants in *E. coli* systems, which produce large amounts of protein and for which good protein purification procedures exist. A review of the literature showed a few studies where similar reproductive proteins produced in this way could elicit strong immune responses in some mammalian species and which, in laboratory animal studies, adversely affected fertility (Gupta et al, 2013, Shrestha et al, 2014).

The design of the first *E. coli* derived recombinant pZP proteins, and their expression were adapted from Shrestha *et al.* (2014). The methodology to produce these proteins is described in detail below. Based on the findings of Experiment 1, recombinant pZP3 and pZP4 were developed and these were used in Exp.3 and Exp.4. Recombinant pZP3 was produced first. The construct used consisted of tetanus toxoid epitope, dilysine and pZP3 or pZP4 (see Figure 1). This was expressed in an *E. coli* strain BL21 (DE3) pLysS. A single colony was used to generate a starter culture which was used to seed 5 x 1 litre culture flasks. The cell pellet from one flask was suspended in lysis buffer and sonicated in a Branson sonifier on ice. The inclusion bodies were isolated in the insoluble fraction after centrifugation at 18,000 x *g* for 45 mins at 4°C. They were washed twice in wash buffer before final suspension in solubilization buffer, and then sonicated for 3 x 20 seconds. They were incubated at room temperature on a roller mixer for 7 hours before being pelleted at 18,000 x *g* for 30 minutes at 4°C. The recombinant pZP3 was in the soluble fraction. This was diluted by 10 times volume overnight in a dropwise manner at 4°C, transferred to Slide-a-Lyzer dialysis flasks and dialysed for 2 x 4 hours at 4°C against 20mM Tris pH 6.0 at a sample to buffer ration of 1:20 to facilitate refolding of the recombinant pZP3. The following confirmatory and quality assurance tests were performed:

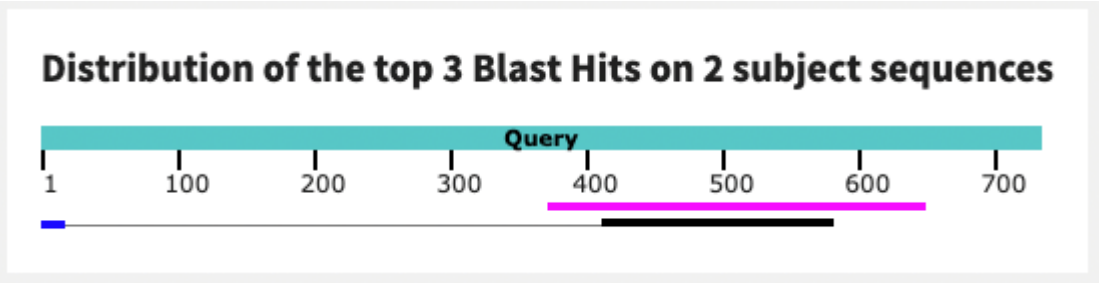
1. SDS-PAGE analysis: Samples were loaded onto 4-12% NuPAGE Bis-Tris protein gels (Thermo Fisher Scientific) and run in MES buffer under denatured and reduced conditions. Gels were stained using SimplyBlue™ SafeStain (Thermo Fisher Scientific) and imaged with a Chemi-Doc™ XRS+ imaging system (Bio-Rad Laboratories, Inc., CA, USA).
2. Western blot analysis: The SDS-PAGE gel was transferred onto PVDF membrane using iBlot® Western blotting system (Life Technologies) and probed with primary antibody MA451 (pZP3) at a dilution of 1:200 for 2 hours. Membranes were washed then probed using Immun-Star goat anti-mouse (GAM)-HRP conjugate (Bio-Rad) secondary antibody at a dilution of 1:15,000 for 1.5 hours. Chemiluminescent signals were developed using ECL chemiluminescent substrate reagent kit (Life Technologies) and visualized on a Chemi-Doc™ XRS+ imaging system.
3. Target protein concentration was estimated by absorbance at 280 nm using an Implen NanoPhotometer N120, taking into consideration the protein's extinction coefficient. Concentration measurements were adjusted for sample purity. Sample purity was estimated by densitometry analysis. The target protein molecular weight was calculated using ExPASy ProtParam tool (http://web.expasy.org/protparam/).
4. Endotoxin measurements were performed using an Endosafe-PTS Limulus Amebocyte Lysate (LAL) test kit (Charles River Laboratory).

**Figure 1.** Design of recombinant pZP4 used to vaccinate heifers in Exp.4

| Protein | Construct Design | Expression System | Molecular Weight |
|---|---|---|---|
| Porcine pZP4 | Bovine RNAse (94-104) epitope – dilysine linker – Protein of interest | Bacteria | 50.2 kDa |

As there was some evidence of infertility induced by vaccination against pZP2 (Experiment 1), an attempt was made to produce recombinant pZP2. Unfortunately, no obvious expression of pZP2 was detected by either SDS-PAGE or Western blot analysis. Although ZP2, ZP3, and ZP4 are of the same protein family and are subcellularly located together in the plasma membrane, they are all *different* proteins. Note differences in the amino acid sequences of ZP3 and ZP4 and ZP2 (Figure 2); the amino acid composition of ZP2 is 2× greater than ZP3. These distinct differences mean their expression behaviour in a recombinant host is independent of each other.

**Figure 2.** Comparison of similar and different protein sequences in pZP2, pZP3 and pZP4




- The cyan coloured query represents the protein sequence of pZP2.
- The pink represents pZP4 sequence alignment that has a close alignment to pZP2, where 39% of the pink region is identical.
- The blue region is the tetanus toxin and dilysine linker, a common construct design element shared with pZP2 and pZP3.
- The black region represents pZP3's sequence closely aligned to pZP2, where 23% of the black region is identical.

Several factors may have contributed to the absence of pZP2 expression in the *E. coli* system used (Table 1).

**Table 1.** List of reasons to explain failure of expression of ZP2

| Factors | Likelihood | Comments |
|---|---|---|
| Difficulty to express protein | High | • Target protein origin is from a higher eukaryotic organism. *E. coli* may lack the necessary cellular machinery to express it.<br>• Common eukaryotic alternatives include baculovirus/insect cell and yeast expression systems. |
| Codon bias | Med | • Construct was codon optimised for *E. coli* expression. |
| Target protein is toxic to cell | Low | • Cell strain and vector promoter features are tightly regulated.<br>• No evidence of impeded cell growth during expression optimisation. |

As a result of the failure of the Shrestha-pZP3& pZP4 protein vaccines to induce infertility in Experiments 3 and 4, investigation of other possible recombinant constructs and production systems were investigated. The project team was aware that a research group at Chiba University in Japan, led by Professor Naoto Yonezawa, had conducted a series of in-vitro studies to determine the sperm binding site in the cattle zona pellucida. These researchers have provided convincing evidence that ZP4 contains the major sperm binding sites. Further, they have developed a co-infection baculovirus system to produce a so-called recombinant bovine ZP3&4 heterocomplex which shows strong bovine sperm binding activity in-vitro (Yonezawa *et al.* 2005; Kanai *et al.* 2007).

A series of experiments were conducted by UQ-PEF to attempt to produce the ZP protein heterocomplex reported by the Chiba University Laboratory. Design of recombinant bZP3 and bZP4 was adapted from Dilimulati *et al.* (2021) and Kanai *et al.* (2007). Design of co-expressed bZP3&4 heterocomplex was adapted from Dilimulati *et al.* (2021), Shrestha *et al.* (2014), and Kanai *et al.* (2007).

**Attempt 1**

The design of the recombinant bZP3 and bZP4 constructs and the co-expressed bZP3&4 constructs are shown in Figure 3.

**Figure 3.** Design of first bZP3&4 heterocomplex based on reported Chiba University constructs

| Protein | Construct Design | Expression Vector | Molecular Weight |
|---|---|---|---|
| ZP3 | gp64 Signal Peptide – 6x Histidine tag – Thrombin cleavage site – S tag – Enterokinase cleavage site – Protein of Interest | pBac-1 | 42.1 kDa (Full length)<br>40.1 kDa (Secreted) |
| ZP4 | gp64 Signal Peptide – 6x Histidine tag – Thrombin cleavage site – S tag – Enterokinase cleavage site – Protein of Interest | pBac-1 | 55.3 kDa (Full length)<br>53.3 kDa (Secreted) |

From the attempted production of co-expressed bZP3&4, less than 0.07 mg of the bZP4 was purified from 200 mL baculovirus/insect cell culture supernatant. No co-expression of bZP3 was observed.

**Attempt 2**

As there was evidence that the majority of expressed protein in the first attempt was intracellular, to increase the yield of target proteins, the insoluble cell pellet was solubilized and on-column folding by IMAC chromatography was attempted. Unfortunately, once again the recovery of the target proteins was very low.

**Attempt 3**

Following a forensic review of the outcome of the second attempt to produce the Chiba pZP3&4 heterocomplex, the leader of the Chiba University team, Prof Yonezawa, was contacted and asked if he could provide UQ-PEF with the promoter (gp64) used in his team's experiments. This promoter is not commercially available and appeared to be a key difference between the UQ-PEF recombinant bZP3 and bZP4 constructs, and the Chiba constructs. Prof Yonezawa stated that he had found that the gp64 promoter is more suitable for the expression and secretion of ZP3 and ZP4 than the commercially available polyhedrin promoter used by UQ-PEF. Further, it was concluded that failure to detect a ZP3 protein band in the culture supernatants when ZP3 and ZP4 were co-expressed in baculovirus cells, was probably because the full region of mature ZP3 had attempted to be expressed. Research conducted by Prof Yonezawa's team has shown that co-expression of a specific fragment of bZP3 (Arg32-Glu178) and the mature full length of bZP4 (Lys25-Arg464) resulted in a complex with significant sperm binding activity, and overall, a higher yield of recombinant ZP3&4 protein. Figure 4 compares the design of the previous and new recombinant bZP3&4 constructs. Finally, based on previous results in which the purified ZP4 displayed low purity (22.3%), UQ-PEF added an additional step in their purification strategy (size exclusion chromatography, SEC) to increase the sample purity. With these changes, a third and final attempt was initiated to produce the Chiba University bZP3&4 heterocomplex.

**Figure 4**. Comparison of previous (2022) and final (2023) recombinant bZP3&4 constructs

| Protein | Construct Design | Promoter | Expression Vector |
|---|---|---|---|
| ZP3 (Arg32-Glu348)<br>ZP4 (Lys25-Arg464) | gp64 Signal Peptide – 6x Histidine tag – Thrombin cleavage site - S-tag – Enterokinase cleavage site - Protein of Interest | Polyhedrin | pBac-1 |
| ZP3 (Arg32-Glu178)<br>ZP4 (Lys25-Arg464)[1] | gp64 Signal Peptide – 6x Histidine tag – Thrombin cleavage site - S-tag – Enterokinase cleavage site - Protein of Interest | gp64 | pBACgus-6 |

Prof Yonezawa shipped to UQ-PEF plasmids of the recombinant bZP3 and bZP4 his laboratory had developed (Figure 5). He also provided details of the DNA sequence of each plasmid. Sequencing at the Australian Genome Research Facility verified the DNA sequence of both plasmids.

**Figure 5.** Detailed description of the Chiba University recombinant bZP3 and bZP4 proteins.

| Protein | Construct Design | Expression Vector | Molecular Weight |
|---|---|---|---|
| ZP3 (Arg32-Glu178) | gp64 Signal Peptide – 6x Histidine tag – S-tag – Protein of Interest | pBACgus-6 | 22.7 kDa (Full length)<br>22.0 kDa (Secreted) |
| ZP4 (Lys25-Arg464) | gp64 Signal Peptide – 6x Histidine tag – S-tag – Protein of Interest | pBACgus-6 | 55.5 kDa (Full length)<br>53.4 kDa (Secreted) |

The optimum protein expression conditions for the co-expressed ZP proteins were determined. Two insect lines (*Spodoptera frugiperda* - Sf9 and *Trichoplusia ni* - High FiveTM) were co-transfected with the recombinant baculovirus constructs. Two different culture temperatures, harvest time points and times of infection were evaluated. Both ZP proteins were co-expressed and detected in the supernatant fraction, rather than the intracellular fraction as occurred in previous attempts to produce these proteins. This was a critically important finding. The optimal condition for ZP3 and ZP4 secreted protein expression was in the High FiveTM cell line cultured at 27°C, 48hr post infection.

Initial scale up of production of the new ZP protein constructs was successfully achieved. A ~90 kDa band consistent with the expected molecular weight of a heterocomplex of ZP3&4 was detected in the SDS-PAGE gel. Mass Spectrometry (Peptide Mass Fingerprinting Intact Mass Analysis) was conducted on excised bands (~90 kDa, ~60 kDa, ~30 kDa) from the gel. The bands at ~60 kDa and ~30 kDa were identified as ZP4 and ZP3 respectively. No high-confidence peptide hits corresponding to ZP3 or ZP4 were identified for the band at ~90 kDa. The apparent absence of any evidence of a ZP3&4 heterocomplex was very concerning and thus it was decided that before any cattle were vaccinated with the co-expressed ZP3&4 proteins, the sperm binding activity of these proteins must be determined.

The Chiba University team has a well-established in-vitro competitive inhibition assay (Yonezawa et al, 2005; Kanai et al, 2007), which quantifies the inhibitory effects of purified or recombinant ZP proteins on bovine sperm-bovine zona binding. The methodology is described in Appendix 8.4. Prof Yonezawa offered to compare the inhibitory effects of the UQ-PEF recombinant ZP3&4 proteins with the same co-expressed proteins produced in his laboratory. A sample of the UQ-PEF co-expressed ZP3&4 proteins was sent to Chiba University. Subsequently, Prof Yonezawa reported that the UQ-PEF proteins had inhibited the binding of bovine sperm to plastic wells coated with bovine zona pellucida protein, and the degree of inhibition was greater than that achieved by the Chiba recombinant protein (89% v's 49%). Based on these findings, it was decided to proceed as planned with cattle Experiment 5.

### 3.1.4 Evaluation of candidate vaccine delivery systems

A thorough review of published reports of development and use of single-shot annual vaccines was conducted. Initially of particular interest was the Spay-Vac™ delivery system (adjuvant), which is manufactured in the USA, and has been used in pZP vaccines to successfully prevent pregnancy in a broad range of mammals from grey seals to wild horses. This delivery system is based on polylactide-glycolide incorporation of antigen which is then released over time. However, there are some significant regulatory issues affecting potential use of this system. Spay-Vac Inc. produces the delivery vehicle on licence from the patent holders. The company then incorporates the antigen as desired. The licencing arrangement prevents the delivery system being provided by Spay-Vac Inc. to a third party. Therefore, pZP produced here would need to be sent to the USA for incorporation into

the delivery system, and then the vaccine returned to Australia for injection into animals. The following regulatory 'hurdles' were identified:

1. AQIS indicated that the vaccine would need to use pZP produced from certified disease-free animals.
2. The pZP would require provenance to ensure delivery in the USA.
3. The Spay-Vac Inc facilities would need to be inspected by the USDA on behalf of AQIS and given clearance.
4. An export certificate from the USDA would be required and AQIS would need to issue an import permit.
5. Use of the vaccine would require APVMA approval.

Some of these obstacles would be obviated or minimised if pZP was replaced by recombinant ZP protein(s). However, the recombinant ZP protein(s) would need to be produced by a laboratory certified to meet GLP standards, of which few exist and even fewer are commercially available in Australia. As a consequence of the above and likely costs of accessing this delivery system, Spay-Vac™ was rejected as a suitable delivery system.

A second novel delivery system developed as part of the Polymer Cooperative Research Centre by Swinburne University of Technology and The University of Queensland was also considered a prime candidate for use in the pZP vaccine. This delivery system has undergone evaluation initially to deliver a tick vaccine in sheep and more recently in cattle, where significant immune responses were generated, comparable to those achieved with the current leading commercially available vaccine (B.AHE.0316). However, in late 2019 the research team was made aware of some side-effects in cattle that were revaccinated using this delivery system. As it is very likely that some cattle vaccinated to prevent pregnancy in extensive management systems may be vaccinated a second time to prolong their period of infertility, the risk of these side effects resulted in this delivery system also being rejected.

A number of commercially available delivery systems which have been used in livestock vaccines were considered. The proprietary product of CSL ISCOMATRIX (poly L glutamic acid micro particles which rely on different physico-chemical methods to incorporate the antigen) was considered but CSL informed us that this product had been withdrawn, and they recommended we consider use of one of the Montanide™ products (highly refined mineral oil emulsion adjuvants) produced by SEPPIC. These adjuvants have been recently investigated for use in Johne's disease vaccines in Australia (Thakur *et al*. 2018; Begg *et al.* 2019) and for single-shot Foot and Mouth Disease vaccination (Ibrahim *et al.* 2015). After consultation with scientists at SEPPIC, it was decided to trial use of Montanide™ ISA VG 61, a mineral oil based adjuvant which has been developed for the formulation of water-in-oil (W/O) emulsions. It is based on a specific enriched light mineral oil and a highly refined emulsifier obtained from mannitol and purified oleic acid of vegetable origin. Montanide™ ISA VG 61 is recommended by SEPPIC for use in single annual vaccination formulations and where induction of a high antigen specific cell mediated immune (CMI) response is required. Evidence that pZP vaccination induces a CMI response further supported selection of this adjuvant for use in the planned cattle experiments in this project. Also, the project team was aware that the SingVac (Virbac) single shot vaccine for prevention of botulism is formulated as a water-oil-water emulsion.

### 3.1.5 Formulation and administration of zona pellucida protein vaccines

Experimental vaccines were formulated aseptically in a standard laboratory laminar flow facility. Details of each vaccine formulation are listed in Table 2. The volumetric ratios (v/v) of ZP protein to adjuvant were 50:50 and 40:60 for Freund's and Montanide™ ISA VG 61, respectively. The required volumes of proteins (purified or recombinant) were adjusted by adding phosphate buffered saline. Each formulation was emulsified by vortexing for 2 minutes. Individual doses of vaccine were then drawn into 5 ml syringes and a 20G one inch needle attached.

Each animal was restrained in the crush and vaccinated by subcutaneous injection either in front of or behind the shoulder.

**Table 2. Vaccine formulation for different treatment groups in each experiment.**

| Experiment | Protein | Adjuvant | Total Quantity (µg) | Priming Vaccine (µg) | Booster 1 (µg) | Booster 2 (µg) |
|---|---|---|---|---|---|---|
| Exp 1 | pZP | Freund's# | 400 | 200 | 100 | 100 |
| | pZP2 | Freund's# | 350 | 150 | 100 | 100 |
| | pZP3&4 | Freund's# | 350 | 150 | 100 | 100 |
| Exp 2 | pZP | Montanide* | 200 | 200 | N/A | N/A |
| | pZP3&4 | Montanide* | 200 | 200 | N/A | N/A |
| Exp 3 | pZP | Montanide* | 500 | 500 | N/A | N/A |
| | rZP3 | Montanide* | 500 | 500 | N/A | N/A |
| | rZP3 | Freund's# | 500 | 200 | 150 | 150 |
| Exp 4 | rZP3+rZP4 | Montanide* | 200 | 100+100 | N/A | N/A |
| Exp 5 | rZP3&4 | Montanide* | 200 | 200 | N/A | N/A |

#Complete Freund's for 1st vaccination, then incomplete Freund's for 2nd and 3rd vaccinations

* Target protein suspended in Montanide™ ISA VG 61

## 3.2 Monitoring response to ZP vaccination of tropically adapted cattle

### 3.2.1 Cattle selection and management

A total of 93 tropically adapted rising 2-year-old heifers broadly representing the common genotypes (Brahman, Brahman cross, Composite) found across northern Australia were purchased from two commercial beef cattle properties in southern and central Queensland for the project. All cattle were quarantined upon arrival at The University of Queensland Pinjarra Hills Research Precinct and were subsequently vaccinated against tick fever and bovine ephemeral fever. Pastures grazed by experimental cattle were typical of coastal southern and central Queensland. Cattle grazed improved mixed sub-tropical pastures consisting of Callide Rhodes grass (*Chloris gayana)*, Green Panic (*Panicum maximum var. trichoglume*) and Setaria (*Setaria sphacelate)* and were managed to maintain good body condition. Depending on seasonal pasture conditions, cattle were supplemented with forage sorghum hay and molasses-urea blocks. The health and well-being of the cattle were checked daily.

