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Vaccine for Control of Flystrike

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EXECUTIVE SUMMARY

CSIRO has explored a range of approaches in engineering vaccines and tested them for the control of flystrike. Since the start of the project in 2019, 93 different formulations of vaccines have been tested in ~ 500 sheep under strict animal ethic's guidelines. The approach taken has been to use genomic and molecular tools to produce protein antigens for the formulation of prototype vaccines that target key proteins in the blowfly larvae. These vaccine antigens were identified through sequencing and analysis of the transcriptome of the three larval stages and the adult fly in addition to the cardia/anterior midgut and salivary gland organs of mature larva. Secreted proteins identified from the transcriptome analysis identified 6 key protein families that were selected for vaccine antigen production and testing. The 6 protein families include cardia/anterior mid-gut produced peritrophins, peritrophin-mucins, mucins, chitinases, serine proteases and predominantly salivary gland produced serine proteases and CRISP/non venom Antigen 5-like peptide proteins. Foundation research undertaken by CSIRO ~25 years ago also identified the potential of the peritrophin and peritrophin-mucin cardia/anterior midgut proteins as vaccine antigens for flystrike vaccine development. The use of next generation sequencing and molecular technologies has allowed the comprehensive repertoire of these proteins to be identified. Proteins from these protein classes have been identified as external to the cell and putatively secreted, thus amenable to antibody targeting. In addition, their relative high abundance, presumed important role and specific larval expression were important criteria for their selection for vaccine antigen production and testing.

Two approaches in production of candidate vaccine antigens were undertaken in this project. An approach whereby native protein antigens from the peritrophic matrix was produced by specialised laboratory culture of blowfly larvae was pursued as a "Type N' native vaccine approach. Not unlike the successful native antigen approach used in the development of BarberVax™ vaccine, the exploration of this approach has proven to be a good benchmark and a potential avenue of approach for flystrike vaccine development. The second approach has been the development of 'Type R' recombinant vaccines using molecular technologies and specialised cultured bacterial or insect cell production systems. The challenge of this approach has been producing protein antigens in a form that replicate the structure of the native larval proteins and herein replicate their relevance and efficacy in vaccination trials. A ~75% larval growth inhibition results as assessed using laboratory *in vitro* larval feeding and growth assays was demonstrated using 'Type N' native blowfly larval protein antigens derived from laboratory cultured larval peritrophic matrix. The results from the 'Type N' vaccine formulation whilst generally effective has been variable and initial investigation using glyco-proteome profiling is shedding light on the fact that specific culture parameters effect the protein abundance and glycans present on the cultured peritrophic matrix antigen cocktail. This presents previously unknown knowledge on native antigen production that will guide future production procedures of native antigen for flystrike vaccine development.

Engineering and production of vaccine antigens that perform as or exceed that of native antigens derived from blowfly larvae remains a significant challenge. We have investigated and tested several recombinant antigen production approaches including bacterial and several insect cell systems including two lepidopteran and a dipteran cell lines in the production of the 'Type R' recombinant vaccine. Results have been highly variable from the 'Type R' vaccine formulations however one formulation using five independent recombinant protein antigens in a combination antigen cocktail achieved a ~75% reduction in larval growth when assessed in laboratory *in vitro* larval feeding and growth trials. Repeat trials demonstrated larval growth inhibition effect but have not been able to repeat this same level of efficacy. Further formulation and vaccine delivery investigation is required.

Bacterial expression of the antigens whilst economical and relatively straight-forward has not produced suitable nor effective antigens and has been ruled out for further investigation. The lepidopteran insect cell lines have been used to good effect and multiple lead antigens have been produced by the University of Queensland Protein Expression Facility. Importantly, the recombinant protein antigens have been produced as soluble proteins allowing for glycans to be attached via the protein secretion pathway. Work is underway to characterise the glycans to determine whether they reflect those of the native larval proteins. A dipteran cell line derived from Drosophila, an insect/fly from the same insect order as *Lucilia cuprina* has also been investigated but its utility was found to be limited and not suited for large scale production. CSIRO is also investigating development of a novel antigen engineering and production approaches based on specific glycan modifications and novel presentation to the immune system.

Additional studies undertaken in this project assessed the impact of several adjuvants, vaccine antigen dose, depot of injection and longevity of the immune response to the lead trial vaccine formulations. Results based on antibody titre levels indicate the ability to achieve an optimal immune response with 2 vaccinations, 4 weeks apart, and demonstration the titre can be maintained for a period of up to 6 months. Efficacy of the vaccine was however shown to reduce after 2 months and did not correlate long term with the measured antibody titre. A single annual boost dose was shown to be sufficient to reinstate peak antibody levels. This indicates the potential of an initial 2 dose and subsequent annual vaccine dose if antibody titre can be demonstrated to align with anti-larval growth efficacy of the vaccine.

An effective flystrike vaccine remains elusive to date, continued work and approaches will be needed to help achieve this goal. Additional approaches are currently under consideration with some already currently under investigation to move this project to the next stage. This report presents research approaches undertaken, research highlights and provides a roadmap for future approaches and strategies to consider and undertake in flystrike vaccine development.

INTRODUCTION

Currently, the control of flystrike relies on informed management practices utilising integration of improved breech strike resistance genetics, insecticides, and husbandry practices including regular crutching and to a lessening degree mulesing with recent introduction of pain relief. With the use of mulesing being in the spotlight for sheep welfare issues and the evolving increase in insecticide resistance.

Australian Wool Innovation (AWI) through co-funding support of CSIRO and University of Melbourne are endeavouring to develop a vaccine to protect sheep from flystrike caused by the sheep blowfly, *Lucilia cuprina* (*L. cuprina*), a significant welfare threat to the sheep industry, costing an estimated $320M+ a year in control and lost production [1]. Controlling flystrike has been a long-term problem for the sheep industry. The sheep industry through their Research & Development Corporations, AWI and more recently Meat and Livestock Australia (MLA), have for many years actively sought an effective, welfare-friendly, consumer acceptable, rapid, and cost-effective alternative flystrike control and prevention technology. This has led to the development of improved breech-strike resistance genetics in the sheep flock and pain relief technologies for use in surgical breech-modification procedures in the short-term. A flystrike vaccine if successfully developed will help provide whole animal protection, reduce the use and reliance on chemical insecticides, and breech modification. A flystrike vaccine offers a new paradigm for flystrike control that will help garner the support of consumers, retailers, and animal welfare advocate organisations, thereby contributing to the future success, profitability and sustainability of the sheep wool and meat industry.

How can a flystrike vaccine benefit the sheep industry?

Vaccine technology has been demonstrated as an effective prophylactic treatment for a range of bacterial and viral diseases for well over a century. Socially vaccines are widely accepted, viewed positively by both the public and farmers, and are typically highly effective in the prevention of many diseases and zoonotic pathogens in humans and animals. A flystrike vaccine will offer the sheep industry a paradigm-shifting control measure for flystrike that will have substantial positive economic, environmental, health and welfare benefits for the industry. A vaccine will have a near immediate effect as it can be administered rapidly across the entire sheep flock and provide a long-term solution to the flystrike problem. CSIRO was initially responsible for the discovery and development of TickGard II™ a cattle tick vaccine in Australia in the 1990's [2,3]. This vaccine was further developed in South America as the cattle tick vaccine, GAVAC™ and reported in a study conducted in Venezuela in 2016. Within two years of its widespread use in ~ 1.9 million cattle, chemical acaricide use was reduced by greater than 80%, saving the industry from tick control costs and contributing to better health and welfare outcomes for the animals [4]. A flystrike vaccine could potentially have a similar positive effect for sheep blowfly control in Australia, New Zealand, and South Africa. Importantly, as a vaccine stimulates the immune system, it will not restrict protection to just the breech but will provide whole of body protection to the sheep.

Can a flystrike vaccine be developed now?

Significant new opportunities exist with the ease of accessibility to new technologies that can build on previous research in flystrike vaccine development making the development of a vaccine a real possibility. A research program undertaken through the late 1980's to early 2000's by CSIRO livestock scientists helped establish the feasibility of producing effective livestock parasite vaccines including for the sheep blowfly. It was demonstrated that antibodies raised by a prototype vaccine to specific classes of native *L. cuprina* larval proteins could be used to significantly inhibit larval growth (Figure 1). The research relied on numerous purifications of different larval protein extracts and testing in vaccination trials. Protein extracts that demonstrated protection against sheep blowfly larvae by inhibiting their growth, were further purified and

peptide signatures from the protective proteins determined. This information was then used in the highly technical and laborious process of generating DNA probes for screening cDNA (expressed gene) libraries that had to be engineered and synthesised. A gene encoding the protective protein could then be identified and this information used to engineer and produce this protein in expression systems including bacteria, yeast, or insect cells for use as an antigen in a vaccine. The research was difficult, time-consuming, and undertaken before modern next generation genomic technologies and information were available. Expression systems were also in early stages of development and the processes were rudimentary and slow. This research, whilst unable to generate a suitable vaccine for commercial development at the time, did generate core foundation knowledge that identified classes of proteins that could be used for vaccine development in the future when science and technical capability had suitably advanced.

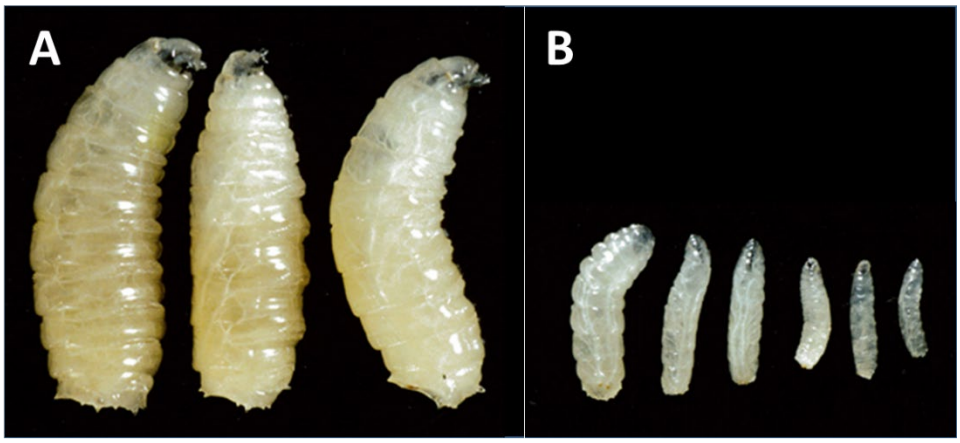


Figure 1: CSIRO results showing Sheep blowfly larvae fed on blood serum collected from sheep that were **A)** unvaccinated or **B)** vaccinated with larval protein extracts. The results demonstrated antibody-mediated larval growth inhibition over a 72-hr period (x10 magnification).

Next generation genomic technologies are now the mainstay of molecular laboratories. A whole genome can be sequenced in a short amount of time as opposed to a gene at a time as was the case twenty-five years earlier. The sheep blowfly (*L. cuprina*) genome has recently been sequenced and annotated by the University of Melbourne, Australia (2017, co-funded by AWI) and Baylor College of Medicine as part of the I5K genome initiative. This is a significant resource as it contains the full gene sequences encoding all proteins in all lifestages of *L. cuprina*. It is now also possible to chemically synthesise a gene, optimise it for protein expression in different cell-based protein expression systems (i.e. bacteria, yeast, or insect cell), and produce proteins for testing in prototype vaccines in a relatively short period of time (i.e. several months rather than years). Using these new technologies, combined with our experience in flystrike vaccine development has generated significant new opportunities for R&D approaches to develop a vaccine for combating flystrike.

New opportunity for development of a flystrike vaccine

CSIRO recognised a new opportunity to re-engage in development of a flystrike vaccine driven by new age technologies and the release of the sheep blowfly genome; information previously unknown. Through generous support and funding by AWI, CSIRO has endeavoured over the past 5 years to progress the development of a flystrike vaccine.

Using a strategy, referred to as 'Reverse Vaccinology', an approach was applied where candidate antigen protein-encoding genes were identified from a range of gene libraries we generated containing genes that are expressed (present) in key gut tissues or life stages of the sheep blowfly. Bioinformatic pipelines have been

used to extensively mine and extract information from the large quantity of generated data. A comprehensive and detailed list was created of genes that are both expressed by blowfly larvae and secreted, representing potential candidates for vaccine antigen production and testing. Prototype vaccine testing has been performed over this 5-year period with ~50 antigens synthetically engineered, produced in bacterial or insect cell expression systems and tested individually and in combination in sheep in over 90 prototype vaccine formulations.

Rationale and pipeline for candidate antigen identification for prototype vaccine development

Sheep blowfly larvae are incredibly tough and resilient organisms that have adapted from feeding on carrion where they were often outcompeted by other fly species, to feeding on live sheep without competition. The larvae spend a reasonably short period of time on the sheep after the gravid adult fly lays its eggs in the fleece. Within ~72-96 hours, the larvae have hatched, undergone three moults and drop off into the dirt to pupate. This reasonably short period of parasite-host interaction, together with their rapid growth, means that a vaccine needs to target and take effect on the larvae immediately they interact with the host sheep.

We previously demonstrated that an antibody-mediated approach, whereby an immunological response is produced by sheep vaccinated with select classes of native larval proteins, results in inhibition of larval growth *in vitro* when larvae feed on immune sheep sera. Inhibition of larval growth was produced principally by antibodies raised to proteins purified from the larval midgut and the semi-permeable membrane that lines the midgut, called the peritrophic matrix (PM). The PM is specifically produced by a small organ called the cardia, located at the start of the midgut. Additionally, excretory/secretory protein extracts, produced principally by the salivary glands and the midgut cells have also demonstrated some larval growth inhibitory effects.

The remainder of the larval gut and its body is lined by an incredibly tough and impermeable polysaccharide polymer called chitin. There is thus a limited opportunity to target the larvae through a vaccine antibody mediated approach except via targeting the midgut and salivary gland produced proteins. These proteins represent the best accessible targets for ingested antibodies. We previously investigated and discerned the mode of action a vaccine directed at the proteins that constitute the peritrophic matrix. Antibodies raised to these proteins bind to the matrix thereby blocking its pores. The blocking restricts the secretion of proteases into the gut lumen, inhibits the passage of nutrients to the underlying midgut epithelial cells, thereby starving the larvae and inhibiting their growth. The antibody mediated blocking is also amplified with a building layer of ingested but undigested sera and tissue proteins (Figure 2).