All experiments and collection of ovaries after slaughter were conducted under Animal Ethics Certificates issued by The University of Queensland:

- **2018/AE000180**: Developing and testing an immunocontraceptive vaccine for cattle
- **2021/AE000372**: ANRFA - Collection of bovine ovaries at slaughter

Further, all experiments were conducted under APVMA Small-scale Trial Permit PER7250 which requires that animals be only sent to slaughter a minimum of 12 months after last treatment.

### 3.2.2 Design of each experiment

In total, five experiments were conducted. Each experiment was designed to achieve specific objectives (Table 3).

**Table 3.** Objectives for each experiment

| Experiments | Timeline * | Experimental objective |
|---|---|---|
| Exp 1 | 3/04/2019 to 26/05/2020 | To determine the impact on fertility of vaccination against different purified ZP proteins formulated with Freund's adjuvant. To determine which ZP protein(s) should be selected for recombinant protein synthesis |
| Exp 2 | 9/01/2020 to 11/05/2021 | To evaluate the impact on fertility of the single administration of a vaccine containing either porcine ZP or a purified mix of porcine ZP3 & 4 formulated with Montanide™ ISA VG 61 |
| Exp 3 | 19/03/2021 to 19/05/2022 | To evaluate the impact on fertility of the single administration of a vaccine containing either porcine ZP or a recombinant ZP3 protein formulated with Montanide™ ISA VG 61 |
| Exp 4 | 13/10/2021 to 30/11/2022 | To evaluate the impact on fertility of the single administration of a vaccine containing a 1:1 mix of recombinant ZP3 and ZP4 proteins formulated with Montanide™ ISA VG 61 |
| Exp 5 | 20/10/23 to 20/10/24 | To evaluate the impact on fertility of the single administration of a vaccine containing a heterocomplex of recombinant ZP3 and ZP4 proteins formulated with Montanide™ ISA VG 61 |

* Starting from the date of first vaccination and ends on slaughter date.

All heifers (n=91) were randomly assigned to experimental groups such that the groups were balanced for body weight, body condition and breed composition (Table 4). Control (unvaccinated) and treatment (vaccinated) heifers were managed together as a single mob throughout each experiment. However, each vaccinated heifer also effectively acted as its own control for all parameters. Heifers selected for each vaccination treatment were examined, blood sampled, and their ovaries scanned several weeks prior to first vaccination and at the time of first vaccination. Further, the heifers which were initially selected as controls were then subsequently selected to be used in later vaccination experiments. As these control heifers had undergone several periods of mating their fertility status had been determined prior to being selected for one of the vaccination experiments.

**Table 4.** Distribution of heifers in treatment groups across five experiments.

| Treatment group | Exp 1 | Exp 2 | Exp 3 | Exp 4 | Exp 5 |
|---|---|---|---|---|---|
| Control | 18* | 8 | - | 4^ | |
| pZP | 5 (F) | 4 (M) | 9 (M) | | |
| pZP2 | 7 (F) | | | | |
| pZP3&4 | 9 (F) | 6 (M) | | | |
| rZP3-Montanide | | | 9 (M) | | |
| rZP3-Freunds | | | 6 (F) | | |
| rZP3+rZP4 (100ug of each) | | | | 12 (M) | |
| rZP3&4 | | | | | 16 (M) |
| **Total** | **39** | **18** | **24** | **16** | **16** |

*These control animals were used in Exp 2, 10 (4+6) for treatment and 8 as controls.

(F)= Freund's adjuvant; (M)= Montanide™ ISA VG 61 adjuvant

Details of the vaccine administered to each treatment group are described below:

**Exp 1** – pZP-F group – Each heifer was injected with 2 ml of prepared vaccine emulsion containing 200 µg of pZP and Freund's (complete) adjuvant at 50:50 v/v. Six and twelve weeks later, each heifer was injected with 2 ml of emulsion containing 100 µg of pZP and Freund's (incomplete) adjuvant at 50:50 v/v.

- pZP2-F group - Each heifer was injected with 2 ml of prepared vaccine emulsion containing 200 µg of pZP2 and Freund's (complete) adjuvant at 50:50 v/v. Six and twelve weeks later, each heifer was injected with 2 ml of emulsion containing 100 µg of pZP2 and Freund's (incomplete) adjuvant at 50:50 v/v.

- pZP3&4-F group - Each heifer was injected with 2 ml of prepared vaccine emulsion containing 200 µg of pZP3&4 and Freund's (complete) adjuvant at 50:50 v/v. Six and twelve weeks later, each heifer was injected with 2 ml of emulsion containing 100 µg of pZP3&4 and Freund's (incomplete) adjuvant at 50:50 v/v.

**Exp 2** – pZP-M group - Each heifer was injected once with 1ml of prepared vaccine emulsion containing 200 µg of pZP and Montanide™ ISA VG 61 at 40:60 v/v ratio.

- pZP3&4-M group - Each heifer was injected once with 1ml of prepared vaccine emulsion containing 200 µg of pZP3&4 and Montanide™ ISA VG 61 at 40:60 v/v ratio.

**Exp 3** – pZP-M group - Each heifer was injected once with 6 ml of prepared vaccine emulsion containing 500 µg of pZP and Montanide adjuvant both at 40:60 v/v ratio.

- rZP3-M group - Each heifer was injected once with 6 ml of prepared vaccine emulsion containing 500 µg of rZP3 and Montanide™ ISA VG 61 at 40:60 v/v.

- rZP3-F group - Each heifer was injected with 2 ml of prepared vaccine emulsion containing 200 µg of rZP3 and Freund's (complete) adjuvant at 50:50 v/v. Two and four weeks later, each heifer was injected with 2 ml of emulsion containing 150 µg of rZP3 and Freund's (incomplete) adjuvant at 50:50 v/v.

**Exp 4** – Each heifer was injected once with 3 ml prepared vaccine emulsion containing 100 µg of rZP3 + 100ug of rZP4 suspended in Montanide™ ISA VG 61 at 40:60 v/v.

**Exp 5** - Each heifer was injected once with 3 ml prepared vaccine emulsion containing 200 µg of co-expressed recombinant pZP3&4 suspended in Montanide™ ISA VG 61 at 40:60 v/v.

### 3.2.3 Monitoring of cattle health

Each time cattle were handled through the cattle handling facilities, their general health and condition was assessed. Following vaccination, the site of vaccination was examined for signs of any swelling and an estimate of the size of any swelling recorded. Body weight and body condition score (BCS 1 = poor, 5 = fat) were recorded approximately monthly when cattle underwent ovarian scanning and blood sampling.

### 3.2.4 Transrectal ultrasound monitoring of ovarian morphology

Heifers were scanned at least once several weeks prior to first vaccination, then again close to the time of first vaccination and then once a month until just prior to being sent to slaughter. All transrectal ultrasonography of the ovaries and uterus was conducted using a SonoSite M-Turbo ultrasound machine (FUJIFILM). Representative images of each ovary were recorded and inbuilt applications were used to measure the Length, Width and Girth, which were then used to calculate the ovarian volume (OV), i.e., OV = Length x Width x Girth. The average volume of both ovaries was used for statistical comparisons between different experimental groups and different time points. Measurement of ovarian volume was discontinued after the first mating in Exp.4 as there was no evidence of any significant change after vaccination.

A short video of each ovary was also recorded during the scanning procedure. Total antral follicle count (AFC) was manually determined (Ireland et al 2008) by visual examination of videos of both ovaries. Total number of antral follicles 3 mm or greater in diameter per pair of ovaries was recorded. The AFC data were used to compare variation among the heifers as well as if there were any significant changes in AFCs after each vaccination in different treatment groups of all experiments.

### 3.2.5 Measurement of Anti-Mullerian Hormone in plasma

Because of the reported strong association (Ireland et al 2008) between the ovarian antral follicle count and circulating concentrations of Anti-Mullerian Hormone (AMH), selected plasma samples from Experiment 1 were assayed using a Bovine AMH enzyme-linked immunosorbent assay AL-114kit (Ansh Labs, USA). This assay has been validated for cattle (Ireland et al 2008). Only samples from Exp.1 were assayed because there was no evidence of a significant change in AFC after vaccination in the other experiments.

### 3.2.6 Measurement of immune response to vaccination

Initially every fortnight until week 6 after first vaccination and then monthly thereafter, blood samples were collected to monitor the immune responses to each vaccine. In most cases blood sampling was conducted at the time of ultrasonography of the ovaries. Blood samples were collected from the tail vein of each heifer in two vacutainers, a 10 ml serum vacutainer (red cap) and 9 ml plasma vacutainer (heparin coated, green cap). Both tubes were centrifuged at 3600 rpm at 4$^{o}$C for 15 minutes. The supernatant from each tube (red and green) were transferred into two labelled tubes, i.e., aliquots of 400 µl from each heifer were sent to different laboratories, i.e., serum for ELISA and plasma for Anti-

Mullerian hormone assays. The remaining volumes of serum and plasma were stored in two sets of labelled tubes (two for serum and two for plasma). One set was stored at -80°C for long term storage and the other was stored at -20°C for short term usage.

Using standard ELISA methodology (Hornbeck, 2015), Professor Ken Beagley (Centre for Immunology and Infection Control, Queensland University of Technology) developed assays to assess the immune response to vaccination against native pZP2, pZP3 and pZP4, and recombinant ZP3 and ZP4. The ELISA procedure developed is as follows:

1. Coat plates with 50 µl of specific pZP protein at 20 ng/well (0.4 µg/ml solution) in 50 mM carbonate-bicarbonate buffer (pH 9.6) and incubate for 1 hour at 37°C.
2. Wash × 3 in wash buffer.
3. Block wells in 100 µl RD for 1 hour at room temperature.
4. Remove block (do not wash).
5. Add 50 µl serum diluted 1:800 – 1:12800* in RD and incubate for 1 hour at room temperature on orbital plate shaker. Assay each sample in duplicate.
6. Wash × 3 in wash buffer.
7. Add 50 µl sheep anti-bovine IgG:HRP (horse radish peroxidase) diluted 1:10,000 in RD and incubate for 1 hour at room temperature on orbital plate shaker.
8. Wash × 3 in wash buffer.
9. Wash × 3 in PBS.
10. Add 50 µl of 100 mg/ml TMB in DMSO (diluted 1:1,000 in phosphate citrate buffer, with 2 µl of 30% $H_2O_2$ added per 10 ml of phosphate citrate buffer) and incubate for 10 minutes.
11. Stop reaction with 50 µl of 1 M $H_2SO_4$.
12. Read plate at 450 nm.

**Wash buffer** = PBS-T (PBS with Tween-20 (0.05% v/v), pH 7.4) **Reagent diluent** = RD (PBS with Tween-20 (0.05% v/v) and Fraction V BSA (1% w/v))

Overall average absorbance values before vaccination were considered as the base to detect the fold change in immune response at different time points.

### 3.2.7 Mating management and pregnancy diagnosis

At the commencement of the project, a rising 2-year-old Droughtmaster bull from The University of Queensland's herd was selected for use in the project. The bull underwent and passed the Australian Cattle Veterinarian's approved breeding soundness examination. The bull had a scrotal circumference of 36 cm and >70% morphologically normal sperm. This bull was used for all matings conducted in the project. Throughout the project, based on the pregnancy rate achieved when mated to unvaccinated control heifers, the bull's fertility was considered normal. Details of timing of matings after vaccination are summarised in Table 5.

**Table 5.** Timing of matings after vaccination and duration of mating for each experiment

| Exp. No | Vaccination* to 1st mating (days) | Duration of 1st mating (days) | Vaccination* to 2nd mating (days) | Duration of 2nd mating (days) | Vaccination* to 3rd mating (days) | Duration of 3rd mating (days) | Vaccination to slaughter (days) |
|---|---|---|---|---|---|---|---|
| 1 | 111 | 56 | 313 | 91 | N/A | N/A | 419 |
| 2 | 32 | 91 | 236 | 83 | 405 | 61 | 488 |
| 3 | 46 | 62 | 182 | 39 | 312 | 45 | 426 |
| 4 | 40 | 35 | 149 | 41 | 323 | 42 | 398 |
| 5 | | | | | | | |

* Days from last vaccination to mating

Experiment 1 matings:

- *Mating period 1*: 112 to 167 days from vaccination (56 days with bull)
- *Mating period 2*: 313 to 404 days from vaccination (91 days with bull)

Experiment 2 matings:

- *Mating period 1*: 33 to 123 days from vaccination (91 days with bull)
- *Mating period 2*: 236 to 319 days from vaccination (83 days with bull)
- *Mating period 3*: 405 to 466 days from vaccination (61 days with bull)

Experiment 3 matings:

- *Mating period 1*: 47 to 108 days from vaccination (62 days with bull)
- *Mating period 2*: 182 to 221 days from vaccination (39 days with bull)
- *Mating period 3*: 312 to 357 days from vaccination (45 days with bull)

Experiment 4 matings:

- *Mating period 1*: 41 to 76 days from vaccination (35 days with bull)
- *Mating period 2*: 149 to 190 days from vaccination (41 days with bull)
- *Mating period 3*: 323 to 365 days from vaccination (42 days with bull)

Experiment 5 matings

### 3.2.8 Slaughter and histopathological evaluation of ovaries

Ovaries from heifers in each experiment were removed at slaughter at a local abattoir. The intervals from last vaccination to slaughter were 419, 488, 426, 398 and 365 for Experiment 1, 2, 3, 4 and 5, respectively. There was some variation in timing of slaughter due to COVID. Further, it should be noted that standard carcass data for each vaccination group slaughtered was collected. The ovaries from a randomly selected group of sixteen rising 2-year-old Brahman and Brahman cross heifers, which had not been vaccinated, was also collected at the same abattoir. The excised ovaries were immediately placed in an esky with a cool brick and then transported to The University of Queensland's School of Veterinary Science pathology facilities. The ovaries were held at 4°C until they were grossly examined, and then sections of each ovary were fixed in neutral buffered formalin prior to being embedded in paraffin blocks. Sections (5 micron) were cut from the paraffin blocks, mounted on glass slides, and stained with haematoxylin and eosin (H&E), before being evaluated under 20x magnification.

The ovaries were assessed histologically in two ways. Firstly, a follicular atrophy scoring system was developed based on the total number of primordial follicles in 10 fields, i.e. Grade I (least abnormal): > 20 follicles in 10 fields; Grade II: > 10 to 20 follicles in 10 fields; Grade III: 5 to 10 follicles in 10 fields; and Grade IV (most severely abnormal): < 5 follicles in 10 fields (Figures 6). Secondly, the number of antral follicles (i.e., tertiary and mature follicles) were counted in one H&E-stained slide from each heifer. Follicles were classified as follows: 1) primordial – single layer of flattened follicular cells; 2) primary – single layer of cuboidal follicular cells; 3) secondary – two or more layers of follicular cells and development of zona pellucida; 4) tertiary – development of follicular antrum; and 5) mature – greater than 10 mm in diameter with a large follicular antrum.

**Figure 6.** Section from control ovary with Grade I atrophy (> 20 primordial follicles per 10 microscopic fields). In this figure, at least 10 primordial follicles (arrows) are observed in the cortex. 20x magnification.

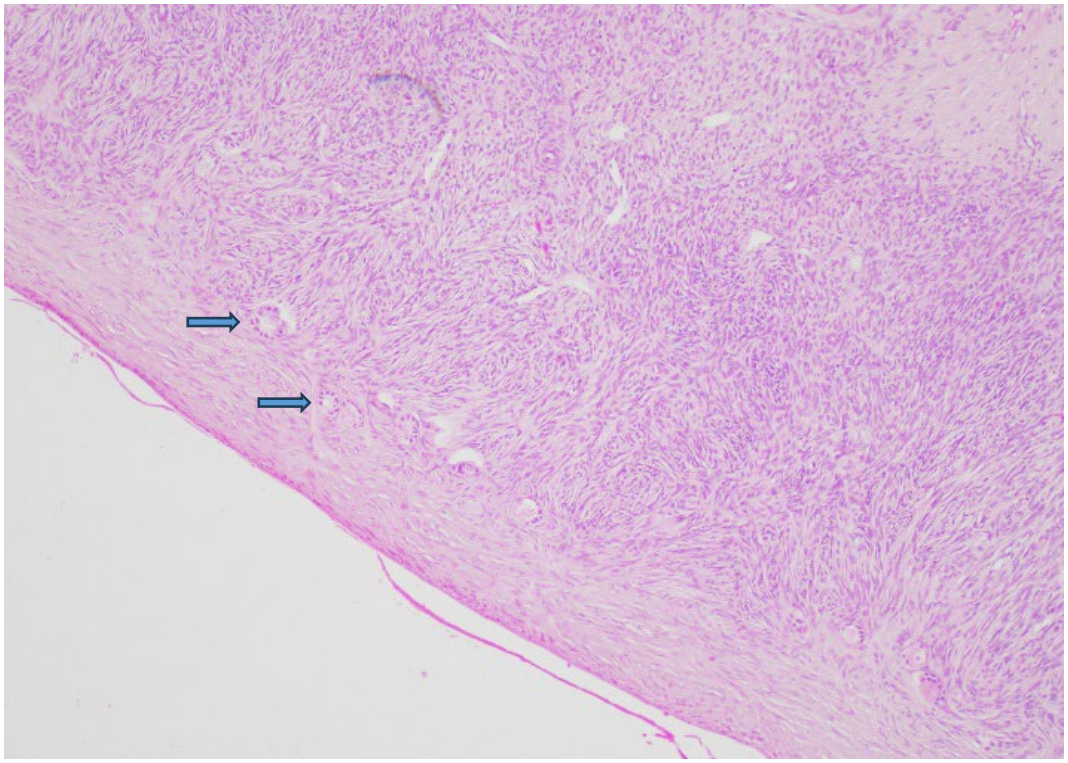


**Figure 7.** Section from vaccinated ovary with Grade I atrophy (> 20 primordial follicles per 10 microscopic fields). Many primordial follicles (arrows) with a well-defined granulosa cell layer are present in the cortical stroma.

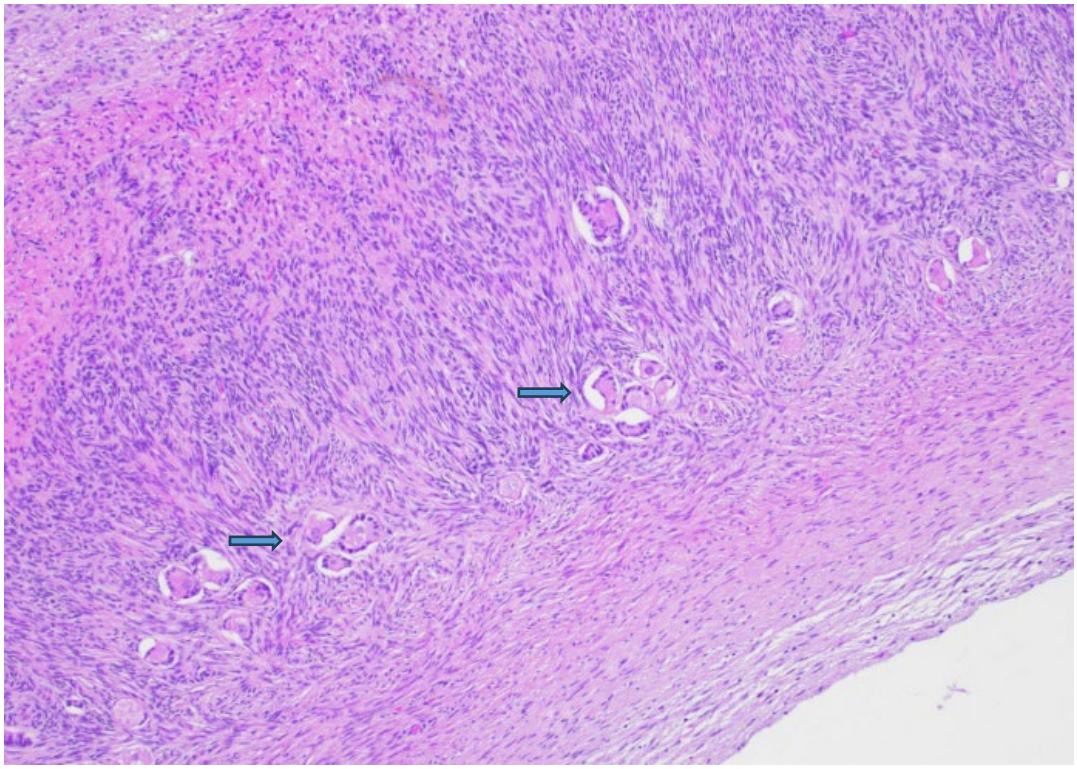


**Figure 8.** Section from vaccinated ovary with Grade II atrophy (> 10 to 20 primordial follicles per 10 microscopic fields). Low numbers of primordial follicles (arrow) are present in the cortex. Note the corpus albicans on the top right.

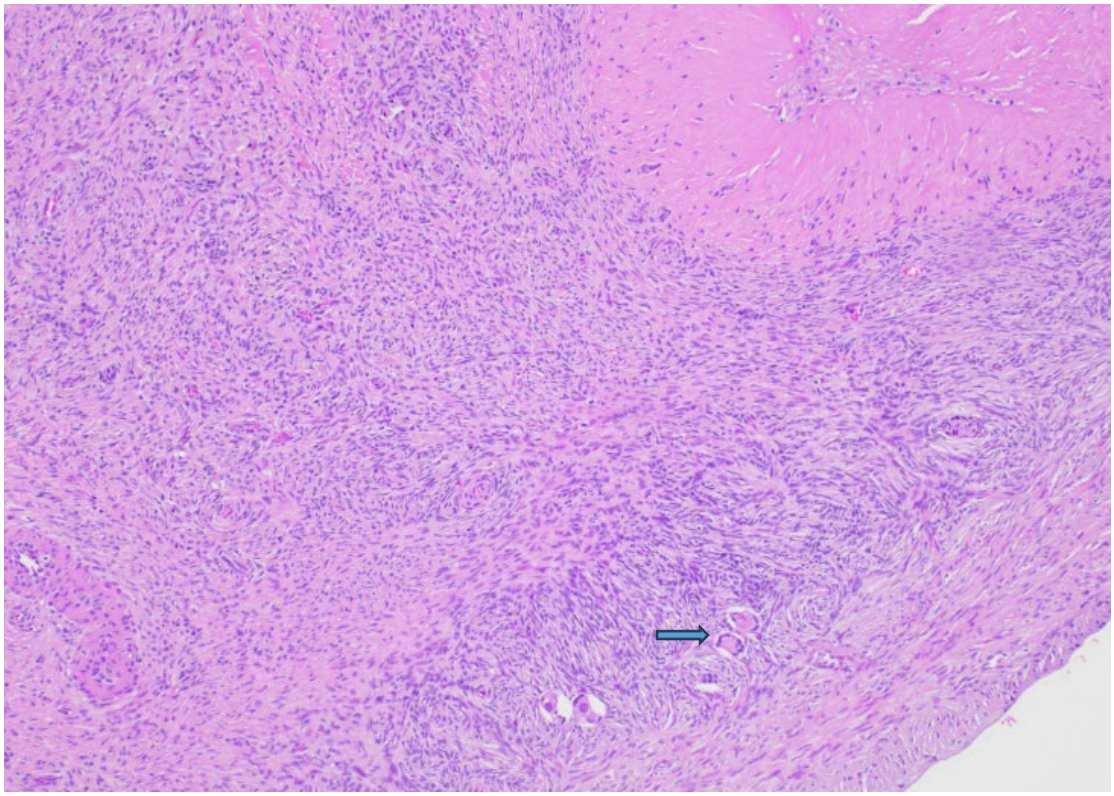


**Figure 9.** Section from vaccinated ovary with Grade III atrophy (5 to 10 primordial follicles per 10 microscopic fields). Only occasional primordial follicles (arrow) can be observed, with the cortex mainly consisting of fibrous tissue and fibroblasts.

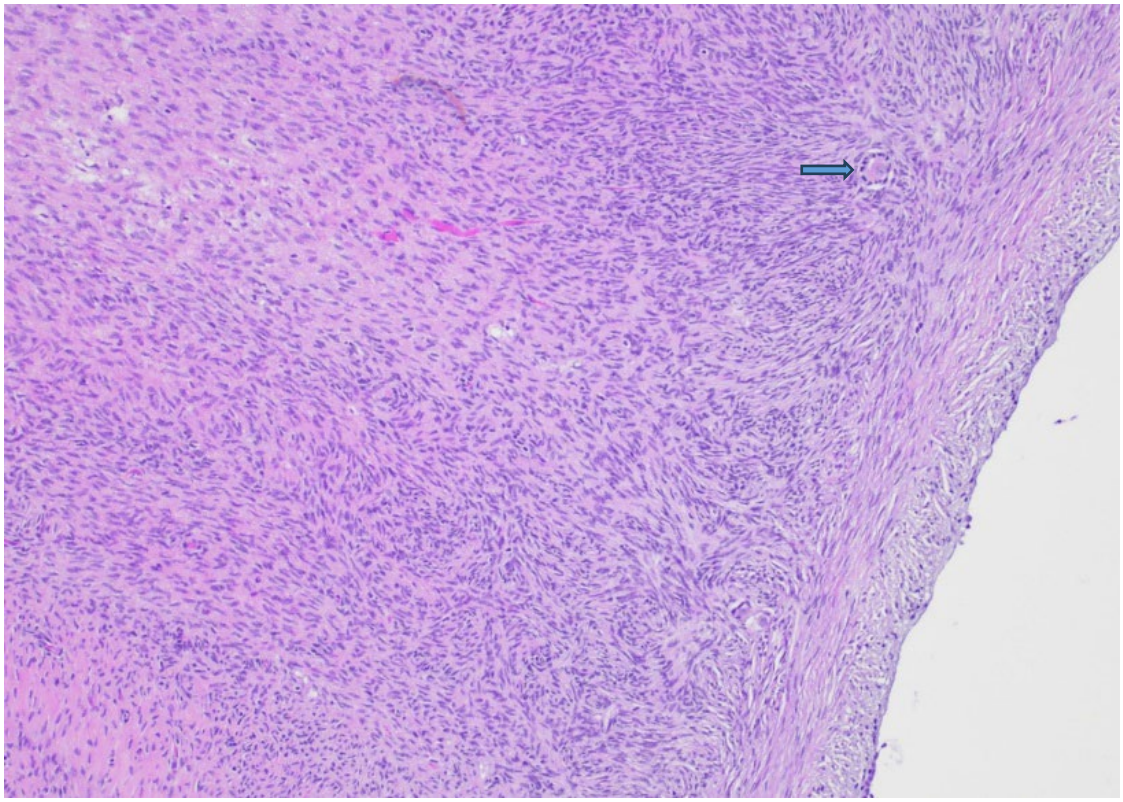
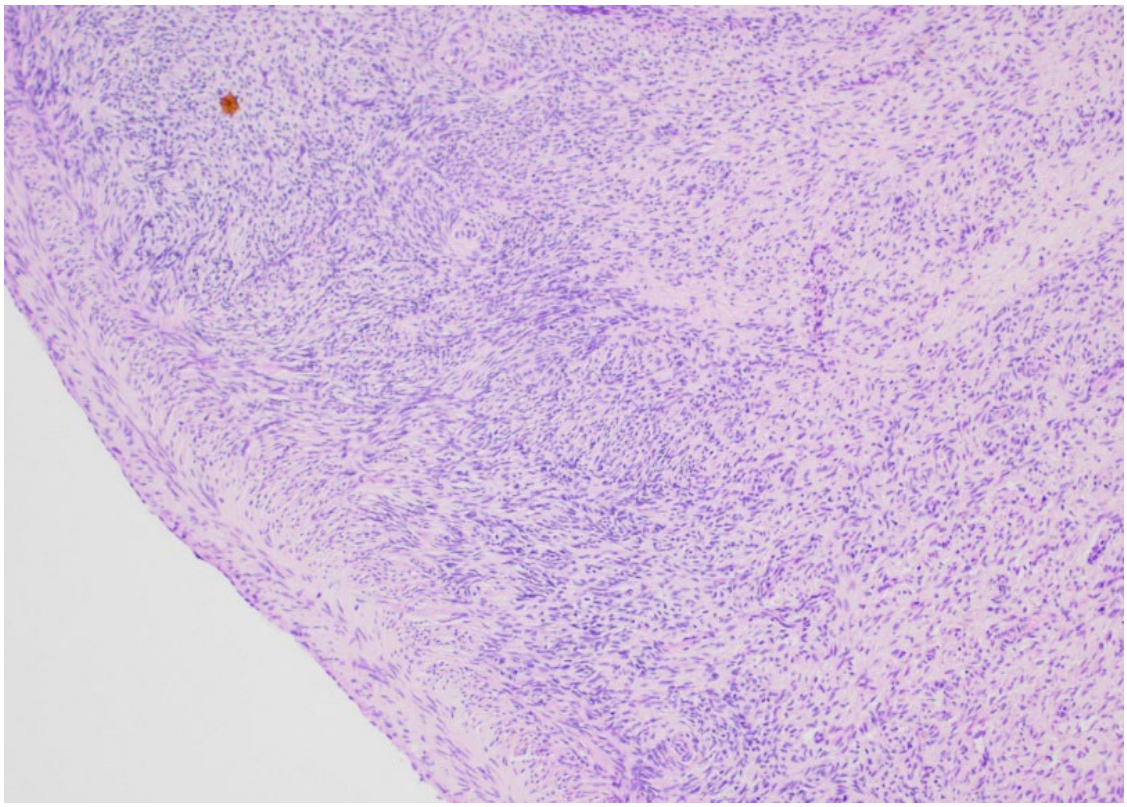


**Figure 10.** Section from vaccinated ovary with Grade IV atrophy (< 5 primordial follicles per 10 microscopic fields). There is a complete absence of primordial follicles.



### 3.2.9 Immunohistochemical evaluation of ovaries

Immunohistochemistry (IHC) was performed on the ovaries to assist in identification of B and T lymphocytes, and to assess ZP3 and ZP4 expression. B-cell-specific activator protein (BSAP) and CD3 were chosen as the B and T cell markers, respectively.

IHC was performed on 4 micron serial sections prepared from one paraffin block from each heifer. The specifications and dilutions of the primary antibodies, and the antigen retrieval solution and positive control tissues used are outlined in Table 6. Positive and negative control slides were included in each run. Negative control slides consisted of one slide where the primary antibody was replaced by antibody diluent (EnVision FLEX Antibody Diluent, Dako) and a second slide where it was replaced with a non-immune, species matched immunoglobulin (Mouse IgG Control Antibody, Vector Laboratories, or Rabbit IgG Control Antibody, Vector Laboratories).

**Table 6.** Specifications, dilutions, antigen retrieval methods, and positive control tissues used with BSAP, CD3, ZP3 and ZP4 primary antibodies.

| Antibody | Company | Clone | Type | Dilution | Antigen Retrieval Solution | Positive Control Tissue |
|---|---|---|---|---|---|---|
| **BSAP** | Dako | DAK-Pax5 | Mouse monoclonal | 1:100 | Low pH | Normal bovine lymph node |
| **CD3** | Dako | | Rabbit polyclonal | 1:200 | High pH | Normal bovine lymph node |
| **ZP3** | | | Mouse monoclonal | 1:1600 | Low pH | Normal bovine ovary |
| **ZP4** | | | Mouse monoclonal | 1:50 | Low pH | Normal bovine ovary |

The slides were deparaffinised and rehydrated, and then heat induced epitope retrieval was performed at 97°C for 20 minutes using antigen retrieval solution (EnVision FLEX Target Retrieval Solution High pH, Dako, or EnVision FLEX Target Retrieval Solution Low pH, Dako) in a Dako PT Link machine. Once the slides cooled to 65°C, they were washed in buffer (EnVision FLEX Wash Buffer, Dako) for 5 minutes. The slides were then incubated with the primary antibody or negative control for 30 minutes, except for the ZP4 slides which were incubated in 5% normal goat serum (Sigma-Aldrich) for 30 minutes prior to incubation with the primary antibody/negative control. The remaining steps were performed on a Dako Autostainer Link machine. The slides were washed in buffer for 5 minutes and then incubated with endogenous peroxidase blocking solution (EnVision FLEX Peroxidase-Blocking Reagent, Dako) for 5 minutes. Next, they were rinsed with buffer before being incubated with labelled polymer (EnVision FLEX/HRP, Dako) for 20 minutes. The slides where then washed in buffer for 5 minutes, incubated with chromogen (EnVision FLEX Substrate Working Solution, Dako) for 10 minutes, washed in buffer for 5 minutes, and incubated in haematoxylin (EnVision FLEX Hematoxylin, Dako) for 5 minutes. Finally, the slides were rinsed with distilled water, washed in buffer for 5 minutes, and rinsed with distilled water again before being dehydrated and mounted.

To assess B lymphocyte numbers, the number of cells expressing BSAP were counted in 10 randomly selected high power fields per slide, and then the average for each slide was calculated. To assess T lymphocyte numbers, the number of cells expressing CD3 were counted in 10 randomly selected high power fields per slide, and then the average for each slide was calculated.

## 3.3 Statistical analyses

The parameters measured in each experiment were classified continuous (body weight, ovary volume, antral follicle counts, IgG titre, ordinal (body condition score), and binary outcomes (presence/absence of corpus luteum) and pregnancy status (yes/no)). The analysis encompassed a time frame where Day 0 denoted the day of vaccination. Outcome variables were documented both prior to and following vaccination, including subsequent days at different intervals up to slaughter.

Statistical analyses were performed to assess significant differences between control and vaccinated heifers within each experiment by implementing a mixed model regression approach. The fixed effect considered was the treatment, distinguishing between the control and vaccine groups, while the random effect accounted for individual variability among heifers for repeated measures at different days following vaccination. The model equation was presented as.

$$\boldsymbol{y} = \boldsymbol{X\beta} + \mathbf{Z}\boldsymbol{\mu} + \boldsymbol{\epsilon}$$

Where $\boldsymbol{y}$ is the outcome variable**,** $\boldsymbol{X}$ matrix of predictor variables, $\boldsymbol{\beta}$ is a vector of the fixed effects regression coefficients; $\mathbf{Z}$ is the design matrix of random effects; $\boldsymbol{\mu}$ is a vector of random effects and $\boldsymbol{\epsilon}$ is a vector of the residuals. Evaluations were conducted within and across various treatments, integrating an interaction term (days from vaccination × treatment) to evaluate changes over time. In experiments 1, 2 and 4, control groups were utilized as benchmarks for parameters with adequate data (e.g., weight and BCS), whereas parameters like OV, AFC and IgG were not recorded in controls, therefore pre-vaccination data were utilized as points of reference.

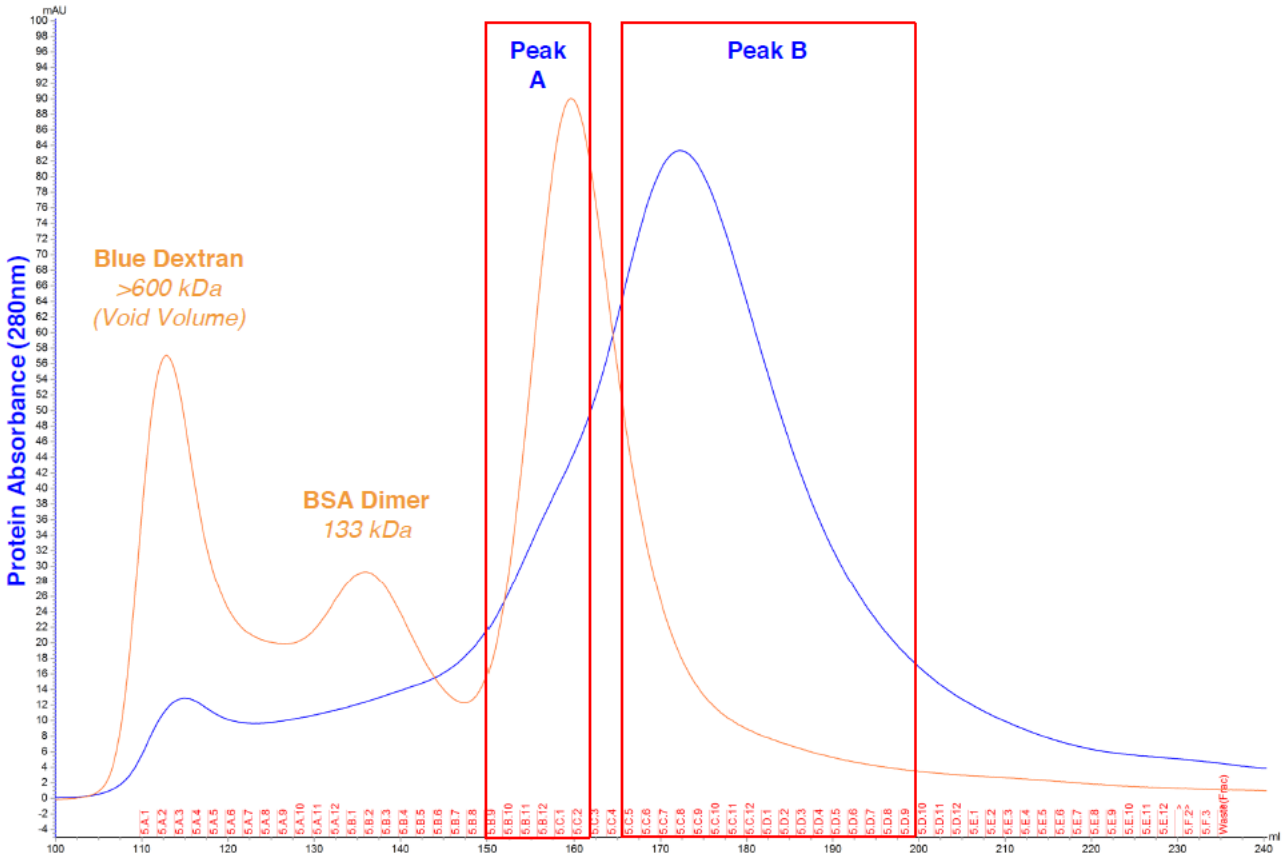
Data management and analyses were performed using the R programming language (R Core Team, 2023). Given the continuous, ordinal and binary types of response variables, the R packages: lme4, ordinal and stats were used, respectively. Total immune response was computed from the area under the curve using the pracma package. Then a generalised linear model was used for pregnancy as an outcome of the total immune response. The fisher.test function was used for Fisher's Exact Test to analyse the significant differences in fertility (proportion pregnant after each mating period) between various groups. The results are provided as figures and tables, and significance threshold of $p = 0.05$ was used.