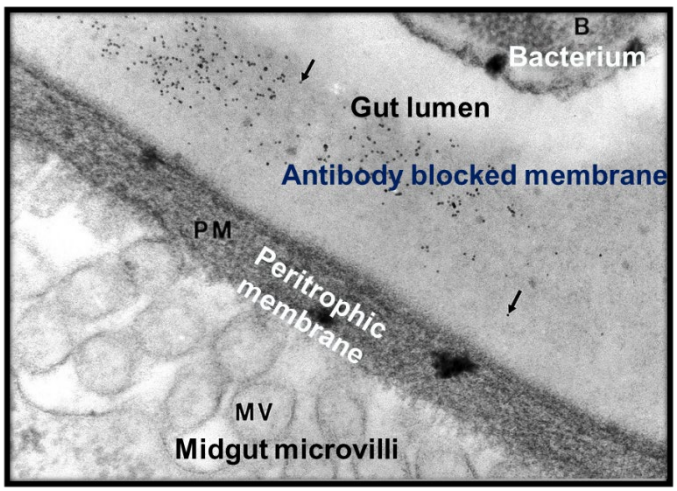


Figure 2: Electron microscope imaging showing the midgut of a *L. cuprina* larvae fed on sera from sheep vaccinated with peritrophic matrix proteins. Antibodies to these proteins cause blocking of the matrix pores. The image shows 6 nm colloidal gold particles (black dots, highlighted with arrows) unable to transgress the membrane as they normally can in larvae fed on normal sheep sera. (image CSIRO)

Target tissue transcriptomics for candidate antigen discovery

The availability of the *L. cuprina* genome sequence, annotated and published by the University of Melbourne, has been highly enabling and facilitated the process of candidate antigen selection. Guided by the foundation work performed by CSIRO and an understanding of how the blowfly larvae interacts with the host sheep, we concentrated our efforts on candidate antigen proteins associated with larval gut and salivary glands. We isolated the key organs of the cardia and salivary gland by micro-dissection of *L. cuprina* larvae, isolated mRNA, (i.e. transcript molecules encoding for the proteins produced in these tissues), and generated RNA-seq transcript expression libraries that were then sequenced utilising the services of the Australian Genome Research Facility (AGRF). Extensive information was generated that details all the genes and putative proteins that are actively produced by the specific tissue at a specific point in time. Using bioinformatics analysis tools, the expression of these protein encoding genes was quantified and those that produce secreted proteins identified. As a result, we identified secreted proteins that are produced in abundance, are associated with structural formation of the PM, or are produced as secreted proteases and other enzymes. It is hypothesised that secreted proteins need to be the target of a flystrike vaccine as they have the best chance of being targeted by antibodies generated from the immunological response to the administered vaccine. There is no evidence to date that demonstrates that antibodies pass through the PM. Hence, the repertoire of candidate antigens that were considered for vaccine development were limited to those that are secreted and available/exposed to ingested antibodies. The process focussed on proteins that constitute the PM or are secreted through it and those produced as excretory/secretory proteins at the site of larval infestation on the sheep skin (Figure 3).

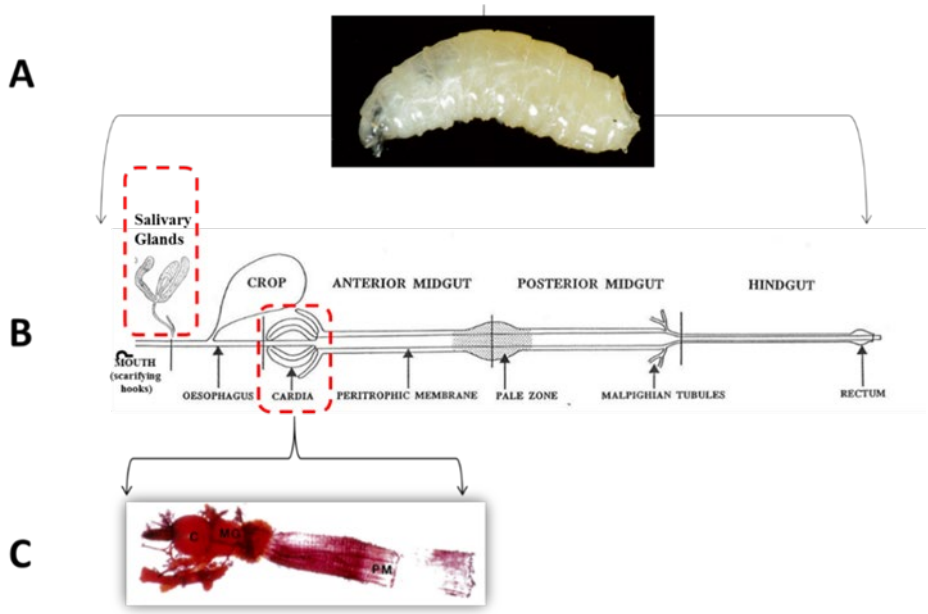


Figure 3: A) *L. cuprina* third instar larvae. **B)** Diagrammatic representation of the internal digestive structure of the larvae with target organs for transcript analysis and vaccine candidate antigen discovery circled in red. **C)** A dissected cardia (C) and small section of attached midgut (MG) cultured *in vitro* showing the organ producing and extruding the peritrophic matrix (PM).

We employed an additional level of analysis and target filtering to assist with identifying lead candidate antigens for vaccine development. To give a vaccine the greatest chance of working, it will need to target the larvae immediately they start to feed and establish on the sheep skin. With this in mind, we produced mRNA transcript libraries from larvae that had just hatched and fed for only 4 hours on either a sheep protein diet or on an artificial non-sheep protein diet. These data inform what protein-encoding genes are produced as the larvae establish feeding and those protein-encoding genes that may be specific to a response from feeding on sheep protein. The data were cross-referenced with the tissue (cardia and salivary gland) data (Figure 4). The process allowed us to refine the selection of candidate antigens for vaccine development. The short-list of candidate antigens represents larval proteins that are (i) secreted, (ii) produced by the key target tissues, (iii) produced as the larvae establish a flystrike, and (iv) are produced in relative abundance.

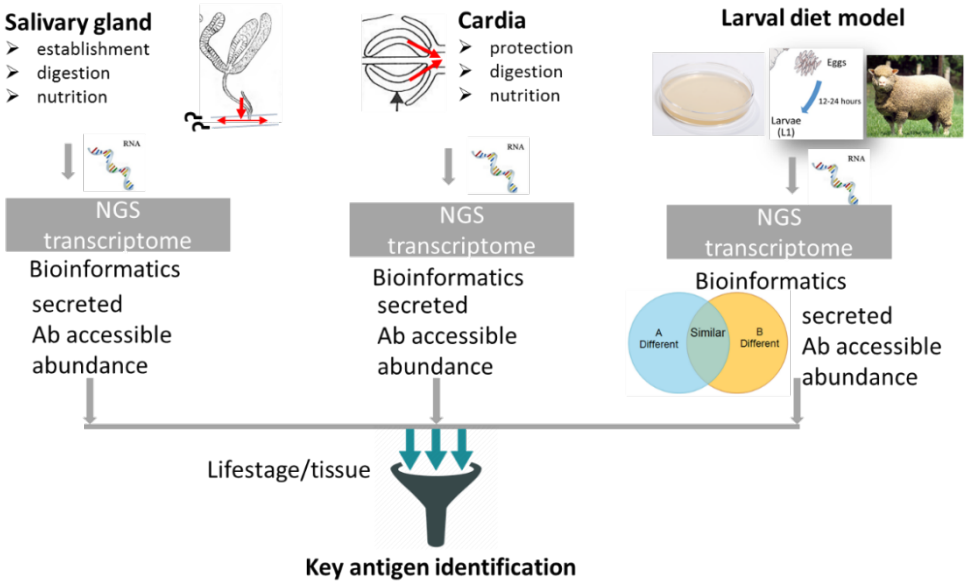


Figure 4: RNA-seq transcript libraries were produced from key tissues and larval lifestages, sequenced by Next Generation sequencing (NGS) technology to produce their transcriptomes. The data was cross-referenced and filtered to produce a short-list of candidate antigens for vaccine development.

Candidate antigen classes

Protein-encoding genes have been identified and short-listed as candidate antigens for prototype vaccine development. These vaccines have been tested to determine whether they can generate an effective immune response in sheep that will inhibit larval establishment and growth on sheep. These classes of proteins represent an informed list of what we considered to represent the best candidates for initial vaccine antigen production and testing. This report details the candidate antigen classes we investigated, the generation of the protein antigens for vaccine testing, sheep vaccination trials and assessment of vaccine efficacy in generating an immunological response and effect on larval growth.

LITERATURE REVIEW

Flystrike or more generally cutaneious myiasis is the infestation of the skin by larvae of predominately Australia Sheep blowfly (*L. cuprina*) on Sheep (*Ovis aries*). In Australia, the economic losses from flystrike myiasis in sheep due to reduction of wool production, body weight loss, preventative measures, and interventions costs more than $320 million annually [5]. Commonly flystrike is referred to by where on the sheep's body it takes place, for example breech strike is the most common and occurs around the rear end (breech) following urine or faeces staining. *L. cuprina* was inadvertently introduced into Australia in the late 1800s and became a reported pest species by the 1880s with the introduction and farming of the Vermont Merino sheep. Work investigating *L. cuprina* and flystrike began in the 1930s by Ian Mackerras at the Council for Scientific and Industrial Research (CSIR) now better known as Commonwealth Scientific and Industrial Research Organisation (CSIRO) [6]. Many high-quality reviews already exist focused on flystrike [5,7], so we here will provide a brief but targeted review into the current work done on controlling flystrike, ecto- and endo- parasite vaccines, and speculate on where the future of parasite vaccines is heading.

How does flystrike and strike-susceptibility occur?

For flystrike, the sheep need to be an 'attractive environment' for blowflies to lay their eggs. One common way sheep become 'attractive' to blowflies is by fleece rot, a bacterial infection, usually *Pseudomonas aeruginosa*, on the skin and fleece causing exudative dermatitis and wool damage [8]. These bacterial infections generate odours attracting sheep blowflies and help provide the perfect environment for laying eggs. Another common way sheep become 'attractive' to blowflies is by moisture or diarrhoeic faeces build up around the breech. Environments with fleece rot and diarrhoeic faeces not only provide the ideal environment for adult flies to lay eggs but also provide larvae rich food sources such as faeces, skin secretions, dermal tissues, and blood. The larvae also use their tough scarifying mouth-hooks to abrade and obtain further nutrition off the sheep skin.

Strike-susceptibility refers to the susceptibility of a sheep breed to flystrike, and there are several factors that influence this. One of the most important factors is the predisposition of sheep to fleece rot, which is influenced by wool characteristics, including wrinkle score, and how wrinkly the skin is [9]. Other wool characteristics also influence strike-susceptibility, including depth of wrinkles around the horn, breech wool coverage and wool colour. Many of these wool characteristics prevent the skin and fleece from properly drying and can lead to bacterial dermatitis or simply ideal environments for blowfly eggs and larvae. Flystrike and fleece-rot are considered a disease complex, where flystrike occurrence is strongly associated with the occurrence of fleece-rot [8].

Prevention measures

The control of flystrike currently relies on mulesing and insecticides. Mulesing was developed in 1931 by JHW Mules, a South Australian sheep farmer and involves the removal of loose skin around the rear end of the animal to permanently stop wool growth around the breech and tail. Recently, the use of mulesing to control flystrike has been in the spotlight for issues surrounding sheep welfare, with the EU looking to ban all wool produced from mulesed sheep [10–12].

Another common prevention method for flystrike is using insecticide treatment which is typically applied via chemical sprays or dips (soaking the target regions to absorb chemicals through to the skin). The treatment of flystrike with insecticides began with the use of arsenic, copper, boron, and phenols [13]. By the 1950's, dieldrin and aldrin, organochloride insecticides were some of the most common methods of flystrike prevention [5]. However, by the late 1950's resistance in the blowfly population to organochloride insecticides had become widespread, linked to a

mutation in the *Rdl* gene [14,15]. The use of organochloride insecticides was quickly replaced with organophosphate insecticides such as diazinon [5]. However, by the mid-1960's resistance to organophosphates emerged, resulting in the need for novel insecticides [16]. Now, cyromazine, dicyclanil, spinosad, and ivermectin are the most common insecticides in use [7,17]. However, due to heavy reliance on insecticides, there is once again growing resistance, and no new or novel and effective insecticides are currently available [18].

Vaccines against *P. aeruginosa* to control fleece rot and therefore body strike have been investigated by the CSIRO in the 1980s and 1990s [8]. These studies showed that vaccinating with *P. aeruginosa* antigens produced a protective immunogenic response [19]. However, controlling flystrike or specifically body strike with fleece rot (*P.* aeruginosa) vaccines is difficult owing to an array of serotypes and as attaining a high level of a disease control of a secondary disease is extremely difficult [8].

With decreasing reliance on mulesing to manage flystrike and growing insecticide resistance, novel methods of presentation are desperately needed. In the mid 1980's the first attempt at developing a vaccine against flystrike was undertaken by the CSIRO [20] following encouraging progress in development of a cattle tick vaccine.

Flystrike vaccine

With the continued development of resistance to insecticides, the key to controlling insect parasites may lie in vaccines. Developing vaccines requires antigens, protein targets. Antigens need to be administered and create an immune response which can affect the growth of blowfly larvae during and importantly at commencement of infestation. As flystrike is caused by an insect ecto-parasite, finding antigens targets is significantly more challenging, as potential antigens are likely 'hidden antigens' and do not interact with the host immune system directly and therefore do not induce a natural and continued immune response. The initial CSIRO vaccine and immunology research showed that the sheep immune system produced antibodies in response to injection with blowfly larval proteins (antigens), however with limited consequences to larval health [21,22]. The peritrophic matrix (PM) lines the gut of blowfly larvae to lubricate the epithelium and protect it from digestive enzymes and pathogens [23]. As digestive systems associated with saliva and the PM are the only point of contact between insect ecto-parasites and host, this limits the targets for potential vaccines.

Peritrophic matrix as the primary vaccine target

In 1993 larval PM harvest from blowflies was first used to vaccinate sheep. This vaccine resulted in antibody production and some protection against larvae, decreasing larval weight by ~50% [21]. After this, specific PM proteins were investigated as potential antigens for vaccines. The peritrophic matrix is made up of chitin microfibrils (~7% of the matrix) [24], and embedded proteins (20 – 55%) in a proteoglycan matrix [25–27]. The specific embedded proteins that make up the peritrophic matrix are known as peritrophins, which typically have chitin binding domains and in a sub-class have mucin like domains. Initial research focused on vaccinating with native and recombinant peritrophins and measuring efficacy (Fig. 5) following proof of concept and has been extended with availability of new research tools in the current attempts to develop a flystrike vaccine.