# 4. Results

## 4.1 Purification of porcine zona pellucida proteins

Two peaks with variable degrees of overlap were detected (Figure 11). Peak A corresponds to a 60-80kD band and peak B corresponds to a 55kD band. Because zona proteins are heavily glycosylated they do not band tightly as protein bands but rather smear making accurate estimations of molecular weight difficult. The smearing is due to heterogeneity in the attached carbohydrate structures. Samples from each of the peaks were run on SDS-PAGE gels under non reducing conditions.

**Figure 11.** Elution chromatography identifies two peaks of ZP proteins




SDS-PAGE analysis of SEC fractions revealed a prominent band at 80 kDa band which is consistent with the molecular weight of ZP2, as well as a prominent smear at the 50 and 60 kDa mark, consistent with the molecular weight of ZP3 and ZP4. Proteins eluting from SEC chromatography in peak A and B appeared as non-discrete bands on SDS-PAGE gel which suggest the presence of post-translational glycosylations. A summary of yields of each protein is presented in Table 7.

**Table 7.** Yield of purified ZP proteins

| Final Protein | ZP2<br>Peak A | ZP3/4<br>Peak B |
|---|---|---|
| Volume | 1.2 mL | 6.0 mL |
| Target Protein Concentration | 0.91 mg/mL | 1.04 mg/mL |
| Target Protein Yield | 1.1 mg | 6.1 mg |

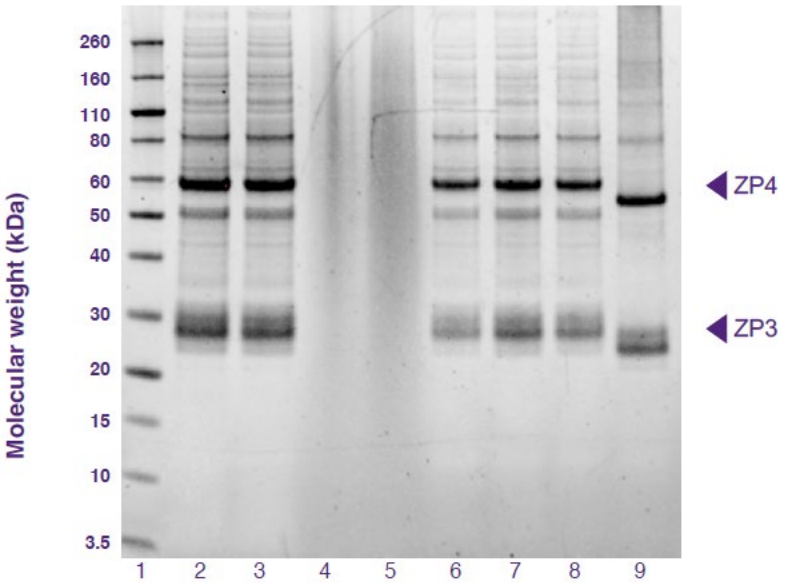
## 4.2 Expression-yield of recombinant porcine zona pellucida proteins

**Exp 3:** 161mg of pZP3 was purified from 533 ml *E.coli* cell culture supernatant for animal studies (total protein concentration was 0.30 mg/mL with a purity of 78%, endotoxin content < 100 EU/mg). An overexpressed protein band corresponding to the molecular weight of the predicted target protein (38kD) was detected in all expression flasks in the total lysate fractions.

**Exp 4:** 10.2 mg of pZP4 was purified from 72.5 ml of *E.coli* cell culture for animal studies (total protein concentration was 0.14 mg/mL with a purity of >99%, endotoxin content 4841 EU/mg). The pZP3 produced for Exp.3 was used in Exp.4.

**Exp 5:** 11.3 mg of bZP3/ZP4 was purified from 20 L baculovirus/insect cell culture supernatant for animal studies (total protein concentration was 0.20 mg/mL with a purity of 74.5%, endotoxin content <1 EU/mg). The bands corresponding to the ~60 kDa and ~30 kDa were identified as ZP4 and ZP3 respectively (Figure 12).

**Figure 12.** SDS-PAGE identification of recombinant ZP3 and ZP4




## 4.3 Response to vaccination against porcine zona pellucida (pZP) proteins

Figure 13 shows the typical vaccine site swellings observed in the first few weeks after vaccination. Overall results for each experiment are presented followed by a series of figures (14-19) and tables

(8-13) describing the changes in each parameter measured. The significance of the impact of ZP vaccination on parameters measured is summarised in Table 14.

### 4.3.1 Experiment 1: vaccination with purified porcine zona pellucida proteins

- ***Impact of ZP vaccination on animal health (body weight, body condition and injection site)***
  - The **body weights** of all heifers gradually increased as expected with the growth rates of 2-3 years old cattle. Overall averages were recorded as 388, 414, 424, 405 and 568 kgs respectively at days -50, -2, 79, 167 and 404 from vaccination. There were some fluctuations of weight, e.g., a decreasing weight of 405 kgs at day 167, which were consistent with seasonal decline in pasture quality. Comparisons of mean weights between the three treatment groups and the control group showed no significant difference at each time point, except for the pZP2 group being significantly higher than all other groups at day 404. The vaccine was ruled out as the cause of this difference as it occurred 14 months after vaccination.
  - The **body condition** of all heifers also improved as expected with growth rate and seasonal fluctuations of pasture quality. Overall mean body condition scores (BCS) were recorded as 3.3, 3.4, 3.6, 3.8 and 3.9 respectively at days 111, 167, 233, 362 and 404 from vaccination. Comparisons of mean BCS between the three vaccination groups and the control group showed no significant difference at each time point.
  - Most heifers showed no detectable **injection site reaction**, with a few having a small lump (Figure X), but in no case did these ulcerate or abscess. No swellings were present 4 weeks after vaccination.

- ***Impact of ZP vaccination on ovarian function (ovarian volume, follicle counts and corpus luteum)***

  No significant impact of vaccination on ovary morphology was observed in any treatment group in experiment 1.
  - The overall mean **ovarian volume** of vaccinated heifers was similar overtime ~14.5 $cm^3$, except for a sudden decrease to 7.5 $cm^3$ at day 111, which occurred after the booster 2 vaccination. Within 100 days mean ovarian volume were similar to prior to day 111. No significant differences were found between the three treatment groups for the average volume of both ovaries.
  - There was a gradual decrease in overall mean AFC between day -50 (44 antral follicles) day 404 (15 antral follicles). However, there was a sudden drop to 11 antral follicles at day 111. Within 100 days mean AFC were similar to prior to day 111. No significant differences were found between the three treatment groups for the average AFCs, although the pZP group was found to have slightly lower AFCs than the other treatment groups.
  - A **corpus luteum** (CL) was consistently present in the majority (> 75%) of heifers throughout the experiment, except at day 111 where only 33% of heifers had a CL.
  - The observed marked decrease in ovary volume, AFCs and CL presence at day 111 is of great interest as it may indicate a destructive cell mediated immune response .

- ***Impact of ZP vaccination on AMH***
  Overall, there were no significant differences between vaccine group in mean plasma AMH concentration. The AMH concentration of eight heifers (1 pZP, 2 pZP2 and 5 pZP3&4; 38% of vaccinated heifers), decreased markedly 111 days after first vaccination but by day 167 their AMH concentrations had returned to close to prevaccination level (Appendix 8.5). Note the mean AFC for each vaccine treatment also decreased at this time and subsequently increased to near prevaccination levels. (Ireland *et al.* 2010) have previously reported that antral follicle count is positively and highly associated with concentrations of AMH in dairy cattle.

- ***Immune response to ZP vaccination***
  - The responses to immunisation against different ZP proteins are presented in Figure 4.5 and 4.6. No significant increase was observed at days 13 and 40 after the priming vaccine; however, a marked 8-fold change was observed following the booster 1 and booster 2 vaccines at days 55 and 97, respectively, in all three treatment groups. A gradual decrease in absorbance values was observed immediately after each peak associated with the booster vaccinations. The levels of immune response remained as high as 2-fold until 200 days after first vaccination. Later, the absorbance values were found to be closer to the pre-vaccination levels although slightly but not significantly higher. Significantly higher levels of mean IgG (area under the curve) were found in the infertile compared to the fertile heifers during mating period 1 ($p = 0.05$) and mating period 2 ($p = 0.01$).

- ***Impact of ZP vaccination on fertility (pregnancy rate)***
  - The **pregnancy rate** was significantly lower in the vaccinated heifers (2 out of 21) than the controls (15 out of 18) in mating period 1. Similarly, mating period 2 also showed very low fertility in vaccinated heifers (4 out of 21) as compared to the controls (7 out of 8). All treatment groups showed similar fertility (no significant differences). Of the two purified proteins evaluated vaccination against the purified mix of pZP3 & 4 resulted in the lowest overall pregnancy rate.
  - Overall, the proportion of control heifers that became pregnant during the 2 mating periods was significantly higher than the vaccinated heifers (83% and 75% versus 9.5% and 19%, respectively). Further, the proportion of heifers in each vaccination group that became pregnant was significantly lower than the control heifers, except for the pZP2 heifers for the second mating.
  - Three heifers (one pZP2, two pZP3&4) that failed to become pregnant during the first mating period became pregnant during the second mating period. This may indicate they initially became infertile as a consequence of vaccination but later recovered their fertility.

### 4.3.2 Experiment 2: vaccination with purified porcine zona pellucida proteins 3 & 4 suspended in Montanide™ ISA VG 61

- ***Impact of ZP vaccination on animal health (body weight, body condition and injection site)***
  - The **body weights** of all heifers gradually increased as expected with the growth rates of 2-3 years old cattle. Overall averages were recorded as 508, 585, 566, 555 and 676 kgs

respectively at days 19, 123, 286, 405 and 446 from vaccination. There were some fluctuations of weight, e.g., a decreasing weight of 566 and 555 kgs at days 286 and 405, which were consistent with seasonal shortage of grazing grass. Comparisons of mean weights between the two treatment groups and the control group showed no significant difference at each time point .

- The **body condition** of all heifers also improved as expected with growth rate and seasonal fluctuations of grazing. Overall mean body condition scores (BCS) were recorded as 3.2, 3.1, 3.6, 3.9, 3.8, 3.5, 3.4, 3.5 and 4.1 respectively at days -48, 19, 67, 81, 123, 202, 236, 286 and 405 from vaccination. Comparisons of mean BCS between the two treatment groups and the control group showed no significant difference at each time point.
- The **injection sites** showed evidence of mild to moderate swelling across all treatment groups during the first 4 weeks, but no ulceration or abscess formation was observed at any time and the swellings resolved within 4 weeks.

- ***Impact of ZP vaccination on ovaries (ovarian volume, follicle counts and corpus luteum)***
  - The **ovarian volume** of each heifer remained consistent with an overall mean of ~13.2 $cm^3$ with a few exceptions, which occurred randomly. In these cases, the subsequent ultrasound scans found all ovaries within normal volume range. No significant differences were found between the two treatment groups for the average volume of both ovaries.
  - Total **antral follicle counts** of each heifer on average ranged between 18 and 26 at each scan from days -48 to 319 from vaccination. No sudden or consistent decline in AFCs was seen after vaccination. No significant differences were found between the two treatment groups for the average AFCs, although the pZP group was initially found to have slightly lower AFCs than the pZP3 & 4group.
  - A **corpus luteum** (CL) was consistently present in the majority (> 75%) of heifers throughout the experiment except at day 67, where only 30% of heifers had a CL.

- ***Immune response to ZP vaccination***
  - The responses to immunisation against different ZP proteins are presented in Figure 4.5 and 4.6. In the pZP group, no significant increase was observed at days 19 and 32 after vaccination, whereas a 6-fold increase was observed in the pZP3&4 treatment group. The pZP group attained a 4-fold peak at day 67, at which time both groups showed similar levels of immune response. A gradual decrease in absorbance values was observed immediately after each peak in both groups. From day 67, the levels of immune response remained as high as 2-fold above prevaccination levels until the end of the experiment, (319 days after vaccination). Significantly higher levels of mean IgG (area under the curve) were found in the infertile compared to the fertile heifers during mating period 1 ($p = 0.03$), mating period 2 ($p = 0.06$) and mating period 3 ($p = 0.06$).

- ***Impact of ZP vaccination on fertility (pregnancy rate)***
  - Overall, the **pregnancy rate** was significantly lower in the vaccinated heifers (3 out of 10) compared to the controls (6 out of 8) in mating periods 1 and 2. The pregnancy rate in mating period 3 was similarly low (3 out of 10) in the vaccinated heifers. Note, all of the heifers used in Experiment 2 had been control animals for Experiment 1 and from a single mating 83.5% had become pregnant.

- From the first mating 87.5% of the control heifers became pregnant and overall 30% of the vaccinated heifers became pregnant (P=0.02); from the second mating 75% of the control heifers became pregnant and overall 30% of the vaccinated heifers became pregnant (P=0.15). One heifer in the pZP group (1/4) became pregnant from the first mating but none became pregnant from the second mating. Two heifers in the pZP3&4 group (2/6) became pregnant from the first mating and three became pregnant from the second and third matings (3/6). However, it should be noted that 2 of the 3 heifers in the pZP3&4 group became pregnant during each of the 3 mating period suggesting they were genuine non-responders to the vaccine.
- One pZP heifer became pregnant during the first mating but failed to become pregnant during the 2nd and 3rd mating periods which may suggest some delay in the onset of the infertility response after vaccination.

### 4.3.3 Experiment 3: vaccination with recombinant porcine zona pellucida protein 3

- ***Impact of ZP vaccination on animal health (body weight, body condition and injection site)***
  - The **body weights** of all heifers gradually increased as expected for 2-4 years old cattle. Overall averages were recorded as 500, 528, 549, 575 and 609 kgs respectively at days -30, -10, 31, 108 and 371 from vaccination. The weight gain in all treatment groups was consistent with ample grazing good quality due to good seasonal rain in the summer of 2021-22. Comparisons of mean weights between the three treatment groups showed no significant difference at each time point.
  - The **body condition** of all heifers also improved as expected with growth rate and seasonal grazing conditions. Overall mean body condition scores (BCS) gradually increased from 3.6 to 4.2 during experiment 3. Comparisons of mean BCS between the three treatment groups showed no significant differences, except a random exception at day 84.
  - The **injection sites** showed evidence of moderate swelling across all treatment groups during the first 4 weeks, but no ulceration or abscess formation was observed at any time and the swellings had resolved within 4 weeks.

- ***Impact of ZP vaccination on ovaries (ovarian volume, follicle counts and corpus luteum)***
  - The **ovarian volume** of each heifer remained consistent. It was noted that two groups (pZP:M and rZP3:M) remained very close to each other at an overall average of ~14 $cm^3$; however, the rZP3:F group showed a relatively higher ovarian volume throughout the experiment, both pre and post vaccination, with a range of 15 – 20 $cm^3$. There were no significant differences in the average volume of both ovaries that could be associated with the treatments.
  - Total **antral follicle counts** of each heifer tended to decline from an average of 24 AFCs at day -10 and finishing at 20 AFCs at day 255. Similar to the ovary volume, no significant differences were found between the three treatment groups for the average AFC of both ovaries, although the rZP3:F group was found to have slightly higher AFCs than the other treatment groups at the first three scans.

- A **corpus luteum** (CL) was consistently present in the majority (> 85%) of the heifers throughout the experiment, except at days 31 and 59 where a CL was present in 54% and 71%, respectively.

- ***Immune response to ZP vaccination***

  The responses to immunisation against different ZP proteins are presented in Figure 4.5 and 4.6. The three groups showed a significant immune response at day 17 after the priming vaccine, with averages of above 8-fold (pZP:M), 4-fold (rZP3:M) and 5-fold (rZP3:F) increases in IgG values. Moreover, up to a 10-fold change was observed following the booster 1 and booster 2 vaccines at day 46 in the rZP3:F treatment group. A gradual decrease in absorbance values was observed after the peak immune response in each group. The levels of immune response remained above a 3-fold increase until the last sampling point at 255 days from vaccination. No significant differences in the levels of mean IgG (area under the curve) were found between the infertile and fertile heifers during the 3 mating periods. There was some variation in the magnitude of the individual heifer immune response to vaccination over time (Appendix 8.6).

- ***Impact of ZP vaccination on fertility (pregnancy rate)***
  - The **pregnancy rate** was very high (above 80%) in both groups of rZP3 vaccinated heifers in all mating periods, suggesting that immunisation against the recombinant ZP3 protein failed to induce infertility. The pZP:M group showed comparatively low fertility (50%); however, it was significantly higher than previously observed in Experiment 1.
  - There was no significant difference between vaccination groups in the proportion of heifers that became pregnant. Four heifers in total failed to become pregnant ( one rZP3-M, one rZP3-F and two pZP) at each mating, of which 3 had been mated before and had become pregnant and 1 had not been mated before. The lack of infertility induced by either 3 vaccinations containing Freund's adjuvants (complete and incomplete) or a single vaccination with rZP3 suspended in Montanide strongly indicates that in cattle, ZP3 alone has only a limited role in sperm binding. The poor infertility response to pZP is of concern as a higher amount of pZP was incorporated into the vaccine used. One possible explanation is an adverse effects of repeated freeze-thawings of the stored pZP.

### 4.3.4 Experiment 4: vaccination with a 1:1 mix of recombinant porcine zona pellucida proteins 3 & 4

- ***Impact of ZP vaccination on animal health (body weight, body condition and injection site)***
  - The **body weights** of all heifers gradually increased as expected for 2-year-old heifers grazing tropical improved pastures. Overall averages were recorded as 354 and 382 kgs respectively at days -16 and 40 from vaccination. Comparisons of mean weights between the vaccination group and the control group showed no significant difference at each time point.
  - The **body condition** of all heifers also improved as expected with growth rate and seasonal fluctuations of grazing. Overall mean body condition scores (BCS) increased from 2.6 to 3.3 as recorded at -16 and 323 days from vaccination. Comparisons of mean BCS between

the treatment group and the control group showed no significant difference at any time point (Figure 4.2).

- The **injection sites** showed evidence of mild to moderate swelling across the treatment group during the first 4 weeks, but no ulceration or abscess formation was observed at any time and the swellings resolved within 4 weeks.

- ***Impact of ZP vaccination on ovaries (ovarian volume, follicle counts and corpus luteum)***

No significant impact of vaccination was noted on ovarian function in the treatment group in experiment 4, as explained below for each variable.