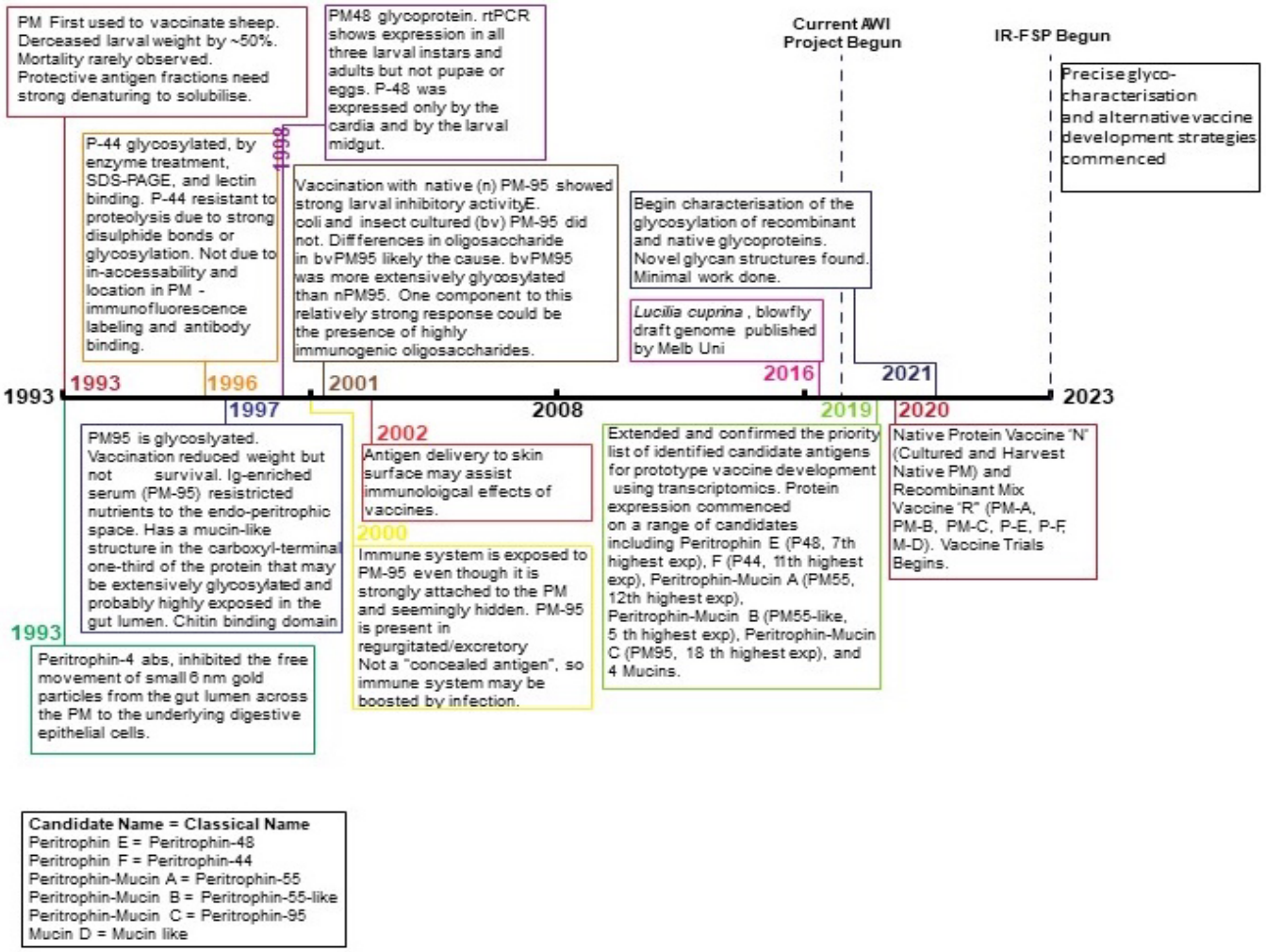


Figure 5: Timeline of key papers and work from CSIRO's flystrike vaccine program.

In 1993 following vaccinations with whole PM, antibodies targeting a highly abundant PM embedded protein, peritrophin-44 were hypothesised to block pores in the PM and result in starvation of larvae [28,29]. In 1997, vaccinations with peritrophin-95 were shown to reduce larval weight but not effect larval survival [30]. Interestingly, it was found that increasing the concentration of antibodies targeting peritrophin-95 were shown to significantly reduce larval survival [30]. When growth/weight is reduced by ~80% then a noticeable effect on larval survival occurs [21,31]. This previous work highlights the importance of peritrophin-95 to the PM and the potentially viability of a vaccine targeting it. In 1998, peritrophin-48, which together with peritrophin-44 make up 70% of the total mass of the PM proteins [29], was characterised and found to be a glycoprotein [32]. Like peritrophin-48, both peritrophin-95 and peritrophin-44 were found to be glycoproteins [29,30]. All antigen candidates were thought to be hidden antigens, that do not naturally encounter the immune system, however Tellam *et al.* (2000) showed that peritrophin-95 was present in the regurgitated/excreted material from larvae. Peritrophin-95 being present in regurgitation/excretion highlights that it is not a hidden antigen and may create some level of natural immunity [33]. In 2001, Peritrophin-95 was produced in Esherichia coli and Sf9 (Spodoptera frugiperda) insect cells [34] and their effectiveness as vaccine antigens investigated. Native peritrophin-95 was shown to reduce larval weight, while vaccinating with E. coli and Sf9 produced peritrophin-95 had no significant effect on larval growth [34]. The question remained as why pure native protein antigen preparations performed much better than recombinant antigen preparations of the same protein.

Glycosylation

Secreted and membrane-bound proteins are almost universally glycosylated. Glycosylation is the modification of proteins with covalently attached oligosaccharides (glycans). There are many types of glycosylation, with two key forms being N- and O-glycosylation. The PM is no different, with many reporting the presence of *N*- and *O*-glycosylated proteins [29,30,32,34,35].

Following differences in vaccination efficacy, native and insect cell (Sf9) produced peritrophin-95 were found to be both glycosylated but with a different composition [34]. Finally, Tellam *et al*. (2001) showed that the inhibitory effect of native peritrophin-95 was associated with antibodies targeting not only the polypeptide but also the glycans. This highlights the important role glycosylation and glycan structure has on antibody production and immune response. After the promising vaccination and growth inhibitory results, research activities on a flystrike vaccine were paused. Not until 2015, when the genome of *L. cuprina* was published [36], did the idea of vaccinating against flystrike re-gain traction.

The blowfly genome

As part of the 5000-insect genome (i5k) project [37], Anstead *et al*. 2015 sequenced the genome of *L. cuprina* and identified and partially characterised several genes involved in insecticide resistance. This genome provides a resource for better understanding the Australian sheep blowfly, including development and reproduction, insecticides, and potential vaccine targets. Since 2015, four more iterations of the Australian sheep blowfly genome have been published, two by the University of Melbourne again in 2017 and 2022, a 2017 annotation by the Baylor College of Medicine and by North Carolina State University in 2022. Interestingly the genome published by North Carolina State University is an inbred strain of blowfly derived from wild type strain LA07 [38], while the strain used by the University of Melbourne and Baylor derived from a continuous culture from a 20-year-old isolate from the Australian Capital Territory in Australia [36].

Vaccine development

Vaccines provide a safe alternative to chemicals and insecticides, reducing environmental impact and potentially being more cost effective in the long term. Australia and in particular the CSIRO have a strong history in ecto- and endo- parasite vaccine research [3,34,39,40]. Here we will briefly describe key ecto- and endo-parasite vaccines and how insect cell lines are being utilised for the development of vaccines.

Commercialised vaccines against ecto- and endo-parasites

Ecto- and endo-parasites pose significant risks to livestock, companion animals, and human health. With the continued development of resistance to insecticides, a key approach to controlling ecto- and endo-parasites is through vaccines. As ecto- and endo-parasites are multi-cellular organisms that exist outside the body or in body cavities, finding antigens targets is significantly more challenging, as potential antigens are 'hidden antigens' and do not interact with the immune system directly and therefore do not induce a natural immune response.

Now sold as Huskvac (Bovillis), in 1960 the first commercial vaccine against an ecto- or endo-parasite was developed against bovine lungworm (*Dictyocaulus viviparus)* [41,42]. Gastrointestinal nematode infection is a major issue in the livestock industry, representing a health, welfare, and production issue [43]. *Haemonchus contortus*, Barber's pole worm, is the most pathogenic nematode in small ruminants. *H. contortus* is the most economically concerning nematode, causing substantial economic losses due to reduction in animal productivity, treatment costs, and increased mortality due to acute infection [43,44]. A recently released native

antigen vaccine, Barbervax® is the only commercially available vaccine against *H. contortus*, providing ~ 80 % protection in young sheep [45,46].

The first recombinant vaccine developed against cattle ticks was in 1989 by CSIRO [39,47] This vaccine targets *Rhipicephalus microplus* a common cattle tick and in 1994 a commercial vaccine known as TickGARD® was produced and released by Hoechst Intervet Australia, using Bm86 antigens. In 1995, TickGARD®PLUS an improved version was released, with Bm86 antigens produced in yeast cells (*Pichia pastoris*). This version produced a stronger immunogenic response likely due to the addition of glycans produced in yeast cells [3]. Both TickGARD®PLUS and Gavac® are based on Bm86 antigen and have varying effects due to genetic diversity [48,49]. TickGARD®PLUS was discontinued in Australia due to the need for frequent boosters, however Gavac® (Heber Biotec; Havana, Cuba) is still produced internationally [3]. Other vaccines targeting *R. microplus* are currently being developed by Beef CRC/MLA [40,50].

Ixodes holocyclus, the Australian paralysis tick has been reported as the most potent of paralysing ticks in the world, affecting ~ 10,000 pets a year and toxic enough to kill a large dog [51–53]. Like with cattle ticks, CSIRO was the first group to begin developing a vaccine for *I. holocyclus*, but practicality of vaccine production was not feasible at the time [54]. The University of Queensland has subsequently identified potential vaccine targets in *I. holocyclus* and have submitted a patent [55].

Vaccine production in insect cell lines

Insect expression systems are widely used to produce recombinant proteins, commonly using baculovirus-insect cell systems (such as Sf9, Sf21, and HighFive) [56] and *Drosophila* expression systems (such as S2). Insect expression systems have also been used to make vaccine antigens. COVID-19 vaccine candidates, such as Novavax®, Sanofi®, and Adimmune® are all produced in baculovirus-insect cell systems [57]. In addition, Cervarix®, an HPV vaccine and Flublok®, an influenza vaccine both use insect cell systems to produce antigens [58,59].

The envelope glycoprotein (Env) tri-mer of human immunodeficiency virus type 1 (HIV-1) is the only surface antigen, responsible for receptor binding and membrane fusion during virus entry [60]. While Env is the primary antigenic target, it exhibits inherent metastability and evades the host immune response by presenting itself in different conformational states. The immune system reacts by creating non-neutralizing antibody epitopes to these non-functional conformations, allowing the functional Env trimer to continue unimpeded [60]. When produced in a modified Sf9 system, the Env tri-mer were heavily glycosylated with a high proportion of oligo-mannose and paucimannose glycans and demonstrated good antigenicity and good immunogenicity [60]. If an insect-based system cannot provide the necessary glycosylation patterns, either a different organism or engineering the insect system to produce the sought after glycans must be chosen. Therapeutics is a major field where insect glycosylation patterns are unacceptable [61]. Due to the desire for human like glycans, significant research has been done on modifying the protein N-glycosylation pathways in insect cells to obtain 'mammalianised' glycoproteins [61–67].

Other factors important to effective vaccine development

There are many factors that contribute to vaccine efficiency, including vectors and production systems, adjuvants, and delivery systems [68]. As we have briefly described the use of insect cell lines in vaccine production, here we will discuss the importance adjuvants and delivery methods have on vaccine efficiency.

Adjuvants, from the Latin word's *ad* and *juvo*, meaning towards and help. Adjuvants are generally described as additions to vaccines that enhance the immunogenicity and are used in almost all modern vaccines [68]. More

specifically, adjuvants have three benefits, 1) to increase or enhance vaccine efficiency, 2) reduce the amount of antigen or dose size needed, along with number of immunisations, and 3) to improve stability of the vaccine [68,69]. There are many reviews that cover in great depths the history and mechanisms of adjuvants [68–71]. The first reported use of a vaccine adjuvant is from 1925, where Gaston Ramon showed that co-immunisation of a diphtheria toxoid with tapioca, starch oil, agar, and other compounds increased anti-toxin response in patients [70]. In 1926, Alexander Glenny found that combining aluminium salts with diphtheria toxoid resulted in a significant increase in immune response [72]. Aluminium salts were the only licenced adjuvants for 60 years, until the 1990's when a number of novel adjuvants were introduced [70]. Currently, there are many available adjuvants on the market, with four key types being aluminium-based, emulsions, lipid-based particles, and polymeric particles all with different benefits and effectiveness depending on antigens, pathogens, and host [73].

Most vaccines are administered by subcutaneous or intramuscular injections [74]. In humans, relatively few vaccines are administered into the skin (epidermis or dermis) [75,76]. There are other delivery methods for vaccines including the nasal mucosa and the gastrointestinal tract [74]. For vaccines to function, dendritic cells need to take up the antigen and present it to T lymphocytes. While, subcutaneous or intramuscular contain few dendritic cells, the epidermis and dermis contain a high number of dendritic cells. Patch delivery methods are an emerging vaccine delivery method that administers the antigen into the epidermis and dermis, results have shown thermostability and enhanced immune response using a patch-based delivery method [77]. The epidermis and dermis have been shown to be effective vaccination sites as they are rich in antigen-presenting cells [78,79], and show advantages in dose-sparing and thermostability of vaccines [77,80,81]. This highlights that delivery of a vaccine and in this case an ecto-parasite vaccine via the skin might be an ideal vaccination approach but will require development [82].

The future of the flystrike and other endo- and ecto-parasite vaccines

With the rise of insecticide resistance and the lack of novel insecticides, the need for vaccines have become critical in the control of endo- and ecto-parasites. However, whilst vaccines have been used or tried to be used for endo- and ecto-parasites, the field has not progressed as quickly as human vaccine research and there are now opportunities to advance their development.

We have shown the importance of glycosylation on vaccine efficiency in research on the Flystrike vaccine and on the cattle tick vaccine TickGARD$^{®PLUS}$. The choice of cell line for production of the various antigens is critical to producing highly antigenic and effective vaccine antigens. While, glycosylation has been a feature here, the impact that glycosylation and other post-translational modifications have on antigen effectiveness is poorly understood in endo- and ecto-parasites. Glycosylation has become critical in the development of human vaccines, with influenza vaccine development considering the changes to *N* glycosylation sequons, the potential immunogenicity of glycans for a HIV vaccine, and the importance of glycans in developing an effective COVID-19 vaccine [60,83,84]. Glycosylation is likely an important focus area for endo- and ecto-parasite vaccine development.

We also discussed vaccines administration by subcutaneous or intramuscular injections compared via the skin (epidermis or dermis). The epidermis and dermis have been shown to be effective vaccination sites [82], due to being rich in antigen-presenting cells [78,79]. Vaccinating against endo- and ecto-parasites has been difficult, one of the reasons for this is the 'hidden antigens' phenomenon. To counter this, strong basal immunity is needed from vaccines. Vaccinations via the skin (epidermis or dermis) has great potential to create a stronger basal immunity compared with subcutaneous or intramuscular vaccinations.

Designing vaccines that target or avoid for glycan structures is likely to significantly improve vaccine efficacy. Developing vaccine delivery methods for the skin could help create stronger basal immunity that produces increased levels of antibodies to effectively neutralise activity or function of hidden antigens. The future of flystrike and endo- and ecto-parasite vaccines may lie in glycosylation, formulation and skin vaccine administration.

PROJECT OBJECTIVES

The goal of this project is the development of a flystrike vaccine using a genomics approach to identify protein encoding genes that meet key criteria as potential vaccine antigens. These candidate antigens will be cloned, engineered, and produced as highly purified recombinant proteins, formulated with appropriate adjuvants into prototype vaccines and used in vaccination trials in pure-bred Merino sheep. Protective efficacy of the vaccines to be determined using laboratory based *in vitro* bioassays and ultimately *in vivo* on-sheep larval implant assays.

Objective 1: Develop a genomics' reverse vaccinology approach to identify potential protein antigens.

- Perform transcriptomics analysis on *L. cuprina* at different life stages (adult fly, 1st, 2nd, and 3rd larval instar) and specialised larval tissue, cardia/anterior midgut, and salivary glands from third instar larvae.
- Determine gene expression profiles for each gene from each life stage and tissue and rank expression based on abundance of transcript.
- Cross reference each transcript library to assist with selection of candidate protein encoding genes to progress to vaccine development.

Objective 2: Identify lead protein-encoding genes to progress as prototype vaccine antigens to produce as recombinant proteins.

- Develop a pipeline for antigen identification and production.
- Categorise antigens and justify for progression as vaccine candidates.