- The **ovarian volume** of each heifer was an overall average of ~9 $cm^3$. No significant changes over time or post vaccination were found for the average volume of both ovaries.
- Total **antral follicle counts** of each heifer consistently averaged 15 AFCs, with a very slight decreasing trend over time. No significant changes over time were observed in the treatment group.
- The **corpus luteum** (CL) presence was initially (pre-vaccine) low (50%) but gradually increased and then remained high (~80%),.
- Overall, vaccination with recombinant ZP proteins (rZP3 and rZP4) did not adversely affect ovarian function.

- ***Immune response to ZP vaccination***
  - A significant immune response of up to a 6-fold increase was observed at days 13 to 40 that subsequently became an 8-fold to day 62 , plateauing by day 83 after vaccination. The mean peak immune response tended to be greater than that observed in the Exp.2 heifers vaccinated with purified pZP3&4. No significant differences in the levels of mean IgG (area under the curve) were found in the infertile (not pregnant) as compared to the fertile (pregnant) heifers during mating period 1 ($p = 0.36$) and mating period 2 ($p = 0.33$). There was some variation in the magnitude of the individual heifer immune response to vaccination over time (Appendix 8.6).
- ***Impact of ZP vaccination on fertility (pregnancy rate)***
  - The **pregnancy rate** was very high in both the vaccinated heifers (83%, 10 out of 12) and the controls (100%, 4 out of 4) after mating period 1. Similarly, mating period 2 and 3 also showed 83% fertility in the treatment group. Only one heifer (#3) remained infertile throughout the experiment for three consecutive mating periods.

### 4.3.5 Experiment 5: vaccination with recombinant co-expressed bovine zona pellucida proteins 3 & 4

- ***Impact of ZP vaccination on animal health (body weight, body condition and injection site)***

  - The mean **body weight** at vaccination was 617 kg ±65. Note due to delays in developing the vaccine used in this experiment the heifers were 4 -years-old at time of vaccination. There was no significant change in body weight in the 100 days after vaccination.

  - The **body condition** score at time of vaccination was between 4.0-4.5 and no significant change was observed after vaccination.

- The **injection sites** showed evidence of mild to moderate swelling across the treatment group during the first 4 weeks, but no ulceration or abscess formation was observed at any time and the swellings resolved within 4 weeks.

- ***Impact of ZP vaccination on ovaries (ovarian volume, follicle counts and corpus luteum)***

  - Total **antral follicle counts**
  - Prior to vaccination 14/16 heifers had a **corpus luteum** (CL). At the time of commencement of first mating (42 days after vaccination) 15/16 had a CL.

- ***Immune response to ZP vaccination***

  - At the time of writing this final report assaying of the samples had not been completed as the original laboratory that had done the assays for Exp.1-4 had closed. A new laboratory has been identified and this report will be updated when the samples are assayed and the data analysed.

- ***Impact of ZP vaccination on fertility (pregnancy rate)***

  - Only data for the **pregnancy rate** following the first mating period are available as there was an unavoidable delay in commencing Exp.5. 56% (9/16) of vaccinated heifers were diagnosed pregnant. This is markedly lower than the pregnancy rate of the heifers vaccinated with recombinant ZP proteins in Exp.3 and 4 heifers. This report will be updated when data from further mating periods becomes available.

**Figure 13.** Typical vaccination site swellings, A – mild; B – moderate

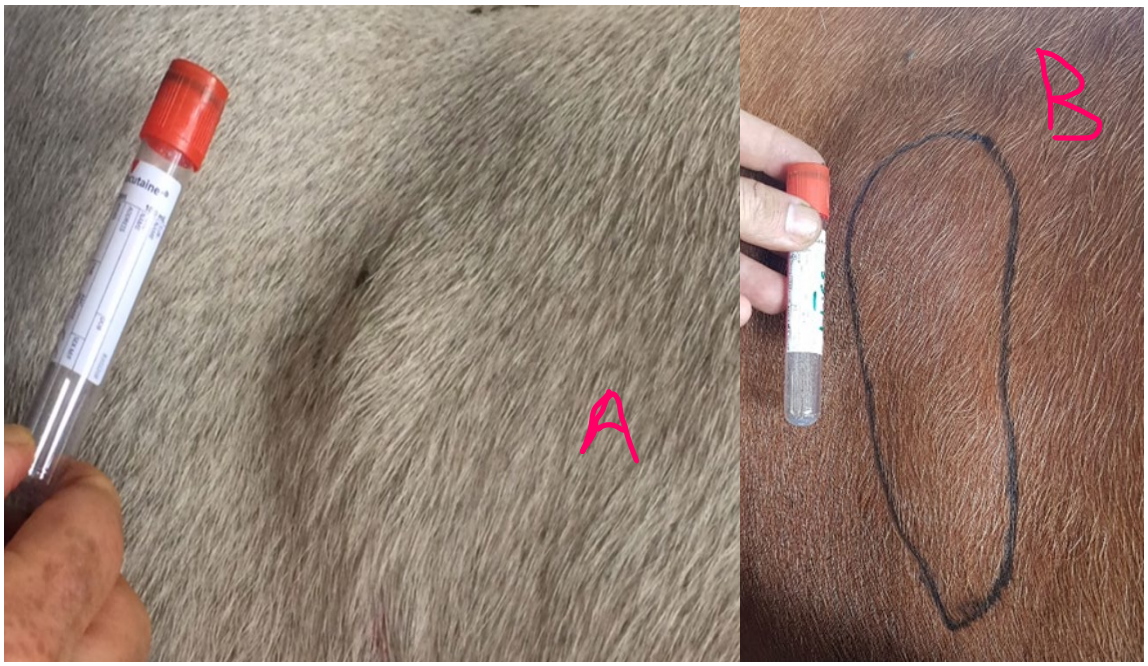


**Figure 14**. Longitudinal changes in average body weights (kg) of vaccinated and control heifers. pZP and rZP respectively indicate types of purified and recombinant ZP proteins, which were formulated in Freund's (:F) or Montanide™ ISA VG 61 (:M) in 50:50 or 40:60 volumetric ratios, respectively.

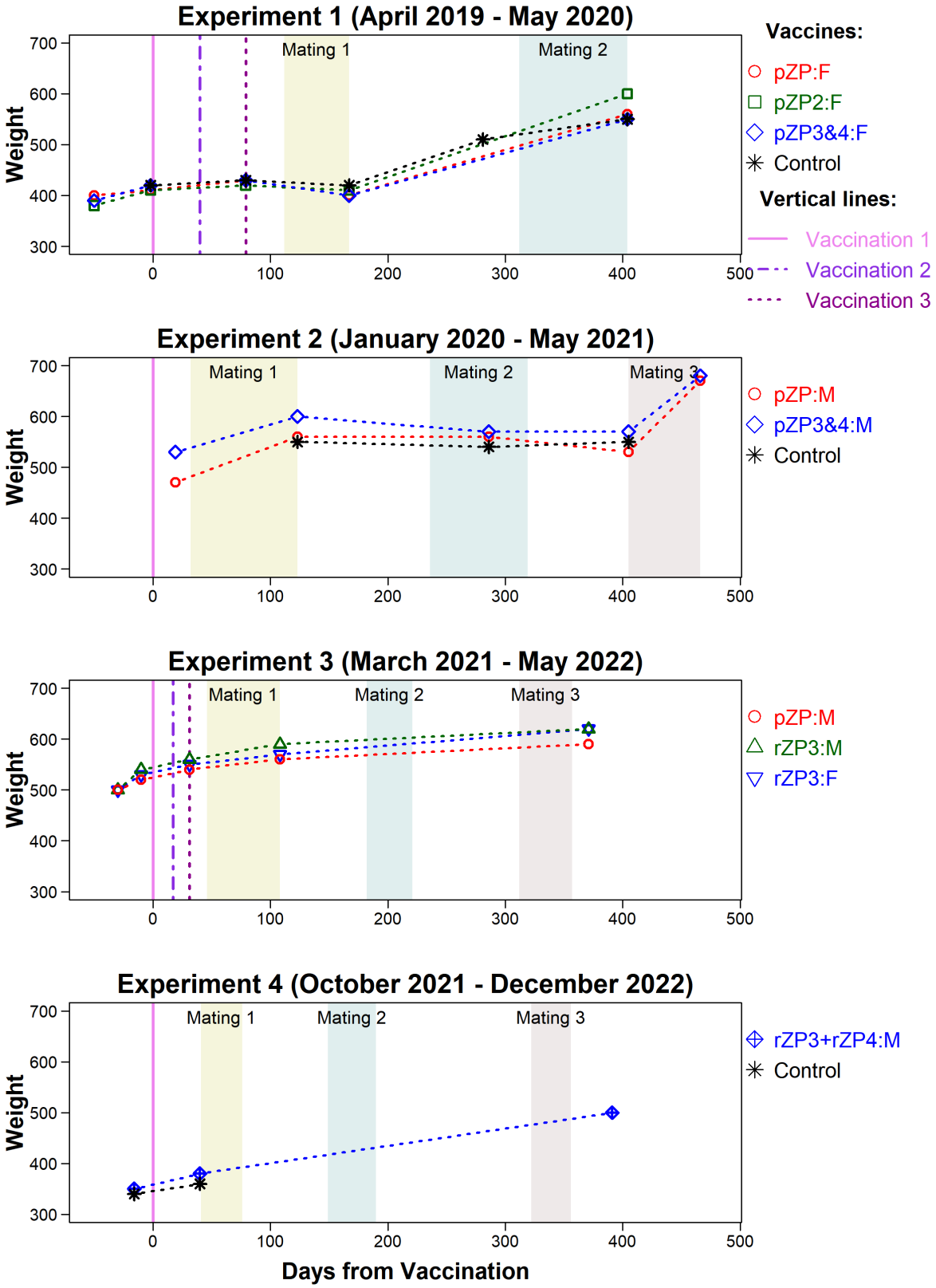


**Figure 15**. Body condition scores (BCS, on a 0 - 5 scale). pZP and rZP respectively indicate types of purified and recombinant Zona Pellucida proteins, which were emulsified in either Freund (:F) or Montanide (:M) in 50:50 or 40:60 volumetric ratios.

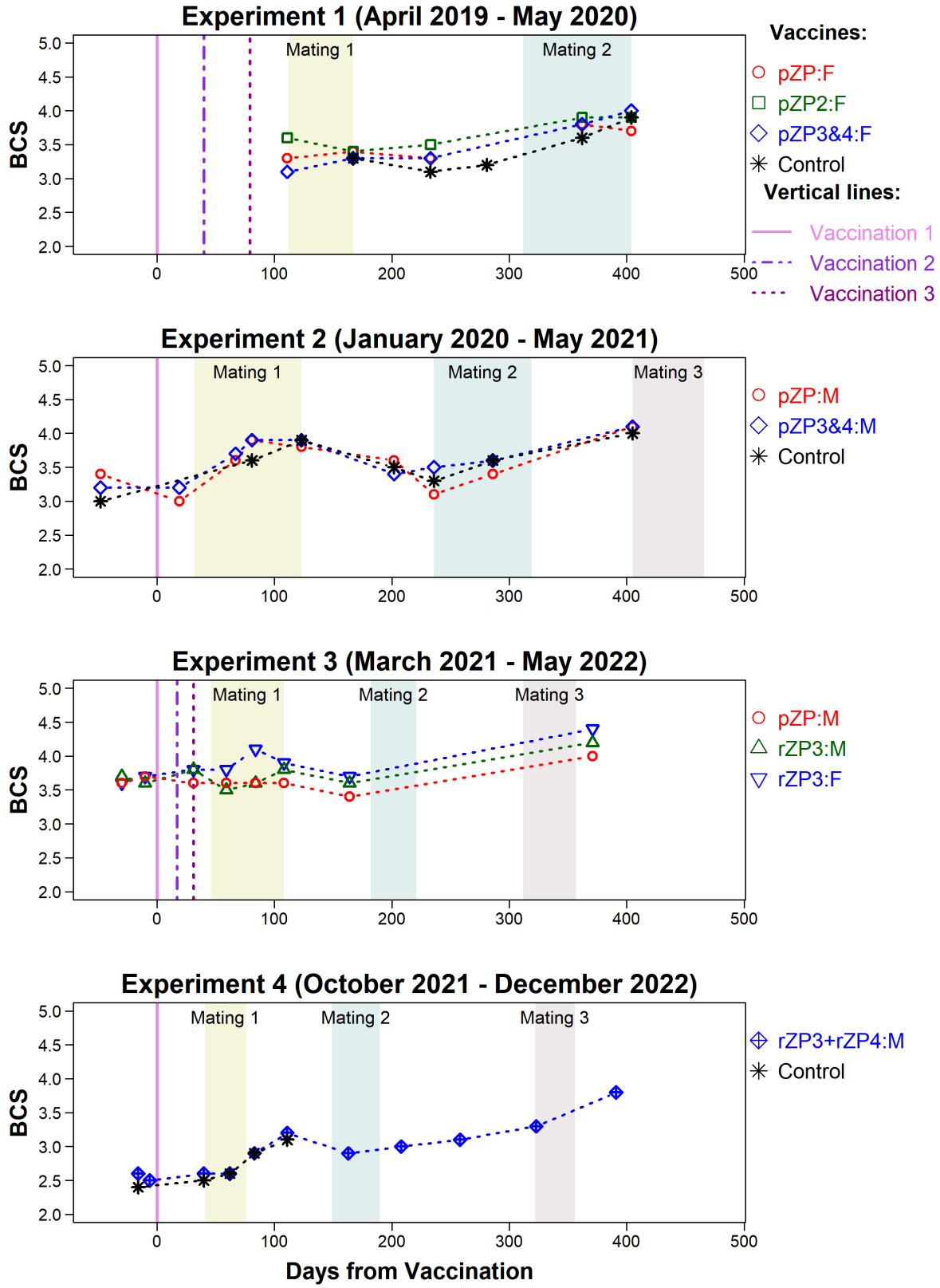


**Figure 16**. Ovarian volume (average volume of both ovaries in $cm^3$). pZP and rZP respectively indicate types of purified and recombinant Zona Pellucida proteins, which are emulsified in either Freund (:F) or Montanide (:M) in 50:50 or 40:60 volumetric ratios.

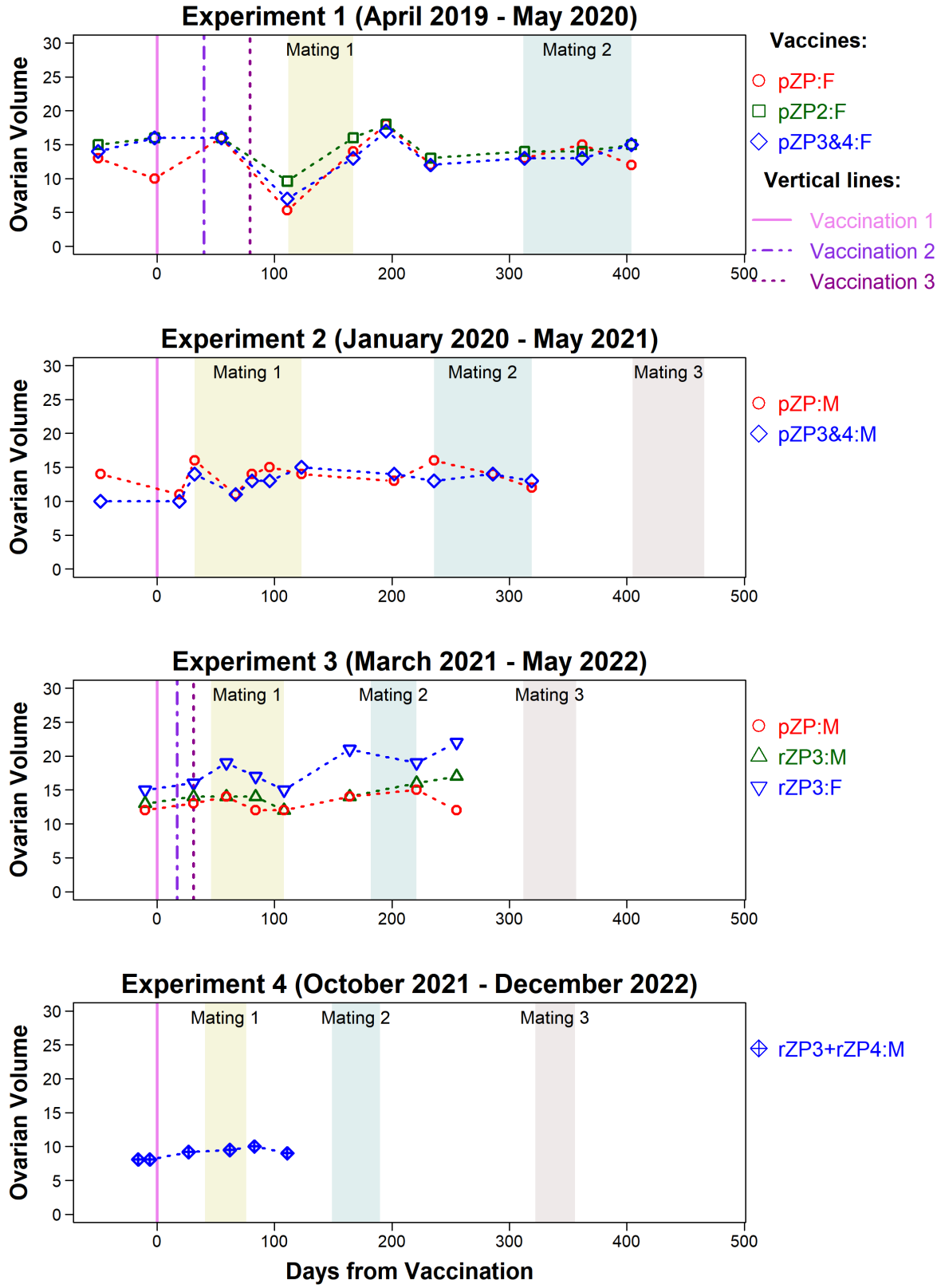


**Figure 17**. Mean antral follicle counts (AFC) before and after vaccination. pZP and rZP respectively indicate types of purified and recombinant Zona Pellucida proteins, which are emulsified in either Freund (:F) or Montanide (:M) in 50:50 or 40:60 volumetric ratios.