Objective 3: Design, engineer and produce short-listed vaccine candidates as recombinant proteins in bacterial, yeast and/or insect cell production systems.

- Identify mRNA protein encoding sequence of candidate antigens and engineer protein encoding plasmid vector for recombinant protein production.
- Produce prioritised vaccine antigens as recombinant proteins in bacteria, yeast, or insect cell systems.
- Produce purified proteins at scale to allow vaccine trials to be performed in sheep.

Objective 4: Produce a native antigen cocktail derived from larval peritrophic matrix for benchmarking recombinant vaccine performance in sheep vaccine trials.

- Devise a method to produce native peritrophic matrix from cultured *Lucilia cuprina* larvae *in vitro*.
- Purify and characterise cultured PM for vaccination trials.

Objective 5: Develop a plan for vaccine dose and longevity of antibody response for benchmarking native and recombinant vaccine performance in sheep vaccine trials.

- Undertake a vaccine dose formulation experiment using a model antigen.
- Assess antibody response in sheep and longevity of response.

Objective 6: Formulate and test recombinant and native protein antigens in prototype vaccines in sheep trials.

- Investigate approach to formulate vaccine antigens with adjuvant suitable for future commercialisation and use in livestock.
- Secure Animal Ethics approval for in and on sheep vaccine testing.
- Test protein antigens in prototype vaccines in sheep trials.
- Assess immunological response to prototype vaccines.
- Identify vaccine antigens that induce strong immune responses in sheep and produce inhibition of *L. cuprina* larval growth *in vitro*.
- Investigate lead vaccine formulation(s) *in vivo* in on-sheep trials to assess efficacy to resist a larval strike.

Objective 7: Propose recommendations and propose next steps on course of action for future development of a Flystrike Vaccine.

- Identify lead vaccine antigens to progress to patenting, testing and commercial development with an Animal Health Veterinary Pharmaceutical partner.
- Identify short-comings and challenges in current Flystrike Vaccine development and suggest future direction and options to advance development.

SUCCESS IN ACHIEVING OBJECTIVES

The Flystrike Vaccine Project is an ambitious initiative and has been able to achieve its core objectives over the past 5 years of research. Whilst an effective on-farm vaccine is still to be developed, the learnings from this project will help advise and advance future efforts in delivering a vaccine to the sheep industry. The following are core achievements documenting progress so far.

Objective 1: Develop a genomic reverse vaccinology approach to identify potential flystrike vaccine protein antigens.

We isolated high-quality RNA from 4 hr old neonate blowfly larvae fed on sheep and non-sheep derived media and from dissected 3rd instar larval key tissues, i.e. the cardia and salivary gland. The RNA was sent to the Australian Genome Research Facility (AGRF); to undertake independent sample RNA sequencing on our behalf using the Illumina HiSeq sequencing platform. Eleven independent NGS transcript libraries representing 1) 3rd instar larval cardia, 2) 3rd instar larval salivary gland, 3)-5) three bio-replicate libraries of neonate 1st instar whole larvae growth initiated on non-sheep milk protein, 6)-8) three replicate bio-libraries of neonate 1st instar whole larvae growth initiated on sheep meat and sera protein, 9) mixed L1/L2/L3 instar whole larvae, 10) adult female *L.* cuprina fly and 11) adult male *L.* cuprina fly were independently mapped to two separate annotations of the *L. cuprina* genome available at the time; i) University of Melbourne annotation (Freeze 2) and ii) Baylor College of Medicine annotation (Baylor I5K). Mapping efficiency was excellent with greater than 95% mapping efficiency recorded for all libraries to the annotated gene models.

An expression profile that describes the expression/abundance of a specific gene transcript was calculated for each annotated gene from each genome annotation. The process used a statistical normalisation process to produce an RPKM figure for each gene. RPKM stands for "Reads Per Kilobase of transcript per Million mapped reads" and reports a normalised abundance score whilst removing bias based on the size of the gene transcript. The transcript data for each biological sample was ranked based on RPKM as i) Extreme RPKM>1000, ii) High RPKM>100 to 1000, iii) Medium RPKM 10 to 100, iv) Low RPKM 1 to 10, v) Very Low RPKM 0.1 to 1 and vi) No expression RPKM 0 to 0.1. This ranking allowed categorisation of expressed genes on abundance and potential importance and function.

Objective 2: Identify protein-encoding genes to progress as prototype vaccine antigens to produce as recombinant proteins.

We developed a pipeline for the identification of candidate protein encoding genes for investigation in flystrike vaccine development. This approach was further informed by foundation work CSIRO researchers had undertaken in native antigen identification for flystrike vaccine development. Candidate antigens were identified using a filtering approach based on gene/protein abundance, lifestage expression, emphasis on neonate larvae expression, expression in cardia or salivary glands, and genes encoding for secreted proteins. We mainly concentrated our efforts on exploring genes that were expressed by the larval cardia and expressed neonatally. The cardia produces a suite of proteins which form the peritrophic matrix, a semi-permeable membrane structure that protects the underlying midgut epithelia and helps regulate digestion. Also produced by the cardia are digestive enzymes and a chitin remodelling enzyme. We also investigated potential anti-microbial/defence associated proteins produced by the larval salivary glands from third instar larvae. We identified lead protein classes from the genes that encode these proteins from the transcriptome of the third instar larval cardia and cross validated that this expression was present in neonate larvae. These genes became the key candidates for prototype vaccine development. The life-stage expression data of these genes

allowed us to determine that the proteins we were aiming to target by vaccination are produced when the *L. cuprina* larvae initiate a flystrike event on sheep and throughout their larval growth phase. This approach was to ensure we have a handle on targeting larvae as soon as they come into contact and initiate feeding on the sheep. This early intervention is thought to be critical to the success of a potential vaccine.

Six protein classes were identified having representative genes expressed in abundance by the larval cardia and in neonate larvae at strike establishment. These classes include; 1) Peritrophin-Mucins, 2) Mucins, 3) Peritrophins, 4) Cysteine Rich Secretory Proteins (CRiSPs)/ Non-venom peptide like proteins/Antigen 5-like proteins which are thought to be associated with modifying host "immunological/stress/defence" mechanisms, 5) Chitinases, and 6) Digestive proteases. Genes encoding these six classes of proteins were identified in the top 100 cardia expressed genes and salivary gland genes and flagged for further investigation as potential vaccine antigens. Classes 1), 2), 3) and 5) represent genes encoding proteins associated with peritrophic matrix structure, function and modification, Class 4) larval immune defence, and Class 6) larval nutrition and digestion.

Objective 3: Design, engineer and produce short-listed vaccine candidates as recombinant proteins in bacterial and/or insect cell production systems.

Genes encoding secreted proteins produced by the larval cardia and/or salivary gland were identified and flagged for investigation as antigens for prototype vaccine production and testing. These proteins were determined to be secreted based on the presence of a signal sequence and demonstrated to be abundant throughout the larval growth phase based on transcript abundance. Initial work concentrated on the production of a selection of these candidate genes as recombinant proteins produced using a bacterial protein expression system. This approach was changed early on to a more relevant insect cell expression system. The protein antigens were produced in insect cell lines derived from lepidopteran (caterpillar) cells (Sf9 and Hi5). These cells contain the cellular machinery to post-translationally modify the proteins of interest, thus theoretically providing a better opportunity to emulate the correct folding and glycosylation of the native *L. cuprina* proteins being investigated. In addition, a Dipteran (Fly order) cell line (Schneider S2) derived from Drosophila embryos was investigated as an alternative production system as it presented a potential approach using a cell line evolutionary closer to sheep blowfly.

We engaged the expertise and capability of the University of Queensland Protein Expression Facility (PEF), (St. Lucia, Brisbane, Queensland), to produce our candidate antigens as recombinant proteins using these insect cell expression systems. We provided the protein encoding gene sequences of the candidate vaccine antigens and liaised with the facility to engineer and produce recombinant protein antigens for vaccine testing. Recombinant proteins were produced as highly pure secreted proteins which were validated for integrity and purity. These proteins were then subsequently used for formulation into vaccines and tested in vaccine trials in sheep.

Objective 4: Produce a native antigen cocktail derived from larval peritrophic matrix for benchmarking recombinant vaccine performance in sheep vaccine trials.

We explored developing a native antigen Flystrike Vaccine 'Type N' as a parallel approach to the Insect Cell recombinant Flystrike Vaccine 'Type R' approach. This approach was undertaken to help inform and benchmark the recombinant protein vaccine approach. It also provided an additional avenue of exploration should the recombinant protein approach not deliver a vaccine of required efficacy or should it be determined

to be non-viable to produce commercially. The native antigen production was demonstrated to be readily and inexpensively produced in the laboratory. Undertaking this approach is also supported scientifically by the success of the Barber's Pole worm BarberVax™ vaccine that is formulated using a native antigen preparation derived from sheep cultured *Haemonchus contortus* intestinal worms.

Native protein was produced and purified which allowed the native vaccine sheep trials to be undertaken. Methods to improve yields and purity of the cultured native protein were investigated. The process of native antigen production aligned with potential scale-up applications for future commercial production, the process of production to this stage has been kept as simple and inexpensive as possible. This has involved culturing blowfly larvae in the laboratory and using simple culture vessels and harvest techniques to collect native produced and shed peritrophic matrix. This matrix has been shown and validated through protein chemistry analysis and proteomic analysis to contain a cocktail of proteins. Vaccine trials have been performed with the native antigen cocktail and various formulation strategies investigated. Proteoglycans are a major constituent of this native protein antigen mix and glyco-proteomic analysis to analyse different culturing approaches is under investigation with these results to inform limitations and best culture protocols in future efforts in this approach. It is becoming evident that specific conditions must be met to ensure relevant and robust production of the relevant native proteins for the 'Type N' vaccine.

Objective 5: Assess vaccine dose and longevity of antibody response for benchmarking native and/or recombinant vaccine performance in sheep vaccine trials.

We undertook a vaccine adjuvant and antigen study to assist with understanding sheep immunological response to vaccination with the aim to inform vaccination trials and vaccine formulation procedures for future flystrike vaccine development. This study included assessing adjuvant, antigen dose, number of doses, longevity of immunological response, annual vaccination booster vaccination effects. This helped inform flystrike vaccine development vaccination protocols and provided evidence to inform discussions on what type of vaccine dose regime may be possible for a future flystrike vaccine.

Objective 6: Formulate and test recombinant and native protein antigens in prototype vaccines in sheep trials.

Key to the development of a Flystrike Vaccine is the use of an appropriate vaccine adjuvant that will help produce an optimal immunological response to the antigens being developed and tested. We have undertaken a majority of our vaccination trial studies using Montanide™ ISA61VG adjuvant supplied 'in kind' by biopharma company SEPPIC and its agent Australian agent Tall Bennett. Montanide™ ISA VG61 was specifically recommended by SEPPIC for use in sheep where a high-level humoral antibody response is required in a vaccine. In later studies, the use of aqueous adjuvants including Quil-A, a highly purified saponin adjuvant and Dextran Sulphate were investigated using several lead candidate antigens deduced from initial Montanide™ 61VG formulation and testing. In additional studies using chitosan as an antigen presenting additive and adjuvant in combination with adjuvants tested was also performed and found to be beneficial in some formulation regimes.

Antibody titres produced during and after the vaccination regime were determined for all animals vaccinated using ELISAs to track the efficacy of the vaccine formulation to produce an immunological response. Most antigens were demonstrated to produce significant immunological responses to the target immunogen. However, an immunological response did not always correlate with efficacy of a protective response against

blowfly larval growth. Six of the recombinant antigens investigated were progressed along with the native protein antigen derived from cultured larval peritrophic matrix for further testing. These vaccine formulations are referred to as the 'Type R' recombinant vaccine and 'Type N' native protein vaccine respectively.

The lead 'Type R' and 'N' vaccine formulations were shown to produce high antibody responses in sheep and a significant degree of efficacy in stunting blowfly larval growth when tested in laboratory *in vitro* larval bioassays. These assays involved feeding just hatched neonate blowfly larvae on serum isolated from sheep injected with the vaccines. Results were variable depending on batch of antigen tested and work continues to understand what key parameters are affecting the reproducibility of effect of these prototype vaccines. Preliminary analysis suggests the abundance and glycosylation profile is having significant effect on the efficacy of 'Type N' and 'R' vaccines. Trials were conducted using *in vivo* on sheep larval growth assays using controlled larval implants on the lead 'Type R' and 'Type N' vaccine formulations. *In vivo* testing of the vaccines demonstrated only marginal efficacy in reducing larval growth.

Objective 7: Propose recommendations and propose next steps on course of action for future development of a Flystrike Vaccine.

Preliminary lead protein antigen candidates have been identified and tested as a 'Type R' and 'Type N' prototype vaccines. The vaccines were tested in sheep and immune sera obtained from the animals assessed on growing blowfly larvae using *in vitro* laboratory larval feeding bioassays. A larval growth impact of up to 75% reduction and a significant impact on survival was demonstrated in the best performing vaccine trials. Testing of these vaccine formulations *in vivo* using on-sheep larval implant assessments did not replicate this result with none to only marginal ~ 20 % reduction of larval growth recorded.

Extensive testing of candidate antigens and vaccine formulations have illustrated the difficulty in developing a vaccine against an ectoparasite that has evolved to use the sheep unopposed for propagation of its larval stages of development. The short 72 hr period the larvae spend on the sheep presents a substantial hurdle to vaccine development. The efficacy demonstrated using *in vitro* bioassays is highly encouraging and supports continued research to develop a pathway to optimise antigen design, formulation, and delivery to significantly enhance *in vivo* efficacy. Optimising the immune response at the skin surface where the larvae feed is critical to this development. We outline approaches we plan to undertake to enhance delivery of antibodies at the skin including but not restricted to transdermal vaccine delivery and vaccine formulation approaches. Additionally, we see the need for an approach that may better elucidate an immune response to a range of larval proteins with a concentrated effort on characterising protein antigen post translational modifications with a particular focus on glycosylation. We have started this characterisation and demonstrated the impact of different cell lines and nutritional status on post translational modifications associated with our candidate antigens. We are currently investigating a novel vaccine approach based on antigen glycosylation and refining this approach. A more informed understanding of these key protein features will help us tailor our vaccines and better formulate them for enhanced effect *in vivo*. Alternatively pausing this research for the moment until parasite and host interaction is better understood and vaccine approaches advance is an option to consider, however current momentum and efforts put into resourcing capability, building networks and advancing knowledge in this endeavour would benefit from continued focus and commitment to this research. We further present ideas on enhancing this understanding through research that is currently underway and planned to be undertaken by our team that will assist in advancing this research.

METHODOLOGY & RESULTS

Objective 1: Develop a genomic reverse vaccinology approach to identify potential flystrike vaccine protein antigens.

Preparation of *L. cuprina* biological tissue and RNA extraction

Initial work was conducted using a *L. cuprina* reference laboratory strain for isolation of lifestages and tissues for downstream RNA isolation for transcript profiling. *L. cuprina* were maintained in culture in the laboratory insectary on an agar solidified media containing milk protein, yeast extract and wheat germ for larval culturing. Adults were maintained in fly screen/gauze netted cages on sugar and water supplemented with sheep liver protein feeds. Eggs were collected from gravid flies and allowed to hatch overnight whereupon the neonate larvae were allowed to feed for 4 hr on either i) non-sheep protein-based milk/yeast/wheatgerm media or ii) sheep liver media. Larvae were collected and used for RNA extraction. Larvae were also grown for 72 hr at 24°C on the milk protein media to develop to mature third instar larval stage whereupon larvae were recovered, washed, and carefully dissected to obtain the larval cardia and the salivary glands. Larval tissue was extracted using Qiagen RNeasy RNA isolation kit and the RNA treated with an on-column DNase treatment. Isolated RNA was assessed using an Agilent RNA chip for RNA integrity, certified as high quality, and supplied to the Australian Genome Research Facility (AGRF) for gene transcript library construction and Illumina short read sequencing.