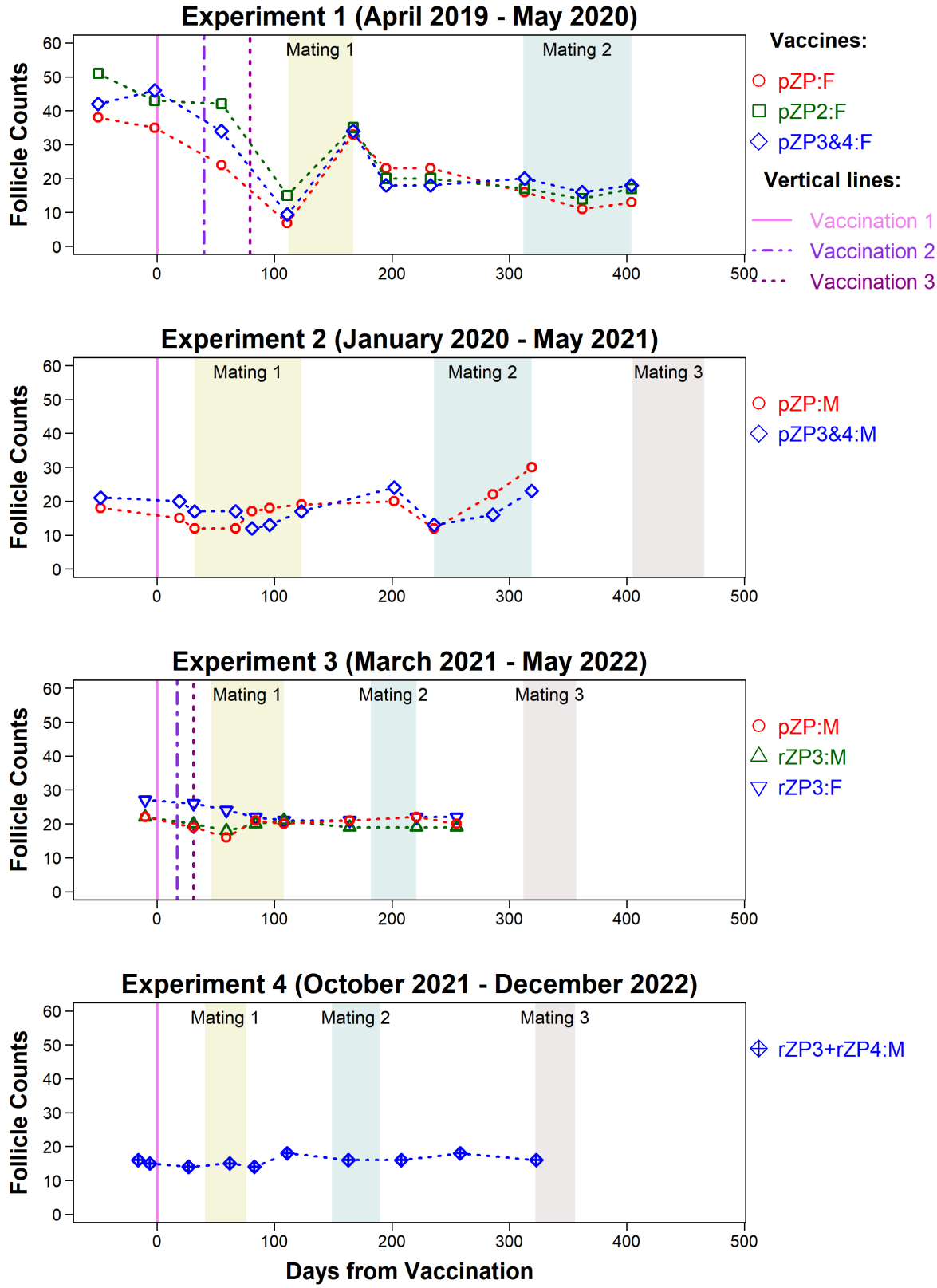


**Figure 18**. Immune response (antibody ELISA) shown as mean optical density (450nM) within each vaccinated group of heifers. pZP and rZP respectively indicate types of purified and recombinant Zona Pellucida proteins, which are emulsified in either Freund (:F) or Montanide (:M) in 50:50 or 40:60 volumetric ratios.

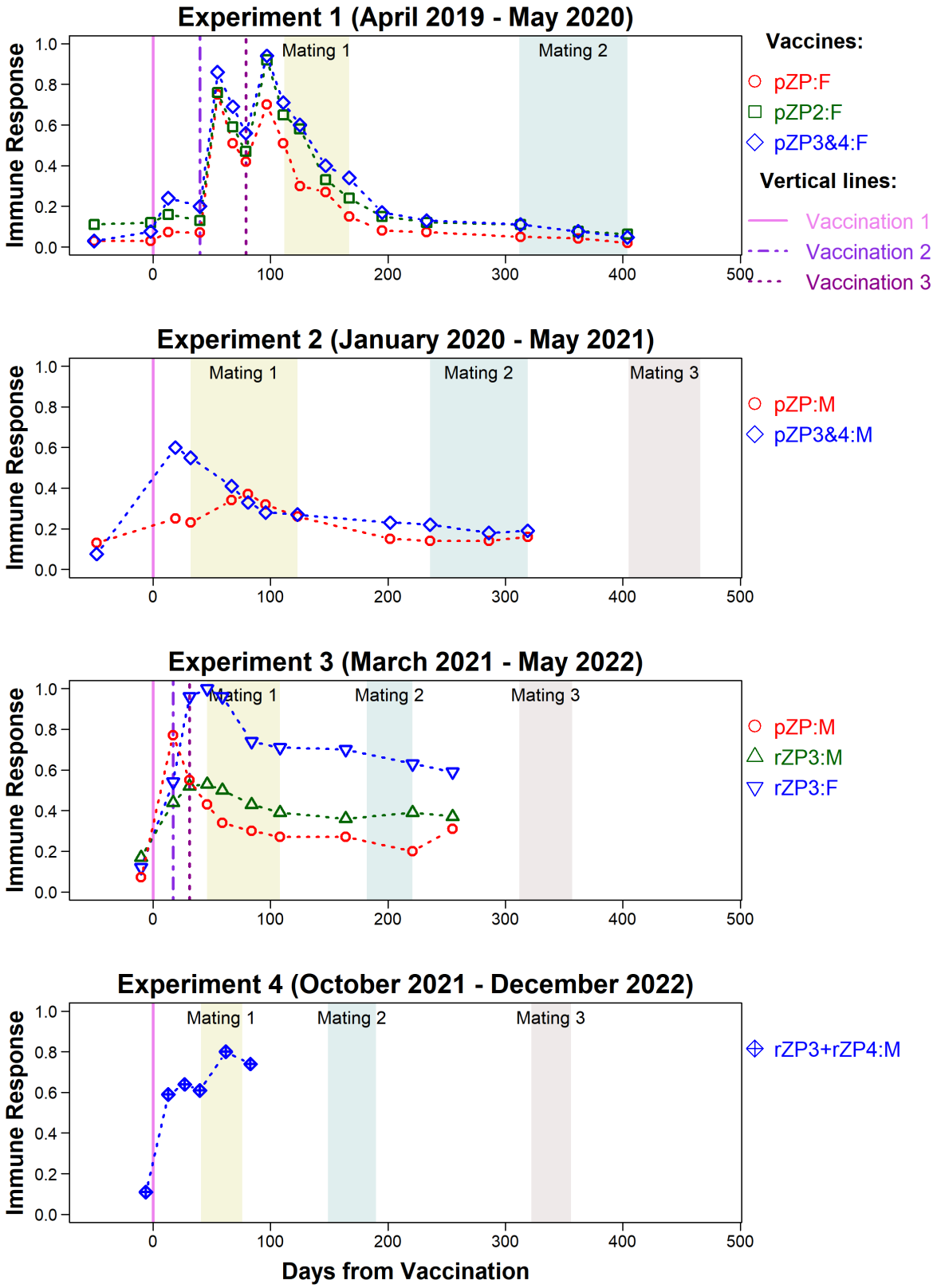
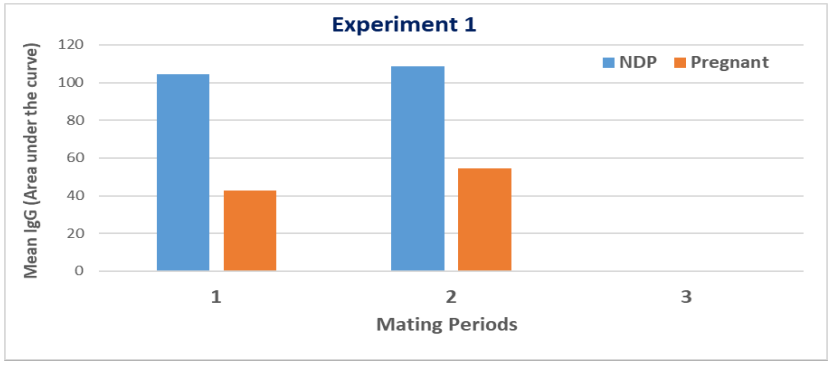
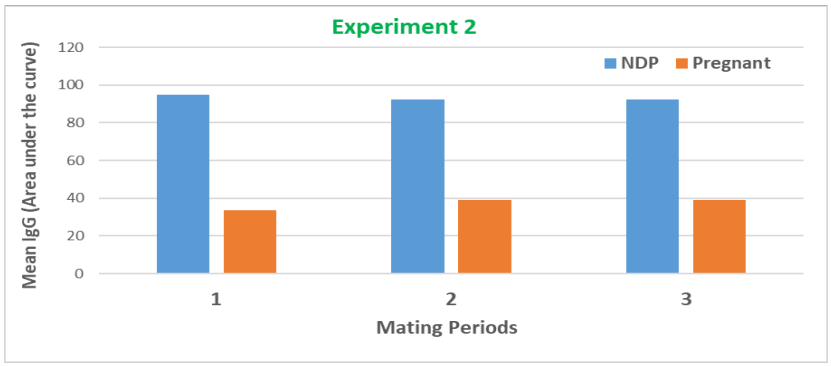
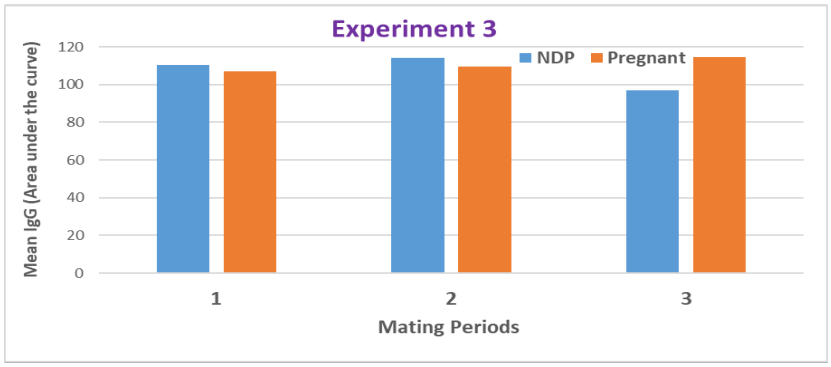


**Figure 19.** The bar graphs show comparisons of means of immune response (IgG, measured as area under the curve) between non-pregnant (NDP) and pregnant heifers within each experiment.



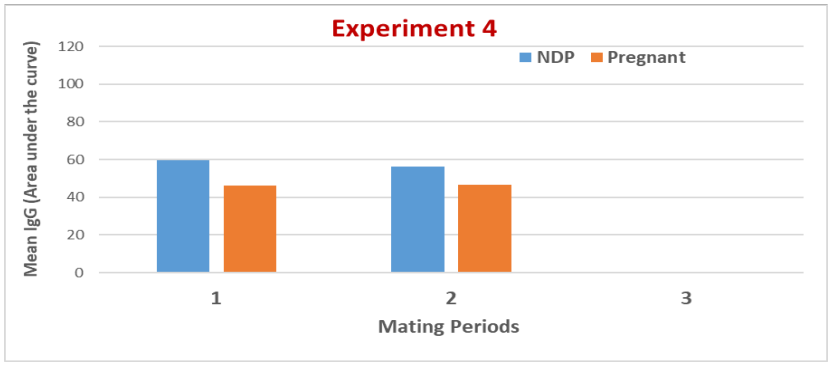



**Table 8.** P-values obtained by Welch Two Sample t-test for comparisons of Immune Response (IgG, Mean area under the curve) between not detectably pregnant (NDP and) pregnant heifers in experiments.

| Mating periods | Exp 1 | Exp 2 | Exp 3 | Exp 4 |
|---|---|---|---|---|
| **1** | 0.0675* | 0.0267 | 0.887 | 0.361 |
| **2** | 0.0106 | 0.0644* | 0.847 | 0.327 |
| **3** | | 0.0644 | 0.470 | NA |

* Significant difference, p-values < 0.1 can be used as suggestive; NA data not available

- Exp 1 & Exp 2 show strong association between the immune response and fertility status (NDP or pregnant) as indicated by P-values and prediction graphs.
- Exp 3 & 4 show no association with fertility status.

**Table 9.** Proportion pregnant by group – Exp.1

| Group | Mating 1 | Mating 2 |
|---|---|---|
| pZP | 0/5 (0%) | 0/5 (0%) |
| pZP2 | 2/7 (29%) | 2/7 (29%) |
| pZP3&4 | 0/9 (0%) | 2/9 (22%) |
| Control | 15/18 (83%) | 7/8 (88%) |

**Table 10.** Proportion pregnant by group – Exp.2

| Group | Mating 1 | Mating 2 | Mating 3 |
|---|---|---|---|
| pZP | 1/4 (25%) | 0/4 (0%) | 0/4 (0%) |
| pZP3&4 | 2/6 (33%) | 3/6 (50%) | 3/6 (50%) |
| Control | 7/8 (88%) | 6/8 (75%) | NA |

**Table 11.** Proportion pregnant by group – Exp.3

| Group | Mating 1 | Mating 2 | | Mating 3 |
|---|---|---|---|---|
| pZP (Montanide) | 5/9 (56%) | 4/8 (50%) | | 5/9 (56%) |
| rZP3 (Montanide) | 7/9 (78%) | 8/9 (89%) | | 5/9 (56%) |
| rZP3 (Freund's) | 5/6 (83%) | 5/6 (83%) | | 5/6 (83%) |

**Table 12.** Proportion pregnant by group – Exp.4

| Group | Mating 1 | Mating 2 | Mating 3 |
|---|---|---|---|
| rZP3+rZP4 (Montanide) | 10/12 (83%) | 10/12 (83%) | 10/12 (83%) |
| Control | 4/4 (100%) | NA | NA |

**Table 13**. Proportion pregnant by group – Exp.5

| Group | Mating 1 | Mating 2 | Mating 3 |
|---|---|---|---|
| rZP3&4 (Montanide) | 10/16 (63%) | | |

**NA – control heifers reassigned to a vaccination group**

**Table 14.** Summary of analysis of significance of observed differences between control and vaccinated heifers for all parameters measured.

| **Parameters** | **Exp 1** | **Exp 2** | **Exp 3** | **Exp 4** | **Exp 5** | **Overall comments and conclusions** |
|---|---|---|---|---|---|---|
| Groups (samples) | Control (18)<br>pZP (5)<br>pZP2 (7)<br>pZP3&4 (9) | Control (8)<br>pZP (4)<br>pZP3&4 (6) | pZP (9)<br>rZP3 (9)<br>rZP3 (6) | Control (4)<br>rZP3+4 (12) | Heterocomplex<br>rZP3&4 (16) | Total 91 heifers typical of genotypes in NAustralia |
| Body weight | NS except pZP2 vs pZP3&4 at 404 days ($p$ = 0.0491) | NS | NS | NS | | Non-significant |
| Body condition score (BCS) | NS | NS | NS | NS | | Non-significant |
| Ovary size | NS 1* | NS | NS | NA | | Non-significant |
| Antral follicles | NS 1* | NS | NS | NS | | Non-significant |
| CL presence | NS | NS | NS | NS | | Non-significant |
| Immune response (IgG) | Significantly higher in NDP heifers | Significantly higher in NDP heifers | NS | NS | | Some evidence of relationship between immune response and fertility status |
| Fertility %pregnant | Significantly higher in controls in 2 mating periods | Significantly higher in controls in 3 mating periods | High fertility in all groups | High fertility and NS between rZP and Controls | | |

NS = non-significant differences at $p$ = 0.05, NA = data not available

### 4.3.6 Histopathological evaluation of ovaries

Overall, the mean histology-derived AFC was similar amongst vaccine treatments and with the mean AFC for the unvaccinated heifers, except that the means for the unvaccinated heifers and the pZP2 heifers in Exp.1 were significantly higher than the mean for the Exp.4 heifers (unvaccinated 16.5, pZP 10.3, pZP2 19.6, pZP3&4 12.4, rZP3 11.1 and rZP3:rZP4 6.7) . There was a strong correlation (r=0.53) between the histology derived antral follicle count (AFC) and the ultrasound derived AFC (mean of last 5 scans before slaughter).

As there was ultrasonographic evidence in some Experiment 1 heifers of a marked reduction in AFC following vaccination, the degree of follicular atrophy in each section was scored. Follicular atrophy scoring demonstrated an increased number of ovaries from the pZP3&4 treatment group with Grade IV atrophy (55.6%), compared to the pZP2 (28.6%), pZP (20.0%) and not vaccinated groups (Table 15). In addition, a small percentage of ovaries from vaccinated animals showed various degrees of morphological changes of the oocytes and zona pellucida, with degenerated or mineralised oocytes, thin or absent zona pellucida, and vacuolation of granulosa cells. Frequent Call-Exner bodies were also observed in the secondary and tertiary follicles. Call-Exner bodies have a follicle-like appearance but are small eosinophilic fluid-filled punched out spaces between granulosa cells, with the granulosa cells usually arranged haphazardly around the space. However, Call-Exner bodies have also been observed in normal bovine preantral and small antral follicles so their presence in the samples under investigation does not necessarily have any pathological implication.

**Table 15.** Percentage of heifers within each vaccination group in Experiment 1 with different follicular atrophy scores.

| Vaccine group | Grade IV | Grade III | Grade II | Grade I |
|---|---|---|---|---|
| pZP (n=5) | 20.0 | 40.0 | 0.0 | 40.0 |
| pZP2 (n=7) | 28.6 | 42.9 | 0.0 | 28.6 |
| pZP3&4 (n=9) | 55.6 | 11.1 | 22.2 | 11.1 |
| Not vaccinated | 37.5 | 31.3 | 18.8 | 12.5 |

### 4.3.7 Immunohistochemical evaluation of ovaries

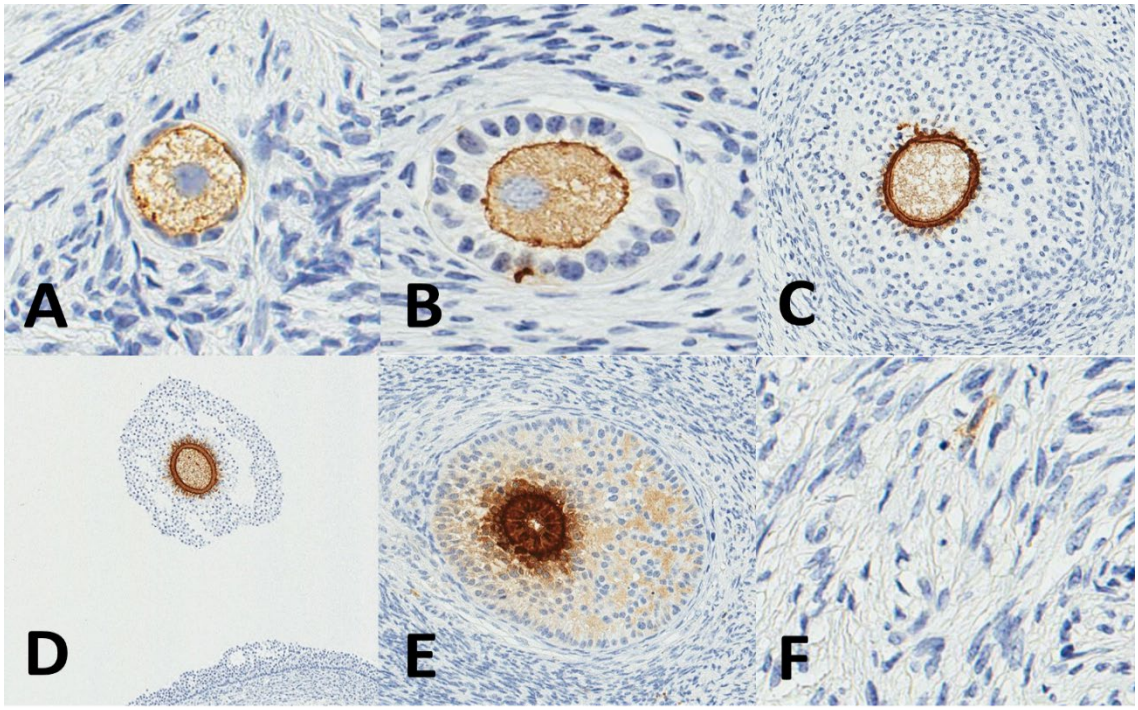
All ovaries from heifers in Exp. 1-4 (includes some controls) demonstrated very low expression of BSAP, with many being negative and an average of < 1 positive cell per high power field in those that were positive. This indicates that very few B lymphocytes were present in the ovaries of both vaccinated and control heifers. BSAP-positive cells were usually found in the ovarian stroma but were also rarely seen amongst the theca cells.