Transcript mapping

Eleven independent RNAseq transcript libraries representing 1) 3rd instar larval cardia, 2) 3rd instar larval salivary gland, 3)-5) three bio-replicate libraries of whole neonate 1st instar larvae growth initiated on non-sheep milk protein, 6)-8) three replicate bio-libraries of whole neonate 1st instar larvae growth initiated on sheep liver and sera protein, 9) mixed L1/L2/L3 instar whole larvae, 10) adult female fly and 11) adult male fly, were mapped to the three annotated *L. cuprina* genome sequences. The transcript data for 9), 10) and 11) were obtained from publicly available transcript data form NCBI created by the University of Melbourne.

Transcript mapping was performed using CLC-Bio Genomics Workbench (Qiagen). Mappings were performed against two independent assemblies of the *L. cuprina* genome available at the time; i) Freeze_2_UOM, Bioproject number: PRJNA419080 produced by the University of Melbourne and ii) The i5k Initiative (December 2017) *L. cuprina* genome assembly Lcup_2.0 undertaken by Baylor College of Medicine. Table 1 outlines the genes and transcripts annotated for the two genomes used in the mapping and abundance analysis. Mapping and cross referencing of the two genomes allowed the identification of all genes that are represented within each library and the identification of key protein classes that were subsequently investigated for antigen production and prototype vaccine development.

Table 1: *Lucilia cuprina* annotated genome builds and gene/transcript statistics.

Genome annotation	Genes annotated		Transcripts annotated
Lc:v2 (freeze 2) UoM	**12933**		**16390**
Lcup_2.0 Baylor I5K	**16651**		**18851**

NB: The difference between "genes annotated' and 'transcripts annotated' is due to some genes being encoding by more than one transcript/splice variant for that gene.

Mapping efficiency, a parameter that describes the overall ability to map the individual sequencing reads to the gene annotated genomes for each transcript library ranged from 93.85 – 99.1% for Lc:v2 (freeze 2) and 95.3 – 98.4% for Lcup_2.0 Baylor I5K genome annotations. The very high mapping efficiency for both Lc:v2 (freeze 2) and Lcup_2.0 Baylor I5k genome annotations supported the high quality of the independent genome builds and annotations. The cross referencing of the independent genome mapping allowed cross-referencing and helped increase the accuracy of the identification of potential candidate proteins ensuring full length and accurate sequence for vaccine antigen design, production, and investigation.

Relative gene expression analysis

An expression profile that describes the expression/abundance of a specific gene transcript was calculated for each annotated gene from each genome annotation using the CLC-Bio software package. The process used a statistical normalisation process to produce an RPKM figure for each gene. RPKM stands for "Reads Per Kilobase of transcript per Million mapped reads" and reports a normalised abundance score whilst removing bias based on the size of the gene transcript. The RPKM figure is a powerful descriptor of the expression of the gene that allows us to accurately identify genes that are expressed, their level of expression and allows for quantitative comparison between different transcript libraries. The transcript data for each biological sample was ranked based on RPKM as i) Extreme RPKM>1000, ii) High RPKM>100 to 1000, iii) Medium RPKM 10 to 100, iv) Low RPKM 1 to 10, v) Very Low RPKM 0.1 to 1 and vi) No expression RPKM 0 to 0.1. This ranking allowed categorisation of expressed genes on abundance and potential relative importance and function.

We concentrated our efforts on exploring genes that are expressed by the larval cardia and the salivary gland. Gene expression data was cross-referenced with the life-stage expression data for these genes allowing us to determine that the proteins we chose to target by vaccination were produced when the *Lucilia* larvae had just hatched and initiate a flystrike event on sheep and produce the proteins as they grow. This allows us to target larvae as soon as they come into contact and initiate feeding on the sheep. This early intervention is undoubtedly critical to the success of a potential vaccine.

Candidate gene selection, informed by the two-genome comparison, at the time progressed to concentrate on the genes described as part of the Lcup_2.0 Baylor I5k assembly. Discussion from this point forward will refer to genes from this annotation. It should be noted that subsequent to this period of time, a further revised and accepted model genome for *L. cuprina* (ASM2521726v1) by University of Melbourne is now available but is not referred to in this report as the description and selection of candidate protein antigens at the time was undertake before its availability.

Secreted protein selection of candidate genes

The top 100 most abundantly transcribed genes from the larval cardia and abundant proteins of the salivary gland with demonstrated expression in the neonate larval stage were further filtered by determining which of these genes encoded for proteins that are secreted. Secreted proteins contain a leader peptide sequence called a signal sequence which directs the protein for extracellular secretion. This means the protein is then present outside of the cell where it can be targeted with antibodies produced by a vaccine. Each candidate gene sequence was analysed for a signal sequence using PrediSi (http://www.predisi.de/) and shortlisted if a signal sequence was found. Our focus on secreted proteins of the cardia/peritrophic matrix and salivary glands over-ruled cell surface and gut lumen proteins as these proteins on the intra-luminal space underlying the peritrophic matrix are considered exempt from immunological targeting as the peritrophic matrix is thought to be impenetrable to antibodies based on prior work.

Objective 2: Identify lead protein-encoding genes to progress as prototype vaccine antigens to produce as recombinant proteins.

Candidate protein antigens were identified from the transcriptomic data concentrating on the top 100 cardia, salivary gland, and neonate larvae expressed gene encoding proteins. The candidates were classified into protein classes based on their physiological and structural characteristics and functions. These candidate genes fall into six protein classes that include 1) Peritrophin-Mucins, 2) Mucins-Peritrophic Matrix associated, 3) Peritrophins, 4) Chitinases, 5) Digestive proteases, and 6) CRISP, non-venom allergen 5-like proteins.

Neonate larval diet contrast analysis

In addition to direct analysis of the genes and proteins produced in key tissues and the *L. cuprina* lifestages, a diet/nutrient comparison was performed to better understand the biological processes at a gene level occurring in *L. cuprina* larvae as they hatch and begin feeding, i.e. establish a flystrike. Freshly laid *L. cuprina* eggs were allowed to mature and hatch on either of two types of nutrition source; i) minced sheep liver supplemented with normal sheep sera or; ii) non-sheep protein diet of bovine milk casein, yeast extract, wheatgerm, and agar. Neonate larvae were allowed to grow after hatching for up to 4 hours after which they were harvested and processed for RNA isolation in triplicate. Unsupervised/unbiased clustering analysis of the gene expression data illustrates the gene expression differences and the clustering of samples by diet (Figure 6).

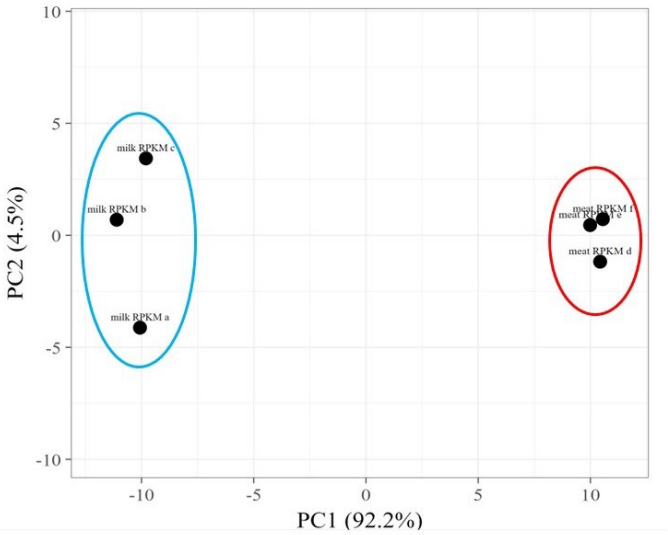


Figure 6: Principal Components Analysis (PCA) using unbiased/unsupervised clustering of gene expression data obtained from neonate larvae within 4 hours post hatching and establishing feeding. **Red**: meat/sera and **Blue**: milk/yeast diet.

Vaccine Antigen Identification

From the more than 14,000 annotated genes, the most abundant 100 genes expressed coding for secreted proteins in the larval cardia and salivary glands were determined and assessed with respect to whole sheep blowfly life-stages expression. From this list of the most expressed protein-encoding genes, the secreted proteins were determined, and classes of protein families identified and investigated for flystrike vaccine development as described in this report. 20 genes encoding secreted proteins produced by the larval cardia were identified and flagged for investigation as antigens for prototype vaccine production and testing.

Objective 3: Design, engineer and produce short-listed vaccine candidates as recombinant proteins in bacterial and/or insect cell production systems.

Production of protein antigens as recombinant proteins was performed using two production systems, i) bacterial and ii) insect cells. Bacterial expression system was used initially by CSIRO as a rapid and inexpensive approach but due to it being a prokaryotic system and lacking the necessary post-translational processing machinery to modify complex proteins, it was therefore replaced with an insect cell system. The insect cell expression system was used to produce the protein antigens for the prototype vaccines utilising a eukaryotic cell line derived primarily from lepidopteran (caterpillar) cells. This system used Sf9 cells derived from ovarian tissue of Spodoptera frugiperda (Fall Army Worm), or High Five cells derived from embryonic cells of Trichoplusia ni (Cabbage Looper Moth). A preliminary investigation of a Dipteran cell line derived from late-stage Drosophila melanogaster embryos, Schneider S2 cells was also undertaken. These cells contain the cellular machinery to post-translationally modify the protein of interest, providing a better chance to emulate the correct folding and glycosylation of the native L. cuprina proteins being investigated. It was anticipated that this approach would provide a better opportunity to produce an effective vaccine response by helping produce relevant antibodies that would better target the larval proteins thus producing a protective response by the sheep immune system against flystrike establishment. The expert services of the University of Queensland Protein Expression Facility (UQ-PEF) were engaged to undertake the construct engineering, cell production, purification and validation of the insect cell produced recombinant antigens. The work described in this report concentrates solely on the insect cell produced proteins for vaccine testing.

Insect Cell line production of candidate antigens

The insect cell expression system used in the production of recombinant protein antigens in this project utilised baculovirus transformed immortalised cell lines, Sf9 or High Five, and analysis performed to determine which line produced optimal expression yield and characteristics consistent with the native protein based on solubility and size. The best performing cell line was then chosen for scale-up production of the vaccine antigens. The key aim was to produce candidate antigen proteins that closely replicate the characteristics of the native *L. cuprina* larval protein from which the antigen is designed from.

The protein expression strategy involved undertaking a pilot scale expression test in both cell lines with culturing at 21°C or 27°C with 24 hr sampling over a 5-day period. This allowed the assessment of optimal cell line, temperature and time for quality and quantity of candidate protein production. The candidate antigens were designed for extracellular secretion of the protein into the culture media. Cells and media were assayed for presence of the candidate protein to ensure protein expression and secretion had occurred. The presence of candidate protein from the pilot cultures was determined by Western blot technique using an affinity probe for the generic 10xHistidine affinity tag incorporated at the carboxy-terminus of each candidate protein. Optimal temperature and time for production of the recombinant protein antigen was determined for both cell lines and samples processed through a trial purification using affinity column chromatography. The optimal binding conditions and cell line providing the best yield was determined independently for each candidate antigen. The best performing insect cell line and culture parameters were selected and then scaled-up (1-4 L) for production of candidate protein antigen for vaccine formulation and trialling.

Methods (as performed and described by the UQ-PEF)

Insect cells *S. frugiperda* (Sf9) and *T. ni* (High Five™) were routinely grown and maintained in ESF 921™ insect cell culture medium.

Recombination & transfection

100 ng of pBac-1 transfer vector carrying the gene of interest, 20 ng of *flash* BACULTRATM and 1 µL of *Trans*IT®-insect transfection reagent (Mirus) was used for co-transfection into 6 x 10^5 adherent Sf9 or High Five cells, which have been grown in 400 µL of ESF 921 in a 24-well tissue culture plate. The transfected cells were incubated for 7 days at 27°C. The culture medium containing the recombinant virus (P1) was harvested and used as seed virus stock for further amplifications.

Virus amplification

Amplification of the recombinant baculovirus in 24-deep well plate format was conducted. Cells were seeded at 2 x 10^6 cells/mL in ESF 921 at a volume of 5 mL/well. An appropriate volume of the recombinant P1 virus seed stock was added. Cell density, viability and diameter were monitored, and the culture supernatant was harvested (P2) by centrifugation when the cells appeared well infected with the virus.

Expression screen

A small-scale expression screen was set up in 24-deep well plate format. Briefly, 5 mL of insect cells were seeded per well at mid-log phase in ESF 921 and infected with high titre P2 recombinant baculovirus. Cell density, viability and diameter were monitored for signs of infection. Duplicate 0.5 mL samples were collected at different hours post-infection (hpi) for analysis.

Analysis

Cell pellets were resuspended in 1 mL PBS with 2 mM MgCl2 with Benzonase. Total lysates (TL) and supernatant fractions (SN) were loaded on 4-12% Bis-Tris SDS-PAGE gels and run under denatured and reduced conditions. For western blot, the gel was transferred onto a PVDF membrane and probed with an Anti-His-HRP (C-terminal) conjugate antibody (Miltenyi Biotec) at a dilution of 1:5,000 for 1 h. Analysis was performed using a Bio-Rad Chemi-Doc™ XRS+ imaging system and the molecular weight was calculated using ProtParam (http://web.expasy.org/protparam/). The culture supernatant samples (0.5 mL) were incubated with loose IMAC resin and purified. Eluate samples were loaded on 4-12% Bis-Tris SDS-PAGE gels and ran under denatured and reduced conditions. Analysis was performed using a Bio-Rad Chemi-Doc™ XRS+ imaging system and the molecular weight was calculated using ProtParam once more. Densitometry analysis was performed using Image Lab Software to estimate 1 L production yield based on IMAC binding assay results. Best performing cell line and culture conditions were then discerned and used for subsequent scale-up expression and production of insect cell recombinant protein antigen. This process was conducted independently for each protein antigen under consideration for vaccine testing.

Objective 4: Produce a native antigen cocktail derived from larval peritrophic matrix for benchmarking recombinant vaccine performance in sheep vaccine trials.

A native antigen Flystrike Vaccine 'Type N' was investigated in parallel to the Insect Cell recombinant Flystrike Vaccine 'Type R' approach. The native vaccine was generated by culturing of *L. cuprina* larvae in the laboratory and isolating the extruded peritrophic matrix shed from the gut as a source of the native antigen. This approach harnesses the larva's cardia and anterior midgut organs native production system of the peritrophic matrix (Figure 7). This extruded peritrophic matrix tissue was retrieved from cultured larvae and formulated into the native 'Type N' vaccine.