In contrast, CD3-positive cells were found in all ovaries. There was a very wide range of expression from < 1 to > 50 positive cells per high power field. However, comparisons between different vaccine types and control heifers did not find any significant differences in the number of CD3-positive cells. This suggests that vaccination was not responsible for the wide variation in T lymphocyte numbers seen in the ovaries. CD3-positive cells tended to be clustered together rather than spread uniformly throughout the ovaries. They were mostly found in the stroma and amongst the theca cells but were also rarely seen amongst the granulosa cells. There was no correlation between T lymphocyte

numbers detected in ovarian sections and either histology antral follicle count (AFC) or ultrasound derived AFC.

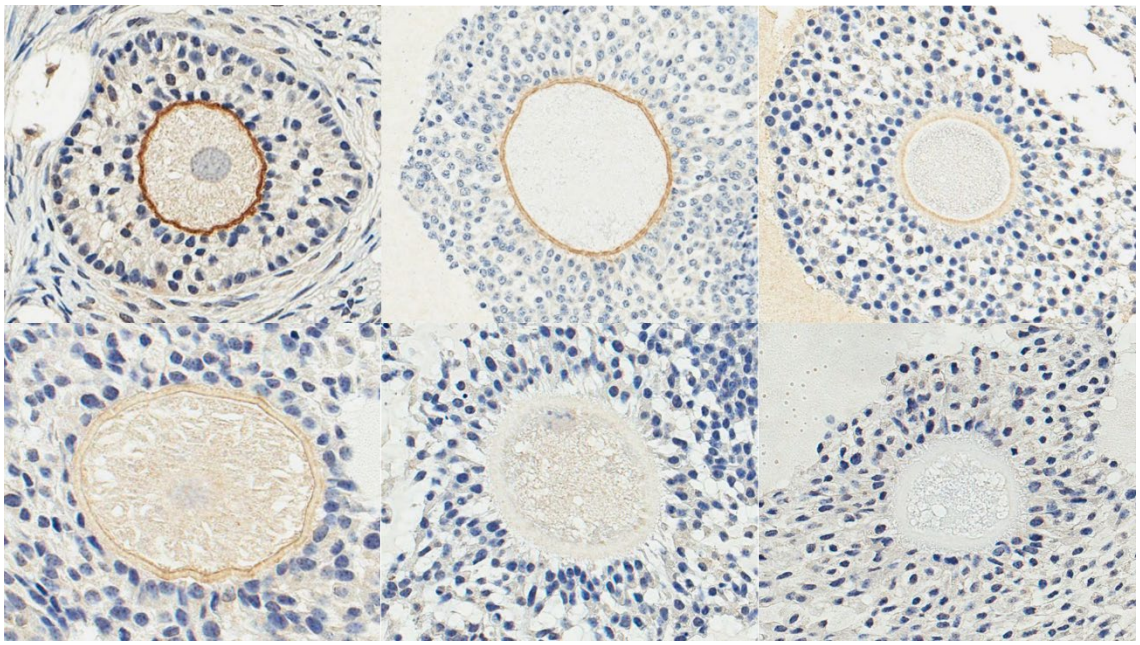
ZP3 was expressed in oocytes at all stages of development, including atresia (Figure 20). Expression was seen in the oocyte cytoplasm, cell wall and zona pellucida. Nearly all oocytes were positive for ZP3, although the intensity and uniformity of expression increased as follicle maturity increased, and so expression was sometimes weak and patchy in primordial and primary follicles. Cytoplasmic expression of ZP3 was also seen in some granulosa cells, as well as scattered cells in the stroma. ZP3-positive granulosa cells tended to be located immediately around the oocyte, except in atretic follicles where they were more widespread. ZP3 was also rarely expressed in the cytoplasm of theca cells in atretic follicles.

**Figure 20.** Examples of ZP3 expression in: A) primordial follicle; B) primary follicle; C) secondary follicle; D) tertiary follicle; E) atretic follicle; and F) stromal cell.




Unfortunately, the ZP4 slides had a lot of background staining that could not be sufficiently reduced, which made interpretation difficult. ZP4 appeared to be expressed in the oocyte cytoplasm, cell wall and zona pellucida, although the strength of expression was highly variable (Figure 21). It also appeared to be expressed in the cytoplasm of granulosa cells, theca cells and cells in the stroma.

**Figure 21.** Examples of variation in ZP4 expression in oocytes.



## 4.4 Evaluation of practical aspects of producing a recombinant ZP vaccine

Based on the initial production of recombinant pZP3 and pZP4 in 5 L shaking flasks an estimate of the cost of producing individual doses of vaccine were determined. To achieve significantly larger scale production requires the use of bioreactors in which aeration is far superior, leading to more rapid and extensive growth of the bacteria, hence improving yields of recombinant protein (Table 14) and reducing cost per vaccine dose. Further small improvements could be made in areas such as ensuring long term protein stability, which would also help increase yield. It should be noted that the costing presented in Table 14 are for the production of the recombinant pZP3 used in Exp.3. This protein was expressed in *E.coli* cell culture whereas the protein produced for Exp.5 was expressed in baculovirus/insect cell culture. The cost of these two production systems are similar.

The cost of producing a vaccine dose (assuming $5 cost for adjuvant plus packaging) using the shaker flask technology would see the recombinant ZP vaccine being cost competitive with spaying, but when produced in the more efficient bioreactors, costs could be significantly below spaying especially when large quantities of antigen are produced. This would only improve with genuine commercial scale production, which would further reduce the costs per vaccine dose. This would require an alliance with a commercial operation in the long-term. Selection of a commercial partner would require consideration of:

1. Size of the company
2. Financial status
3. Technological capability
4. Track record
5. Regulator compliance
6. Cost
7. Timeframe

Few Australian companies, with the exception of Ourofino and Kemwell Biopharma, would meet most/all of these criteria and so overseas partners may need to be considered for commercial scale operation.

**Table 14.** Recombinant protein production costs using different technologies.

| Process | Shake Flask | | Bioreactor | |
|---|---|---|---|---|
| | 5L Shake Flask | 10L Shake Flask | 5L Bioreactor | 10L Bioreactor |
| Upstream Process | $3,000 | $3,500 | $4,500 | $7,000 |
| Downstream Process | $16,500 | $33,000 | $20,000 | $40,000 |
| Final Sample Preparation | $1,000 | $1,500 | $1,500 | $2,000 |
| Protein Analytics | $1,000 | $1,000 | $1,000 | $1,000 |
| Total Production Cost | **$21,500** | **$39,000** | **$27,000** | **$50,000** |
| Projected Yield (mg) | 600 | 1200 | 1000 | 2000 |
| Number of Doses (600µg/dose) | 1000 | 2000 | 1667 | 3333 |
| Cost Per Dose | **$21.50** | **$19.50** | **$16.20** | **$15.00** |

# 5. Conclusion

The findings from Exp.1 and 2 confirmed the earlier PhD research that the project team had conducted. Vaccination of cattle with a crude preparation of porcine ZP induces infertility in most cattle for at least 12 months. Although the project team developed an effective method of producing large quantities of pZP which could readily be scaled up to a commercial level it is very unlikely that APVMA approval would be granted for commercial pZP vaccination of beef cattle in Australia. Although experimental recombinant ZP vaccines have been shown to induce infertility in some non-human primates, mice, dogs, horses, and donkeys there was no evidence of a similar response in the heifers vaccinated with recombinant pZP proteins in Exp.3 and 4. This was a little

surprising given the immune response measured after recombinant ZP protein vaccination was similar to that observed after native pZP protein vaccination. The finding of some evidence of induced infertility in Exp.5 is encouraging but will need to be confirmed with outcome from 2 further mating periods. Development of an in-vitro method of assessing the degree of binding of anti- ZP antibody to bovine oocytes, perhaps similar to what was used by Fayrer-Hosken *et al.* (1999 could be useful for screening potential recombinant ZP proteins before animal experiments are conducted.

## 5.1 Key findings

- A practical method of producing large amounts of pZP suitable for cattle vaccination studies was developed
- pZP3 and pZP4 are apparently tightly bound and were purified as a complex rather than individual proteins
- Administration of vaccines to cycling heifers containing either purified pZP2 or pZP3&4 suspended in Freund's adjuvant (Exp.1) induced prolonged infertility in a high proportion of heifers (71% and 100% first mating, 71% and 78% second mating, respectively), similar to that achieved with native pZP. The anti-ZP immune response was significantly higher in infertile compared to fertile heifers. Infertility persisted in spite of a decline in the anti-ZP immune response to close to prevaccination levels. At slaughter 419 days after the last vaccination there was histologic evidence of some to severe follicular atrophy in the ovarian cortex. The loss of developing follicles is likely to have contributed to the observed long term infertility. Although, most vaccinated heifers became infertile a small proportion remained fertile and became pregnant to one or both matings i.e these animals apparently failed to respond as expected to vaccination.
- Single administration of vaccines containing either pZP or pZP3&4 suspended in an emulsion of MontanideTM ISA VG 61 to cycling heifers, resulted in prolonged infertility in 70% of vaccinated heifers. Again the anti-ZP immune response was significantly higher in infertile compared to fertile heifers.
- However, single administration of vaccines containing recombinant pZP3 or a 1;1 mixture of recombinant pZP3 and pZP4 suspended in an emulsion of MontanideTM ISA VG 61 to cycling heifers, failed to induce infertility. This was despite the fact that the anti-ZP immune response was similar to that achieved following vaccination with native pZP or purified pZP proteins. This suggests that although the recombinant pZP proteins incorporated in each vaccine were immunogenic the antibodies produced failed to effectively bind to the ZP in the follicles of vaccinated heifers. Differences in the glycosylation and conformation between the native and recombinant proteins, which has been reported before with other ruminant recombinant vaccines, is the most likely explanation.
- Single administration of a vaccine containing a recombinant heterocomplex of bZP3&4 suspended in an emulsion of MontanideTM ISA VG 61 to cycling heifers induced infertility in some heifers initially. The outcome of two further mating periods is required to confirm and better understand this finding.
- Although there was evidence in Exp. 1 of a marked transient negative impact on the number of ovarian antral follicles this was not observed in any of the other experiments. This suggests that when cattle are repeatedly vaccinated with a highly immunogenic pZP vaccine

both a humoral and cell-mediated immune response are induced as has been observed with other animal species (Joone *et al.* 2017).

- As with all vaccines, there was some evidence that even where an effective response was obtained there were a few animals which failed to respond.
- There was some evidence that pZP vaccination may be reversible.

## 5.2 Benefits to industry

This project has demonstrated that an immunocontraceptive vaccine targeting the zona pellucida could be developed for use in cattle but more work needs to be done to create better quality recombinant ZP proteins. Vaccination of cattle with either pig derived or recombinant ZP/ZP proteins was shown to have little or no impact on their health, well-being, growth and carcass quality. The only observed impact was transient mild to moderate swelling at the injection site. Vaccination did not alter the normal behaviour or handling of cattle. There was evidence that some vaccinated cattle could recover fertility after approximately 12 months, but this was not examined in detail.

The following are key recommendations provided by Peter Letchford, Veterinary Pastoral Solutions, Kununurra on how a one-shot sterilizing vaccine with 12month efficacy could potentially be incorporated into the management of north Australian beef herds:

1. Best practice vaccination protocol to be used including restraint of each animal in a crush
2. Although some vaccinated animals may remain infertile for a prolonged period many will recover their fertility and thus an alternative to the spay punch hole will be required e.g a designated coloured EID button, to identify vaccinated cattle
3. For herds joining 2-year-old heifers a selection process is often imposed in the middle of the year prior to joining in the coming wet season. This would be an ideal time to vaccinate heifers culled from this draft. The delay in onset of an effective immune response is unlikely to be a significant issue as most heifers at this time are not cycling due to poor nutrition. Further, as most spayed heifers are still required to be pregnancy tested before being exported this enables identification of heifers which failed to respond to the vaccine and became pregnant
4. For yearling mated herds culled heifers could either be vaccinated at time of the selection draft or at time of pregnancy diagnosis after first mating
5. For cows the main issue is their lactation/pregnancy status at the time a decision is made to cull them. A single vaccination at the weaning muster when a decision is made to cull may be reasonably effective for dry pregnant cows which can be weaned first round the following year and then removed at the second round. However, for cows which that will have to be weaned at the second round they would require a second vaccination to prevent them becoming pregnant over the following wet season before being removed from the herd. The potential problem with vaccination of lactating non-pregnant cows at weaning (mainly applies to first round) is the weaning triggered resumption of cycling in some cows with subsequent conception before they have mounted an effective immunocontracpetive response. Thus a proportion of these cows may need to be vaccinated a second time.

# 6. Future research and recommendations

The failure of the administered recombinant ZP protein vaccine to induce infertility in a high proportion of heifers represents the major challenge for future research. The project team built collaborations with two well-published groups working on development of recombinant ZP vaccines for use in animals and identifying the key ZP protein responsible for sperm binding in cattle. However, despite this it seems likely that the failure of the recombinant ZP protein vaccine to induce infertility was due to significant differences in glycosylation and conformation between the native pZP and the recombinant pZP. Glycosylation is a complex and critical process playing an important role in the biological activity, folding, stability and solubility of proteins (Beygmoradi *et al*. 2023). Failure of a recombinant protein to fold into the correct three-dimensional structure typically results in the production of inactive proteins. These are some of the well-recognised challenges in producing recombinant protein vaccines (Pech-Cervantes *et al.* 2020). It is concerning that heifers vaccinated against the recombinant pZP proteins had an immunological response very similar to that observed for heifers vaccinated against native pZP. This may partly be due to our failure to be able to produce recombinant pZP2. The pregnancy rate of heifers vaccinated against native pZP2 in Exp.1 was significantly lower than the control heifers (29% v's 83-88%). There is evidence that ZP2 plays a role in blocking polyspermy, and in humans it has been shown to contain a binding site for acrosome-reacted spermatozoa and to play an important role in secondary sperm binding and penetration into the zona pellucida (Tsubamoto *et al.* 1999). Overall, funding of further research into production of recombinant bZP could be justified on the basis that the estimated cost of producing a single injection ZP vaccine is very similar to the true cost of spaying.

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Professor David Owen and Dr Merce Salla Martret Protein Expression Facility, The University of Queensland – produced the co-expressed recombinant ZP3/ZP4 proteins for the final experiment

Professor Ken Beagley, Institute of Health and Biomedical Innovation, Queensland University of Technology – conducted the IgG assays

Dr Lyndal Hulse School of Environment, The University of Queensland

Michelle Story (PhD student) School of Veterinary Science, The University of Queensland

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Peter Letchford, Veterinary Pastoral Solutions, Kununurra

Ian Braithwaite, Kick Gate Veterinary Services, Mt Isa

# 8. Appendices

## 8.1 Cost of spaying versus immunocontraceptive vaccine

The below table compares the direct costs of use of an immunocotraceptive vaccine with spaying. The spay costs were calculated by Ian Braithwaite (2009). The cost of $20 for the immunocotraceptive vaccine is based on a single vaccination and assumes that the vaccine will have limited sales and will only be available from veterinarians.

| | **Immunocotraceptive vaccine** | **Spay** |
|---|---|---|
| **Cost/hd for vaccine** | $20 | nil |
| **Spay costs/hd** | nil | $4.90 |
| **Stockcamp costs/hd** | $1.20 | $2.73 |
| **Mortalities+** | nil | $18.40** |
| **Total cost/hd** | $21.20 | $26.03 |

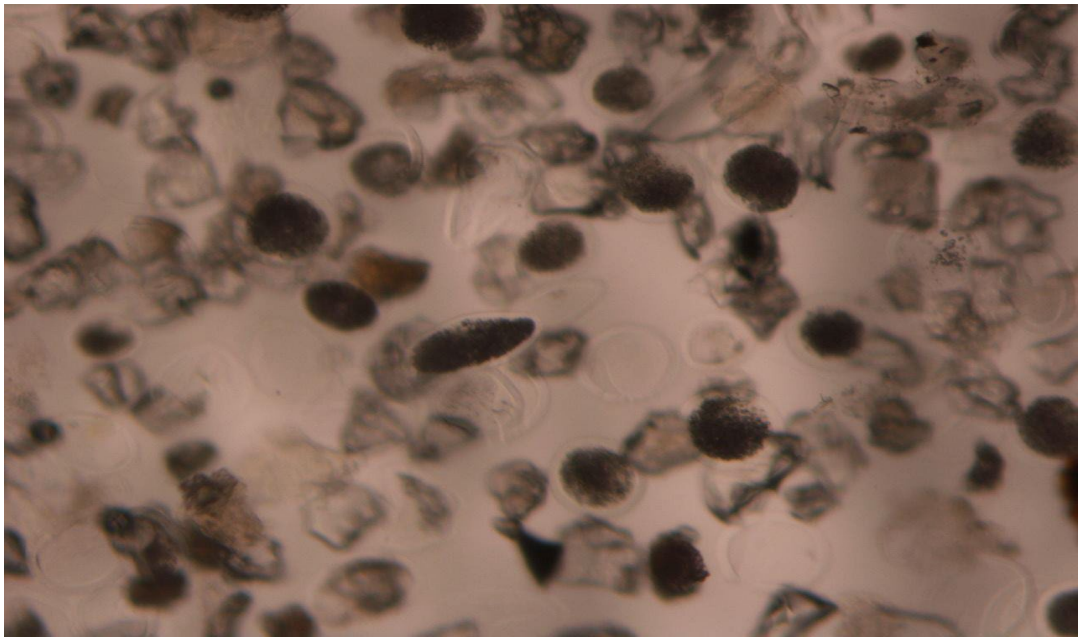
***** total spaying costs calculated as spay cost ($4.50/hd ) plus travel cost ($0.40/hd)

****based on 3.3% loss and dressed value of lost cow of 230kg dressed weight by $2.40/dressed kg

## 8.2 Isolation of porcine zona pellucidae

The sliced ovarian tissue was initially rinsed with Tris-Zona (TZ) buffer (50 mM Tris, 150 mM sodium chloride, 4 mM EDTA, 2 mM sodium citrate and 0.02% (w/v) sodium azide adjusted to pH 8.0), with the filtrate passing through a 1600 µm mesh nylon filter and then successively through 500 µm, 225 µm, 124 µm,70 µm and 48 µm nylon screens, each of which was washed with TZ buffer. Clumps of tissue were removed either on or prior to the 124 µm screen. Intact oocytes were trapped on the 70 µm screen whilst free zona from ruptured oocytes were retained on the 48 µm screen. Smaller material such as individual cells or erythrocytes passed completely through the 48 µm screen. The 70 and 48 µm screens were washed 5 times each to remove intact oocytes and ruptured oocytes. The products retained on these screens were kept separately.

The figure below shows the intact oocytes (dark colour) and the zona ghosts (clear circles) on the 70 µm filter.



The oocytes retained on the 70 µm filter were washed 5 times with TZ buffer to remove all blood cells, small ovarian cells, and follicular fluid. The filter was examined under the microscope to ensure all oocytes were removed, and the retained material was examined and photographed (see above) to record purity. The average protein yield from this filter was 3.35 +/- 1.31 mg zona protein. The 48 µm filter was treated similarly and its product (largely zona ghosts or zona fragments) were kept separately. Recovery from this filter was significantly lower at 0.332 +/- 0.041 mg zona protein.

Intact zona from the 70 µm filter were hand homogenised in a 30 ml Potter-Elvehjem homogeniser using 15 strokes of a Teflon pestle.

The isolated zonae were collected on a new 48 µm filter, washed into 100 ml of 20 mM Tris buffer pH 8.0, and then washed gently for 60 minutes on a magnetic stirrer to remove any contaminating

material originating from the oocyte itself. The zonae were collected on a new 48 µm filter and washed into 25 ml of 20 mM Tris buffer pH 8.0. The zonae were heat solubilised at 73°C for 20 mins. The material was centrifuged at 4°C at 27,000 x *g* for 15 mins. The supernatant was carefully removed and stored at -20°C. The total protein yield was quantified in an Olympus analyser. This colorimetric method uses pyrogallol red combined with molybdite which forms a red complex with a maximum absorbance at 470 nm. When this red complex binds to the basic amino groups on proteins, a blue-purple complex forms with a maximum absorbance at 600 nm. Absorbance at this wavelength is directly proportional to the protein concentration in the sample. In addition to quantifying the protein, a SDS-PAGE gel was run on the material using the Biorad Mini-Protean system and the gel stained with Commaisse Blue G-250 to ensure only proteins of the expected molecular weight were present.

## 8.3 Initial attempts to produce recombinant pZP3 and pZP4

Preliminary experiments were conducted using the mature secreted form of ZP4 with the natural signal peptide replaced by that of IgG kappa to optimise its ability to be secreted in mammalian cell cultures. The majority of the target protein was present in the intracellular fraction and little was secreted into the medium. Whilst purification from the intracellular portion is possible it is not preferable. We therefore conducted a broad based search of the literature for modifications to the construct which might facilitate secretion of the target protein. Two strategies were initially trialled. Firstly , three signal peptides (secrecon, CD-33 and tPA) shown to induce high secretory activity with other proteins were used to replace the IgG kappa signal peptide. Secondly, for each of the three different signal peptides two alanine residues were inserted at +1 and +2 positions as a clear preference for alanine at these positions has been established (reviewed: Guler-Gane et al, 2016). The result is the six constructs shown below, two each with one of the three signal peptides and one member of each 6f pairs also containing the modified alanine residues:

Secrecon signal peptide - POI - 10x Histidine Tag (C-terminal)

Secrecon signal peptide – AA (N-terminal) - POI - 10x Histidine Tag (C-terminal)

CD33 signal peptide - POI - 10x Histidine Tag (C-terminal)

CD33 signal peptide – AA (N-terminal) - POI - 10x Histidine Tag (C-terminal)

tPA signal peptide - POI - 10x Histidine Tag (C-terminal)

tPA signal peptide – AA (N-terminal) - POI - 10x Histidine Tag(C-terminal)

Two cell lines widely used in expression studies, human embryonic kidney cells (Expi293F) and Chinese hamster ovary cells (ExpiCHO-S) were evaluated. Small scale expression was evaluated in 24 deep well plate format. Briefly, 4.3 mls of Expi cells were seeded at 2.9 x$10^6$ cells/ml and 13.5ul of Epifectamine was added. Enhancers were added 24 hours post transfection to a target volume of 5ml per well. Expression was profiled after incubation at 37C for 4 or 5 days. A second incubation was also profiled after initially be5ng 5nc4bated at 37C but on days 5 and 6 the temperature was dropped to 32C before profiling. Cell density, viability and diameter were monitored and samples (0.5ml) were collected on the days indicated for post transfection analysis. Supernatant (S) and cell pellet (CP) were collected at each time point and were loaded onto 4-12% Bis-Tris SDS PAGE Gels whch were run under denaturing and reducing conditions. Western blots were conducted after the gel was transferred onto PVDF membranes and probed by anti-His-Horse raddish peroxidase (Miltenyi Biotech) at a 1 in 5,000 dilution for 1 hr at room temperature. Analysis was performed

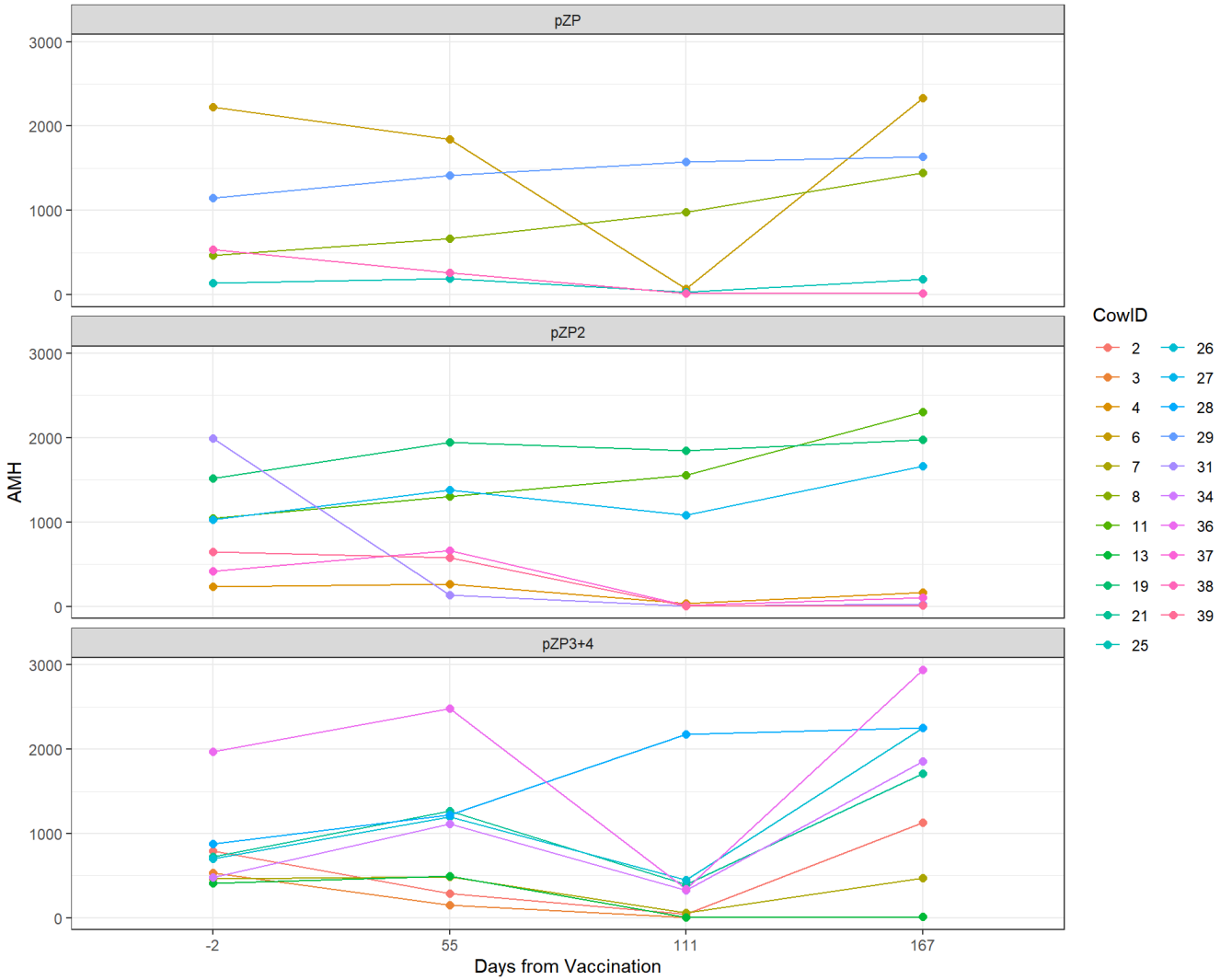
using a Bio-Rad Chemi-Doc XRS+ imaging system and the molecular weight calculated using ProtoParam (http://web.expasy.org/protoparam/).

To attempt to further enhance secretion of recombinant protein co-transfection experiments with three different cDNA enhancers: Vamp8 and empty pcDNA3.1 construct (Papp et al, 2011) and a chemical enhancer phenybutyrate (Estes et al, 2014) were conducted. The conditions of the experiment are as detailed above. The major difference was that these enhancers were added 24 hours post transfection when the incubation temperature was decreased from 37C to 32C. However, again as for Secrecon very little protein was secreted into the supernatant but significant quantities were present in the cell pellet. An additional step was added and at conclusion of the incubation the supernatant was collected and the cell pellet which contained the majority of the protein was resuspended in 1ml of lysis buffer (Phosphate buffered saline containing 1 tablet of Roche complete protease inhibitor and benzonase (130ng/ml)) and lysed on ice (3 x 10 sec) using a Branson sonicator. The lysates were centrifuged at 20,000xg for 15 mins at 4°C to obtain the soluble fraction. To determine whether the recombinant protein was in the soluble fraction SDS-PAGE analysis of both the total lysate and soluble (Sol) fraction were compared. The samples were reduced and run under denaturing conditions on 4-12% Bis-Tris SDA Page gels before being stained with Coomaisse Blue. Duplicate gels were blocked overnight at 4C with PBS containing 0.1% Tween-20 and 5% skim milk powder before being transferred to PVDF membranes in a Western blot apparatus before being repeatedly washed and stained with Anti-his antisera conjugated with horse radish peroxidase. Membranes were analysed in a BioRad Chemi-Doc XPS+ imaging system. (1:5000 dilution for 1 hour at room temperature.

## 8.4 Competitive inhibition assay protocol

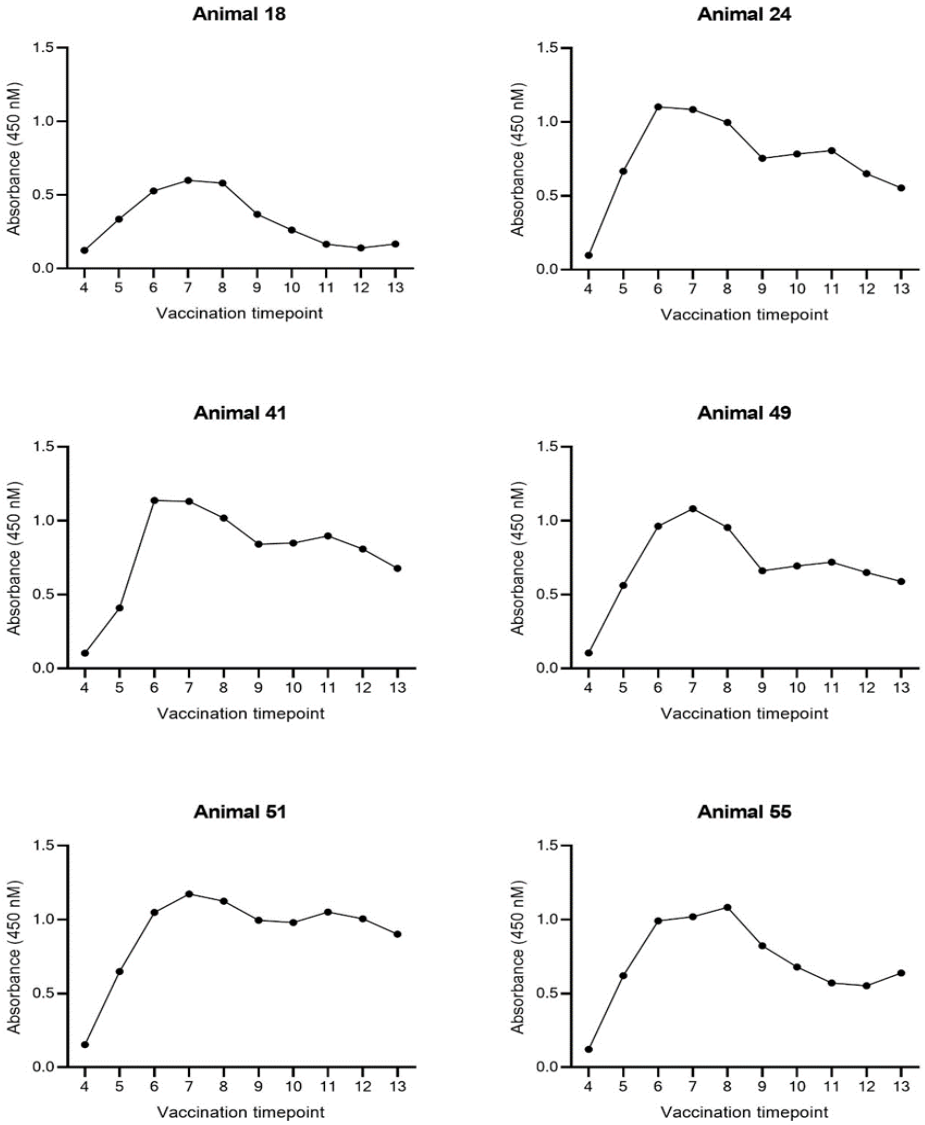
Recombinant bovine ZP4 (1.0 µg in 50 µL of TALON wash buffer containing 150 mM imidazole) was adsorbed to each well of a 96-well plate (Maxisorp, Nalge Nunc) at 4°C overnight. The wells were blocked with 250 µL of 3% BSA in TBS at 38.5°C for 2 h. We used frozen bovine sperm from Holstein bulls. The sperm straws supplied for artificial insemination were purchased from Animal Genetics Japan Co., Ltd. (Mie, Japan). Frozen bovine sperm was thawed and washed three times in pre-warmed (38.5°C) Brackett and Oliphant (BO) solution. The sperm pellet was suspended in 200 µL of BO solution and incubated at 38.5°C for 30 min. In this period, 10 µL of the suspension was mixed with 990 µL of PBS to dilute the suspension, and sperm concentration was determined by counting sperm in 0.1 µL of the diluted suspension using a haemocytometer. 50 µL of TALON wash buffer containing 150 mM imidazole (without inhibitors) or 50 µL of recombinant ZP protein in TALON wash buffer containing 150 mM imidazole (0.6 µg of single ZP protein or ZP protein complex) was mixed with 50 µL of sperm suspension containing $4 \times 10^5$ sperm. Each mixture was incubated at 38.5°C for 30 min. The wells were washed three times with 200 µL of BO solution, and then each mixture was transferred into the wells. The plates were incubated at 38.5°C for 2 h. The wells were then washed three times with 200 µL of BO solution. 50 µL of 70% glycerol in PBS was added to each well; sperm bound to the wells were recovered by 20 strokes of vigorous pipetting. The number of sperm in 0.1 µL of suspension was determined using a haemocytometer. The number of sperm bound to the wells not coated with recombinant bovine ZP4 protein (0 to 6.5) was subtracted from the number of sperm bound to the wells coated with recombinant bovine ZP4 protein. The number of bound sperm in the control experiments without inhibitors is shown as 100% sperm binding.

## 8.5 Individual Anti- Mullerian Hormone profiles (pg/ml) – Exp.1

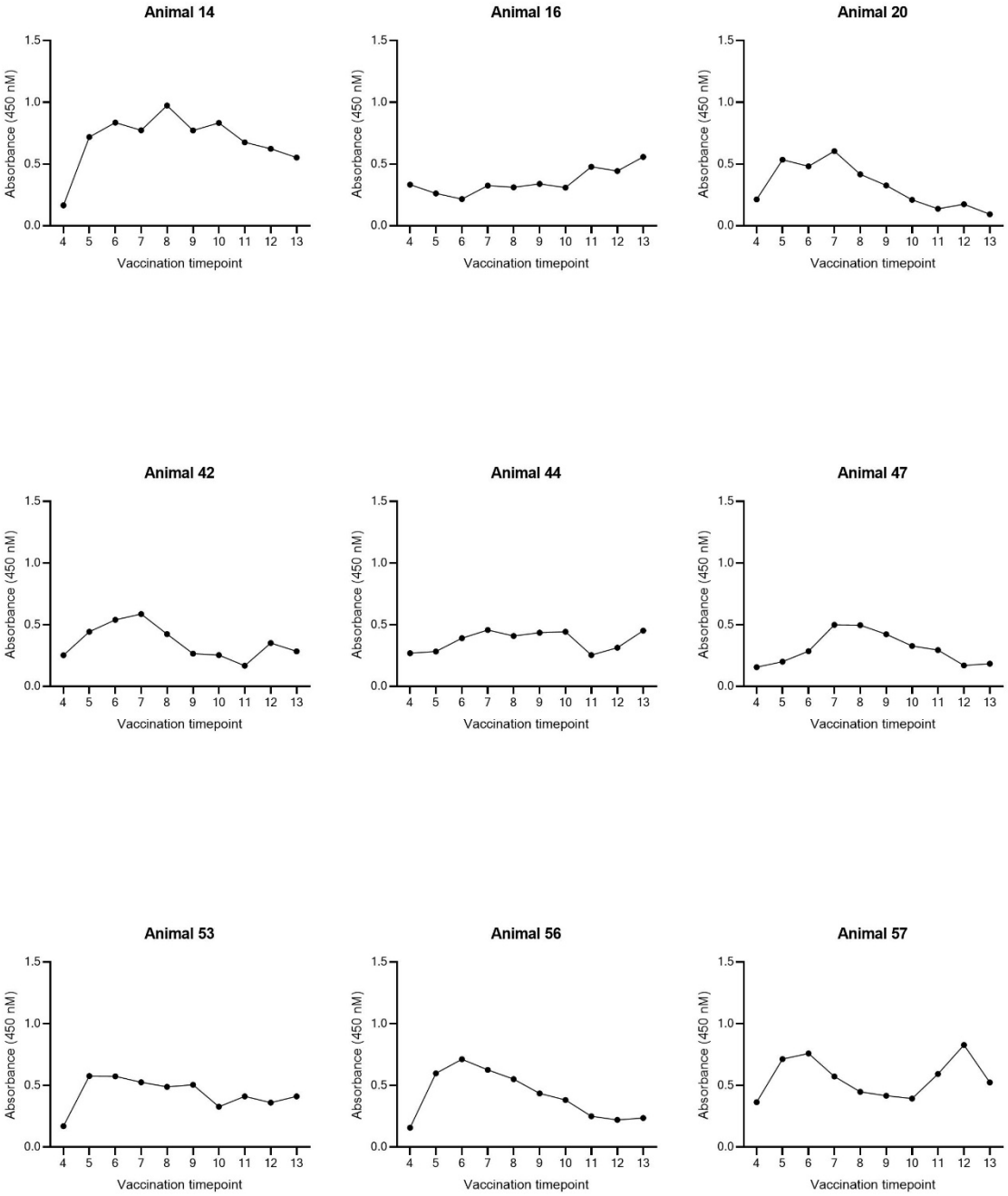


## 8.6 Individual immune response to vaccination Exp.3 and Exp.4

Experiment 3 immune response rZP3 formulated in Freund's complete and incomplete adjuvants



Experiment 3 immune response rZP3 formulated with Montanide™ ISA VG 61



Experiment 4 immune response rZP3&4 formulated with Montanide$^{TM}$ ISA VG 61