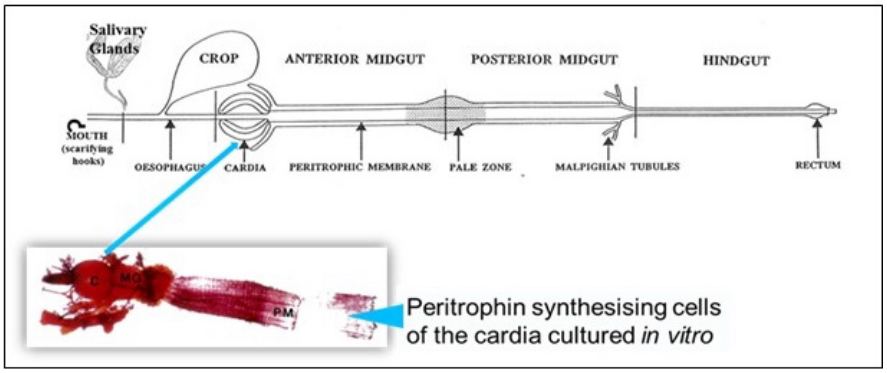


Figure 7: *Lucilia cuprina* larvae are specially cultured under controlled laboratory conditions and used to produce native protein antigen from their cardia and anterior midgut. This native antigen is comprised of a cocktail of proteins from the peritrophic matrix which is then purified and formulated into the "Type N' Flystrike Vaccine being developed.

The 'Type N' prototype vaccine has been demonstrated to raise a strong immune response in sheep when used in a vaccine and shown to significantly reduce larval growth and survival assessed in in vitro larval feeding and growth bioassays. We have demonstrated production of native peritrophic membrane antigen from cultured larvae using a laboratory-based production system with potential to scale-up production at minimal cost (Figure 8). We investigated various methods for PM production based on two broad categories – (1) larvae fed or unfed before and during PM harvest and (2) physical devices to assist in PM/larvae separation and retrieval. Actively feeding larvae have been reported to increase PM production, but this complicates PM extraction due to contamination with residual feed proteins and digesta. Separation of PM from larvae and subsequent concentration methods were also investigated.

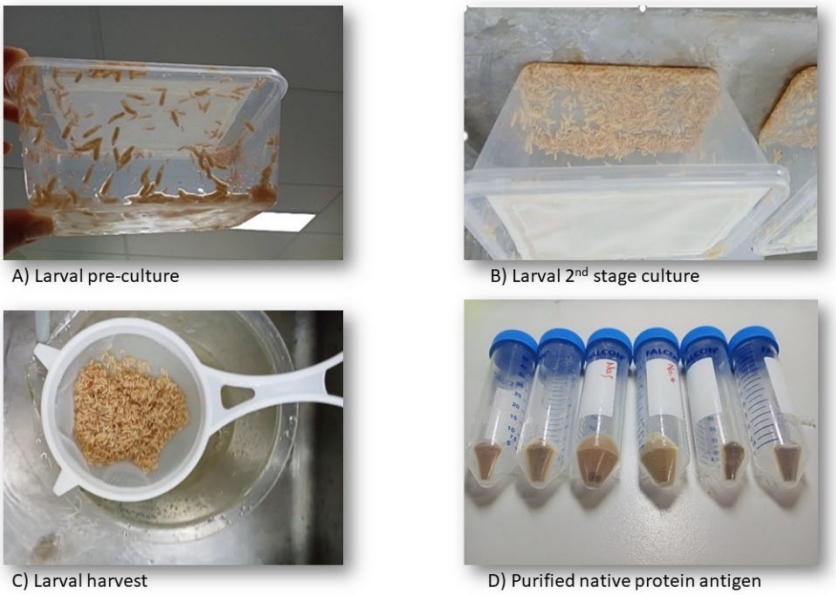


Figure 8: *Lucilia cuprina* larvae in laboratory culture used to produce Peritrophic Matrix (PM) for native protein antigen isolation for the "Type N" Flystrike Vaccine.

We are in the process of characterising the glycoproteome of key proteins derived from the native antigen production using the larval culture and PM isolation. Culture and diet protocols and effects on post translational modifications with specific reference to glycan attachment will help inform future protocols for production of the native antigens for development of the native 'Type N' vaccine.

Objective 5: Formulate and test recombinant and native protein antigens in prototype vaccines in sheep trials.

Animal Ethics Approval

All animal work conducted as part of this research was undertaken in accordance with the review and written approval of the CSIRO Livestock Animal Ethics Committee in accordance with the Australian code for the care and use of animals for scientific purposes.

Australian biosecurity approval for biologicals use in non-laboratory animals

Approval for use of biologicals in sheep was obtained from Biosecurity Australia-DAFF, formerly AQIS, and all animal work and containment undertaken in approved Biological Containment level 1 (BC1) facilities at the CSIRO research farm, McMaster Laboratories, Chiswick, Armidale, NSW, Australia.

Vaccination adjuvant and formulation

The potential success of this vaccination approach requires formulation of the recombinant antigens with a suitable adjuvant that allows optimised presentation of the antigen to the sheep and a sustained slow release. This is to ensure that a high level and prolonged humoral (IgG) immune response is activated in the sheep thereby resulting in high antibody titres. It is well documented that presentation of recombinant protein antigens in water-in-oil adjuvant emulsions can provide this type of immune response. The flystrike vaccine trials used Montanide™ ISA 61VG adjuvant system (Seppic Ltd) water-in-oil antigen formulation and presentation adjuvant. A minimal amount of adjuvant was used in the vaccine formulation (max of 2ml/injection) containing up to 500 µg of purified antigen. Pilot studies with this formulation demonstrated that there were no adverse reactions to the Montanide™ ISA 61VG adjuvant or formulated vaccine. Tissue samples were taken and investigated for lesions at the site of injection. No lesions or abnormalities were found apart from a small amount of residual injected material in the muscle after 2-4 weeks injection that had yet to be absorbed by the body. Quil A, a saponin based aqueous adjuvant and Dextran Sulphate were other adjuvants on which some minor testing was also performed.

Chitosan formulation

We applied a vaccine formulation strategy to attempt to present the recombinant antigens in the recombinant 'Type R' vaccine in a conformation that may mimic that of the native peritrophic matrix. The hypothesis being it would result in a more relevant and enhanced immunological response in sheep to the vaccine. This approach involved taking the recombinant proteins, reacting them with reacetylated chitosan (or similar derivative) and then formulating this with an adjuvant to make the vaccine. Reacetylated chitosan is a chitin derivative that is soluble and can be manipulated as described. Chitin in its super high molecular weight form is non-soluble and inert. Chitosan is produced commercially generally from the exoskeletons of crustaceans, namely shellfish. It is produced by an extensive deacetylation (70% +) of the chitin from the exoskeleton. It can also be prepared from multiple organisms that contain chitin including insects. There was some suggestion that the recombinant 'Type R' protein antigens may bind chitin/chitosan to some degree and the addition of chitosan to the formulation showed an increase in vaccine efficacy. It is likely that the enhanced results observed in the vaccination trials is likely due to chitosan acting as an adjuvant with a small degree of chitin binding present, rather than an absolute specific interaction and binding with the recombinant 'Type R' proteins. This formulation approach will continue to be considered and attention made where possible in future trials to include with and without chitosan formulation.

Vaccine administration

Up to three doses of vaccine was administered over an eight-week period to each animal with dose 1 (priming dose) followed 4 weeks later with dose 2 and after another 2 weeks with dose 3 (a final booster injection). The vaccine was administered by intramuscular injection in the rump (*Gluteobiceps*) where there is a bulk of muscle to accommodate the injection. In general, vaccine trials consisted of a minimum of 4-6 sheep per vaccine formulation group for each vaccine antigen tested and a control group that received only phosphate buffered saline (PBS) formulated with the adjuvant. The control group along with the pre-vaccination timepoint were used as a baseline reference for comparing and assessing the efficacy of the prototype vaccines in *in vitro* bioassay assessment.

Animal blood collection

Blood was collected from the sheep for isolation of serum which was used for ELISA assays to determine antibody titres and for use in feeding to larvae in *in vitro* larval growth bioassay assessment. Blood was collected from each sheep immediately prior to each vaccine injection. The site of blood collection was from the jugular vein in the neck. At conclusion of the vaccine trial 2 to 4 weeks post final vaccine administration, the animals were humanely killed, exsanguinated, and disposed of by deep burial in accordance with Biosecurity regulations governing use of experimental biologicals in non-laboratory animals.

ELISA antibody titre assessment

Enzyme-linked immunosorbent assays (ELISA) were performed for all vaccination trials. An ELISA measures the immune response of an animal to the vaccine antigen however it is important to note that an immune response to the antigen does not always correlate with vaccine efficacy. The general protocol for an ELISA is to bind one microgram of vaccine antigen irreversibly to a clear 384 well, high bind, polystyrene plate, the bound antigen then captures the antibodies in the sheep serum by adding the serum to individual wells. These captured antibodies are then detected using a secondary antigenic probe, and subsequently visualized using a specialised dye, and converted to a numerical value or optical density (OD) using a spectrophotometer.

To quantitate the immune response of experimental sheep, serum taken from the sheep at four time points: Bleed 1, (before vaccination), Bleed 2 (4 weeks post first dose), Bleed 3 (4 weeks post second dose), and Bleed 4 (2-4 weeks post third dose). The serum from each time point is serially diluted starting at 1:500 and ending at 1:64000 and analysed using the ELISA method described above. The antibody titre is determined to be the greatest dilution at which the optical density of the serum from bleed 2, 3, and 4 is twice the value of bleed 1. The greater the dilution of the antibody titre, the stronger the immune response.

In vitro larval feeding bioassay assessment

We used several *in vitro* larval feeding and growth bioassays for testing the efficacy of the prototype flystrike vaccines on *L. cuprina* larvae (Figure 9). These assay systems include feeding of sheep serum isolated from vaccinated or control animals from the vaccine trials to neonate larvae and monitoring their growth and survival up to a 72 hr period. Generally, a period of 48 hr, at which larvae will normally have undergone two larval moults and be at early third instar larval development was used in the *in vitro* assessments. The three assay systems used to assess the effectiveness of the vaccines included 1) a method where sera was mixed in a matrix with agar, 2) sera absorbed into a preformed skin matrix using cross-linked denatured collagen simulating sheep skin, and 3) a direct feeding of the sera to larvae on an inert adsorbent cellulose matrix. To necessitate effective growth of the larvae in the assays, yeast extract, buffering salts and an antibiotic were added to all assay methods. 25 neonate larvae were gently counted under water with a pipette and seeded into

an assay vessel containing the sera and matrix. Each vessel contained up to 4 ml of matrix and sera and five replicate tests were routinely performed for each sheep sera collected. The assays were performed in a constant temperature (28°C) and humidified incubator (75%) for the length of the assay period. Larval growth was measured by weight and number surviving, compared, and normalised to a control sample that was either from no antigen vaccinate controls or from the same sheep with serum collected immediately before the initial vaccination injection for the trial was administered.

In vitro assessments as described, allowed many sheep vaccine trials to be performed without the need for on sheep larval implants and associated sheep welfare and ethics implications. *In vitro* assay assessments are generally a good indicator of vaccine induced immune response with regards to larval growth. This system was used to filter and determine which prototype vaccine formulations would progress to *in vivo* on-sheep larval implant assessment.

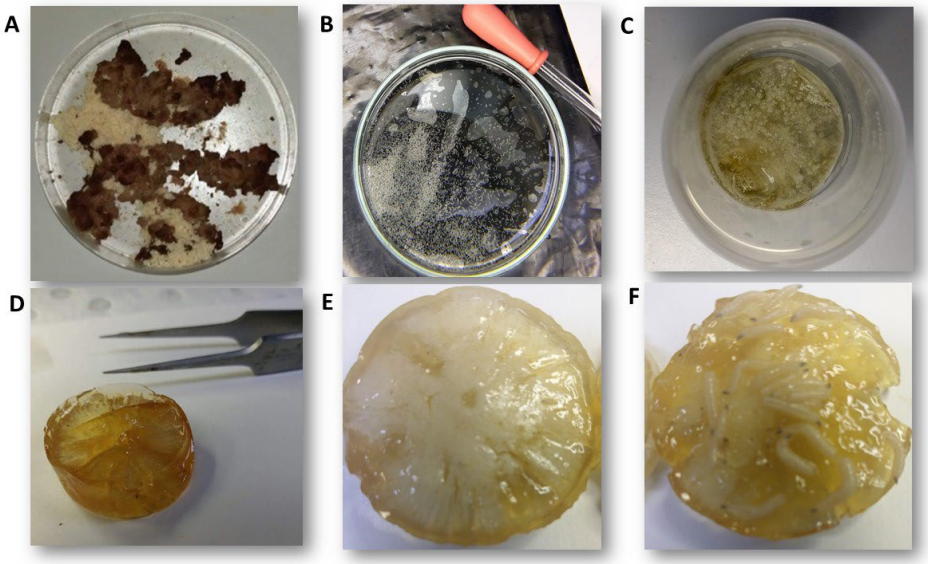


Figure 9: An *in vitro* bioassay has been developed that closely simulates ovine skin. **A)** *L. cuprina* eggs are collected from lamb liver laid by a field strain maintained in the CSIRO insectary, **B)** neonate larvae are gently counted under water and then transferred to vessels containing the 'skin' matrix **C, D** and **E)**. The matrix is saturated with sheep serum collected from sheep vaccinated with the prototype Flystrike vaccine. **F)** Larvae are recovered from the matrix at specific time intervals for growth/viability analysis. Shown is an example of healthy larvae after 72 hrs growth on non-immune sheep serum.

In vivo Implant Method

We developed and adapted a protocol approved by the Animal Ethics Committee, whereby we can reproducibly initiate a strike on sheep skin and contain the growing larvae to a small area. The procedure involves clipping wool to the skin line, affixing a plastic vessel for larval containment and maintenance of critical humidity, and implanting the slightly abraded skin with neonate larvae (Figure 10). We undertake this procedure with up to 500 larvae per implant site. The larvae are covered with a damp sponge and the vessel is sealed with a vented lid. Larvae are allowed to grow and feed on the skin of the sheep for a period of up to 72 hr after which they are harvested, counted, and weighed. Initially this approach involved affixing two such devices and implants to the midline of the sheep's back between shoulders and hips. Subsequent trials utilised a single implant device allowing more secure placement and reduction of larval implant stress on the animal. The trials utilised six animals per vaccine group and animal adjuvant only control group. At the conclusion of the trial,

total larval biomass recovered as measured by total combined larval weight per implant, mean larval weight, and larval survival were recorded and normalised to the control no vaccine antigen trial group.

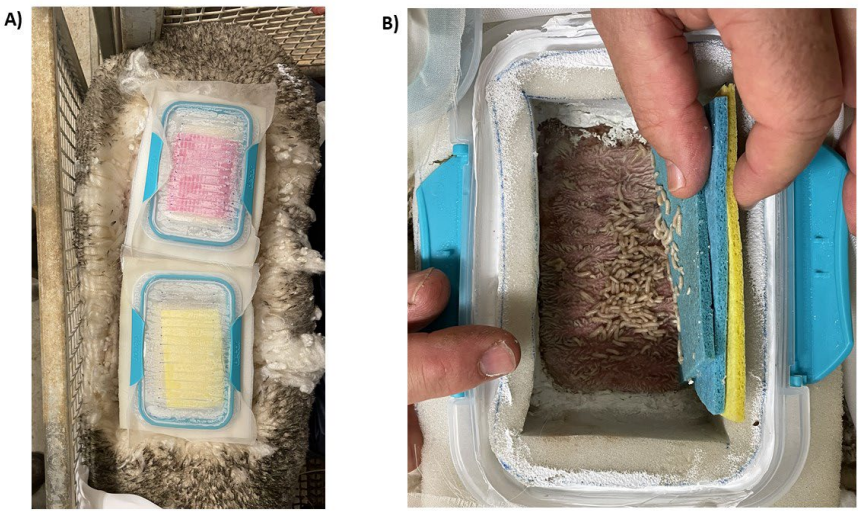


Figure 10: Larval implant device used for assessing larval growth *in vivo* on sheep and ascertaining effects of vaccine induced immune response of the sheep on larval growth.

Sheep vaccination trial assessment of recombinant protein antigens

Peritrophin-Mucins

A vaccination trial was conducted where sheep were vaccinated with either Peritrophin-Mucin candidate antigens A, B, or C produced in insect cells as described previously. A trial group also received a combination of the three antigens. An immune response was measured for all of the vaccination trial groups (Figure 11). *In vitro* larval feeding and growth assays showed a small but not significant effect on larval growth (Figure 12).

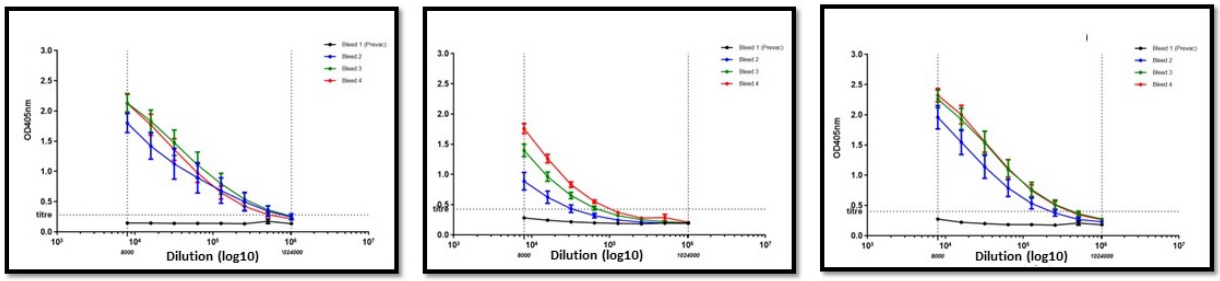


Figure 11: Mean ELISA results of sheep groups (4 sheep per group) vaccinated with either Peritrophin-Mucin Candidate A, B or C. Black = pre-vaccination sera, Blue = post 1st vaccination, Green = post second vaccination and Red = post third and final boost vaccination.

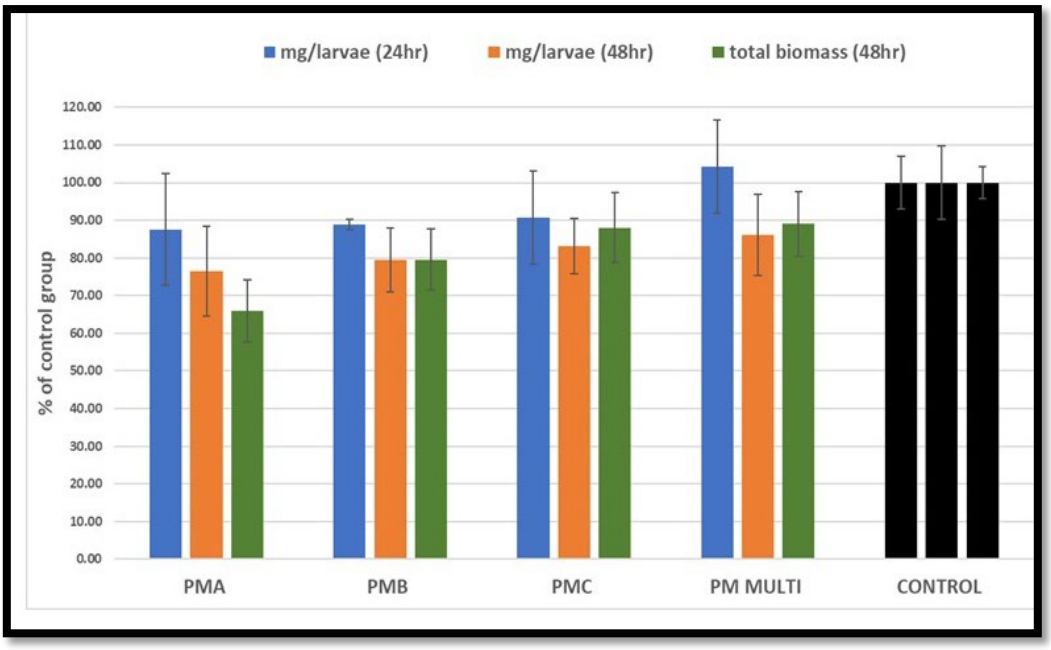


Figure 12: *In vitro* larval growth bioassay assessment of sera from animals vaccinated with Peritrophin-Mucin Candidate A, B, C or combined multiple antigen A, B and C. Results shown are group mean values (n= 4 sheep, 5 replicates each) for larval weight measured 24 or 48 hr growth and total larval biomass recovered at 48 hr. Results are normalised as a percentage of the control adjuvant only vaccinated animal group.

Peritrophins

Three insect cell line produced recombinant Peritrophin candidate antigens were tested in sheep vaccination trials, Peritrophin Candidate CD, E, and F. An immune response was measured in all sheep vaccinated (Figure 13) and a degree of larval growth inhibition observed for all three vaccine candidates as compared to adjuvant only vaccination control group (Figure 14). Peritrophin Candidate E performed the best with respect to immune response and larval growth inhibition effects with up to a 42% reduction in larval weight after 48hours of feeding on immune sera (Figure 14).

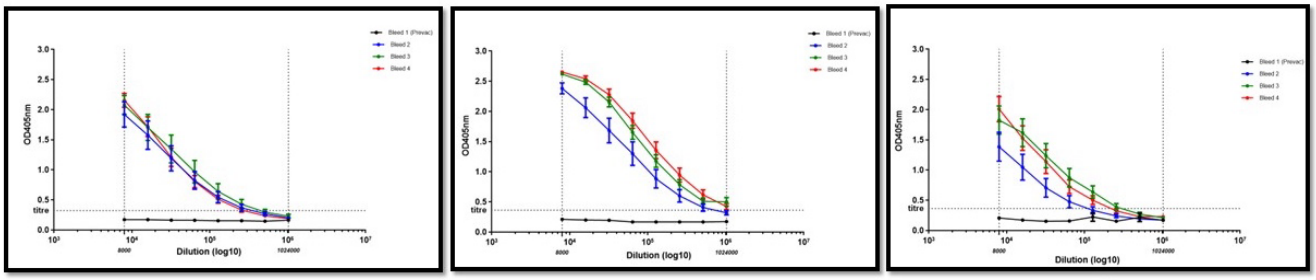


Figure 13: Mean ELISA results of sheep groups (4 sheep per group) vaccinated with either Peritrophin-Candidate CD, E or F. Black = pre-vaccination sera, Blue = post 1st vaccination, Green = post second vaccination and Red = post third and final boost vaccination.

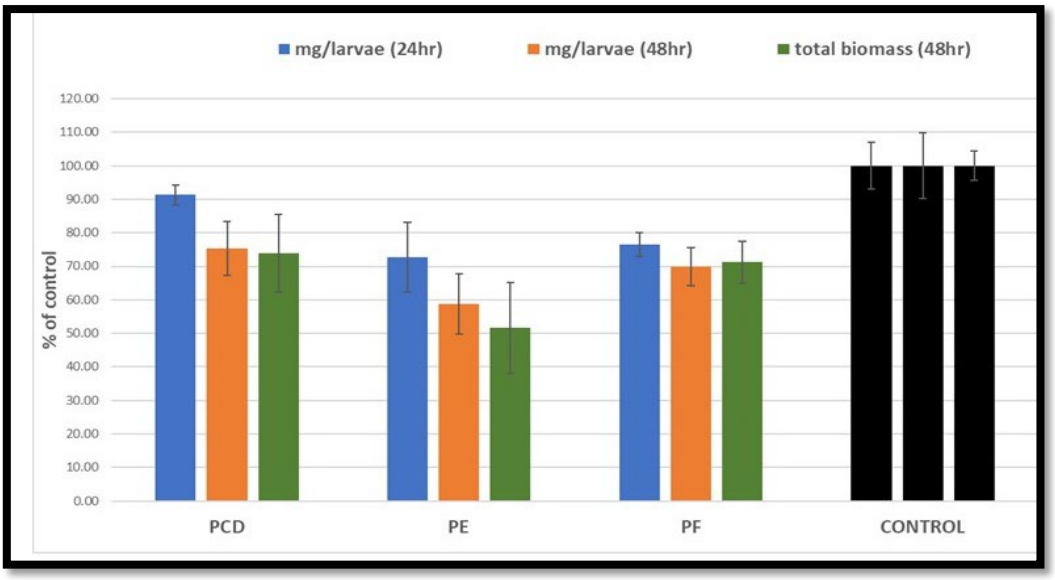
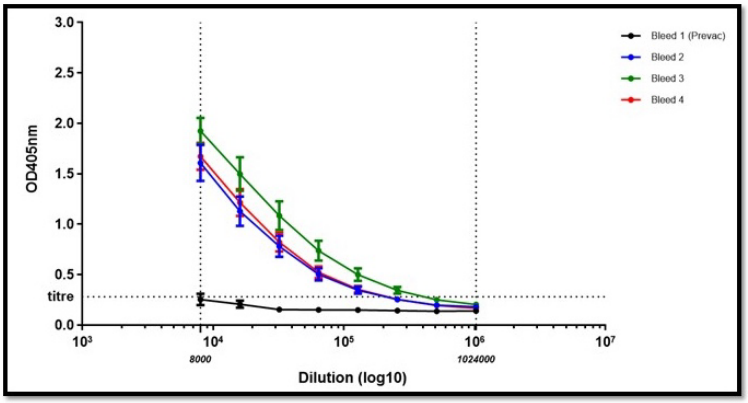


Figure 14: *In vitro* larval growth bioassay assessment of sera from animals vaccinated with Peritrophin-Candidate CD, E or F. Results shown are group mean values (n= 4 sheep, 5 replicates each) for larval weight measured 24 or 48 hr growth and total larval biomass recovered at 48 hr. Results are normalised as a percentage of the Control adjuvant only vaccinated animal group.

Chitinase

An immune response in sheep to the insect cell recombinant Chitinase candidate A was observed (Figure 15) with only a marginal effect on reducing larval growth in in vitro feeding assay assessment (Figure 16).



Chitinase candidate A

Figure 15: Mean ELISA results of a sheep group (4 sheep per group) vaccinated with Chitinase Candidate A. Black = pre-vaccination sera, Blue = post 1st vaccination, Green = post second vaccination and Red = post third and final boost vaccination.

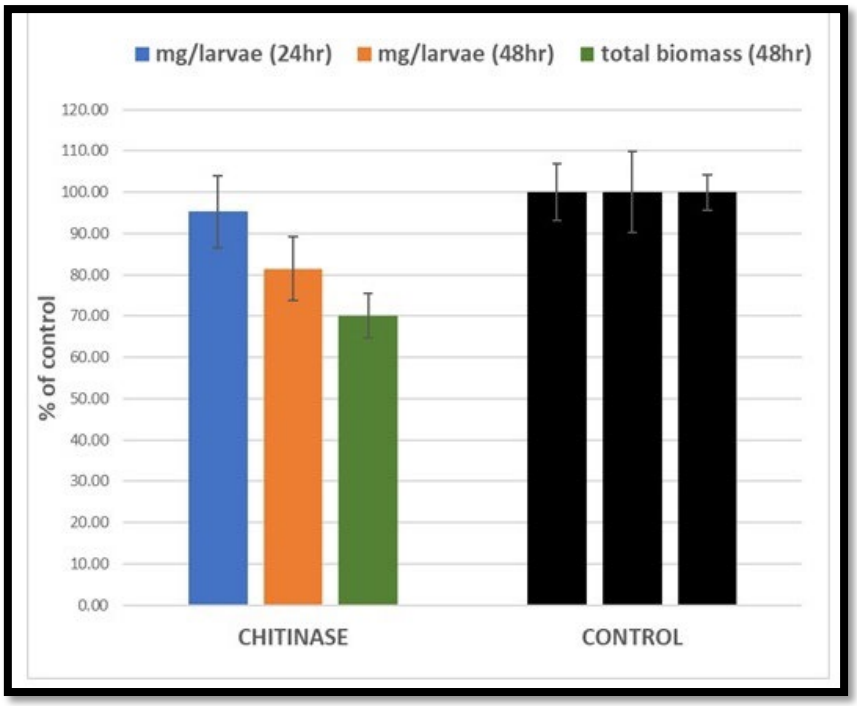
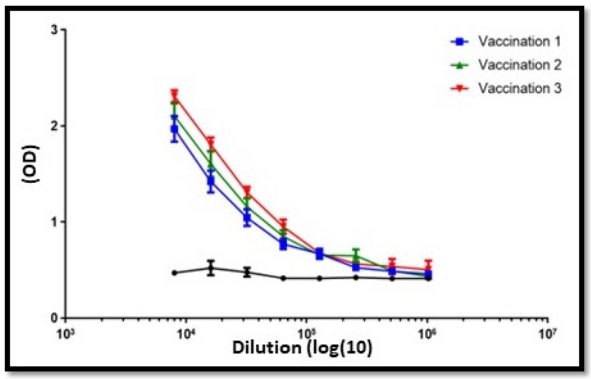


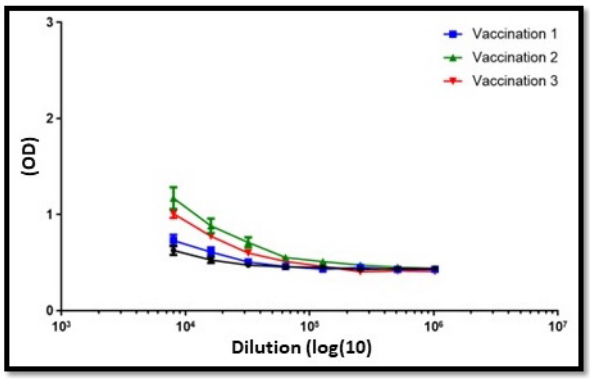
Figure 16: *In vitro* larval growth bioassay assessment of sera from animals vaccinated with Chitinasen-Candidate A. Results shown are group mean values (n= 4 sheep, 5 replicates each) for larval weight measured 24 or 48 hr growth and total larval biomass recovered at 48 hr. Results are normalised as a percentage of the Control adjuvant only vaccinated animal group.

Mucins

A single Mucin Candidate D was produced in secreted and intracellular form in insect cell expression and assayed in vaccination trials in sheep. Mucin Candidate D produced a good immune response in the sheep trial group whilst the intracellular recovered recombinant protein produced a limited immune response in sheep (Figure 17). *In vitro* larval feeding bioassay assessment displayed limited efficacy in reducing larval growth with best result recorded for the secreted protein.



Mucin D (secreted)



Mucin D (intracellular)

Figure 17: Mean ELISA results of a sheep group (4 sheep per group) vaccinated with Mucin Candidate D. A secreted and intracellular trapped protein form of the insect cell produced recombinant protein was tested. Black = pre-vaccination sera, Blue = post 1st vaccination, Green = post second vaccination and Red = post third and final boost vaccination.

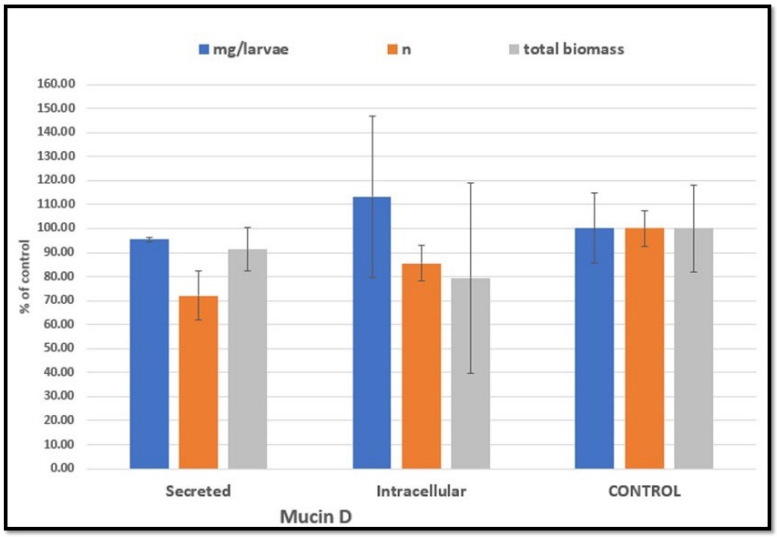


Figure 18: *In vitro* larval growth bioassay assessment of sera from animals vaccinated with Mucin- Candidate D secreted and intracellular isolated recombinant protein. Results shown are group mean values (n= 4 sheep, 5 replicates each) for larval weight measured 24 or 48 hr growth and total larval biomass recovered at 48 hr. Results are normalised as a percentage of the Control adjuvant only vaccinated animal group.

Serine Proteases

Serine protease candidate protein antigens were produced as bacterial recombinant proteins and did not progress to insect cell production. The five successfully produced protein antigens all invoked an immune response in sheep (Figure 19). The *in vitro* bioassay assessments showed a minor but variable reduction in larval growth after 24 hours of feeding (Figure 20).

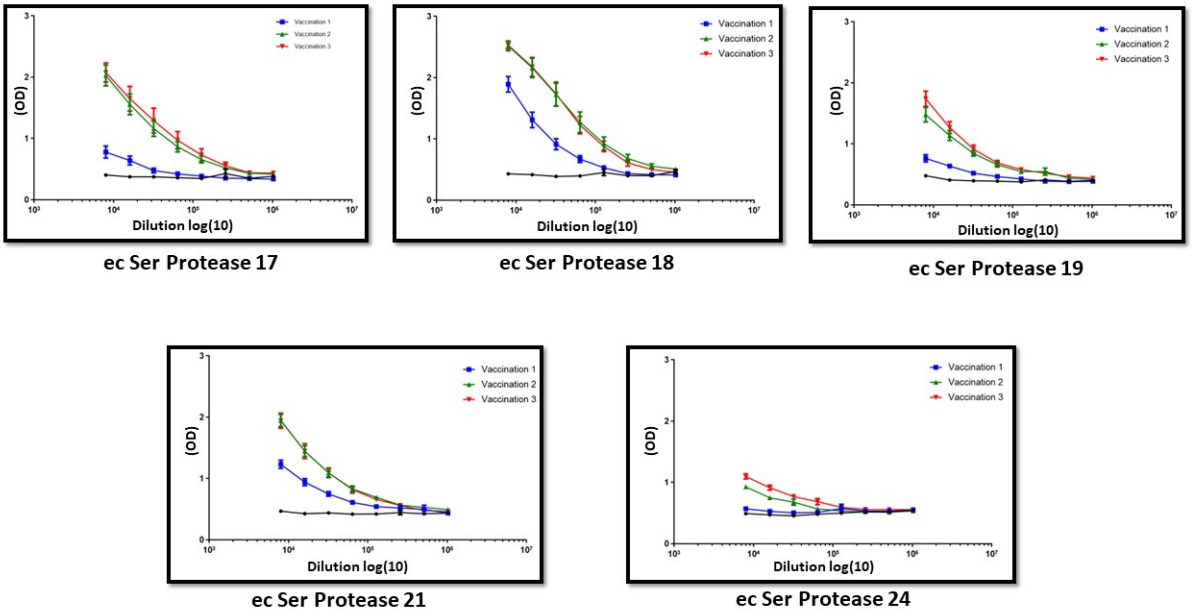


Figure 19: Mean ELISA results of a sheep group (4 sheep per group) vaccinated with bacterial produced recombinant Serine Protease candidate antigens. Black = pre-vaccination sera, Blue = post 1st vaccination, Green = post second vaccination and Red = post third and final boost vaccination.

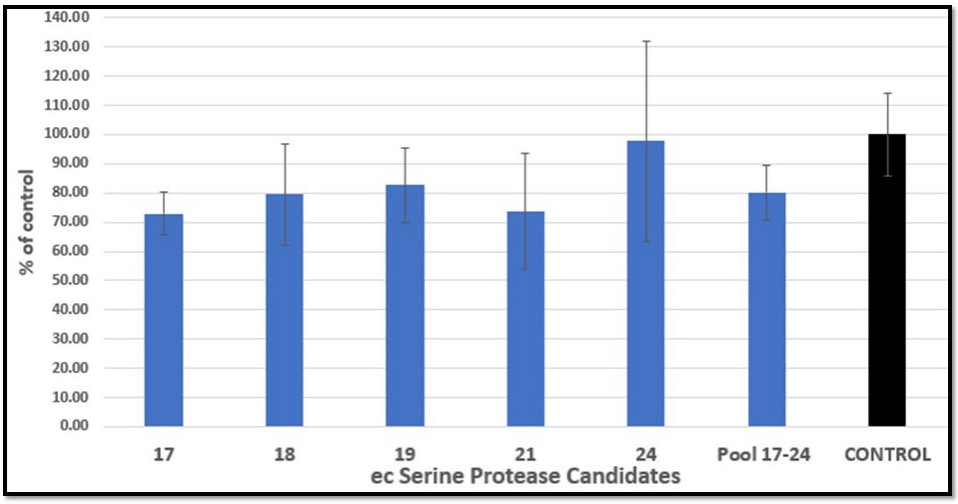


Figure 20: *In vitro* larval growth bioassay assessment after 24 hours of sera from animals vaccinated with five Serine Protease-candidate recombinant proteins and a combined (n=5) formulation. Results shown are group mean values (n= 4 sheep, 5 replicates each) for larval weight measured after 24 hr. Results are normalised as a percentage of the Control adjuvant only vaccinated animal group.

CRiSPs

Two CRiSP candidate proteins, Candidate 1 and 2 were produced as recombinant proteins in insect cells. Both CRiSP candidates produced good immune responses (Figure 21). *In vitro* bioassay testing of larval growth demonstrated a 50% reduction in larval growth over 24 hours for Candidate 2 consistent across the 4 animals in the vaccine group (Figure 22). Candidate 1 response was variable but also indicated a degree of larval growth reduction for this 24 hour period.

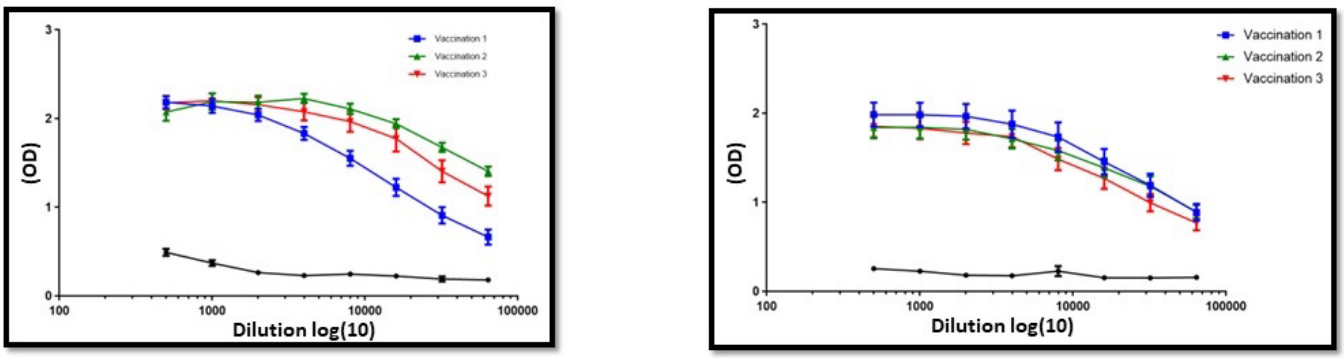


Figure 21: Mean ELISA results of a sheep group (4 sheep per group) vaccinated with insect cell produced recombinant CRISP candidate antigens 1 and 2. Black = pre-vaccination sera, Blue = post 1st vaccination, Green = post second vaccination and Red = post third and final boost vaccination.

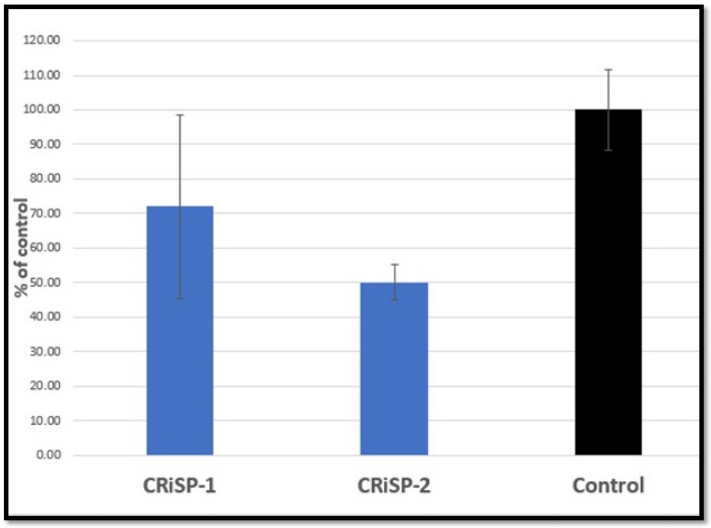


Figure 22: *In vitro* larval growth bioassay assessment after 24 hours of sera from animals vaccinated with insect cell produced CRISP Candidate 1 and 2 recombinant proteins. Results shown are group mean values (n= 4 sheep, 5 replicates each) for larval weight measured 2 after 4 hr growth. Results are normalised as a percentage of the Control adjuvant only vaccinated animal group.

Drosophila S2 Peritrophin

Three vaccine formulation trials were conducted with the Drosophila S2 insect cell produced Peritrophin candidate E antigen. The recombinant antigen was formulated and tested independently in sheep with three different adjuvant mixes including i) Montanide ISA 61VG, ii) QuilA, and iii) a mixture of Montanide ISA 61VG and Dextran Sulphate 500K. A good immunological response was recorded for each vaccine formulation and for all trial group animals with maximum titre achieved 4 weeks post the second dose of vaccine. There was no discernible difference with reference to antibody titre between trial groups (Figure 23).

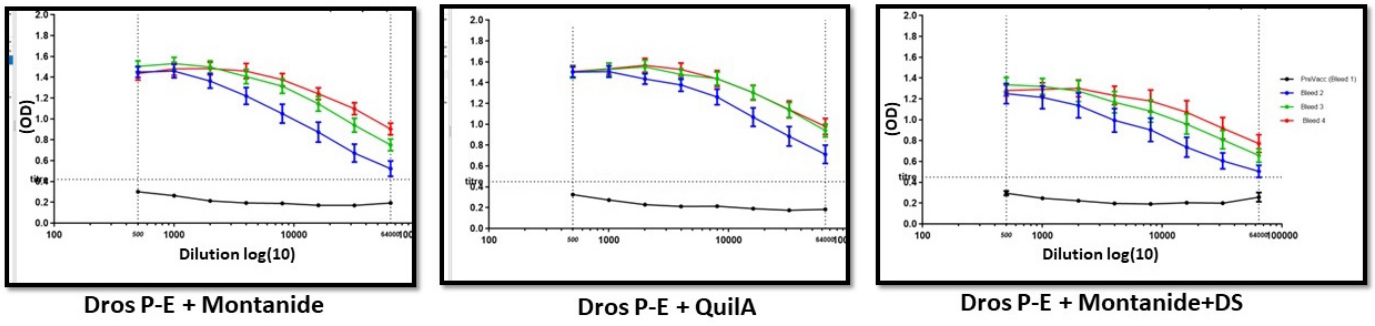


Figure 23: Mean ELISA results of a sheep group (4 sheep per group) vaccinated with Drosophila S2 insect cell produced recombinant Peritrophin candidate E antigens 1. Black = pre-vaccination sera, Blue = post 1st vaccination, Green = post second vaccination and Red = post third and final boost vaccination. Three adjuvant formulations were tested.

In vitro larval feeding and growth bioassays on sheep serum collected from the trial sheep 4 weeks after final boost vaccination did not show any significant effect on larval growth or survival for any of the three trial groups as normalised to the control adjuvant only group (Figure 24).

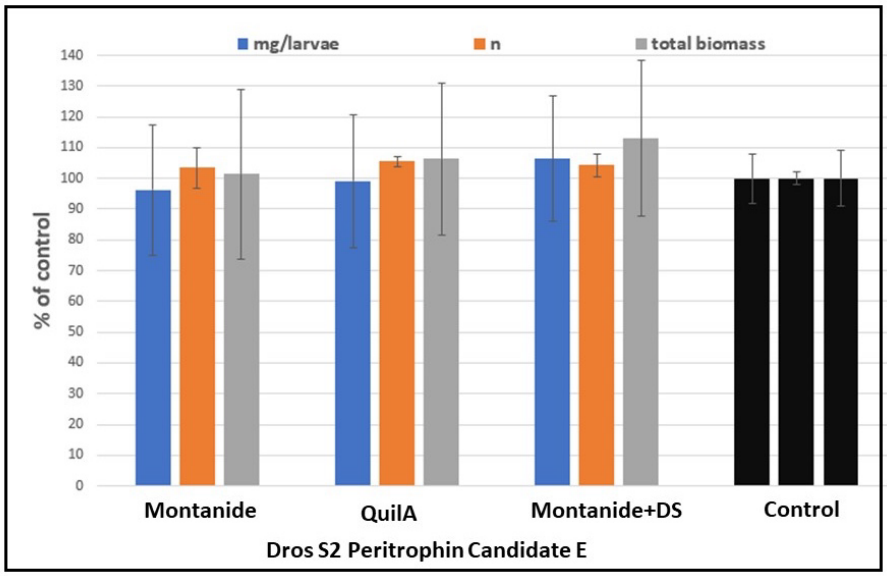


Figure 24: *In vitro* larval growth bioassay assessment after 24 hours of sera from animals vaccinated with Drosophila S2 insect cell produced Peritrophin Candidate E. Results shown are group mean values (n= 4 sheep, 5 replicates each) for larval weight measured after 24 hours growth. Results are normalised as a percentage of the Control adjuvant only vaccinated animal group.

The three Dros S2 Peritrophin candidate E groups formulated with different adjuvants and a Control adjuvant only group consisting of 4 animals per group were assessed for vaccine efficacy using an in vivo on sheep implant test. There was a difference observed for the mean larval weights recovered from Dros S2 Peritrophin E recombinant antigen formulated with Montanide ISA61VG alone (Figure 25). A mean reduction in the weight and size of the larvae growing on the sheep of ~25% for this group alone was observed. It must be noted that this is only a small group and the absence of an effect in the in vitro larval feeding and growth bioassays (Figure 24) and no difference observed in the other groups may suggests this effect whilst encouraging may also be arbitrary. There was no reduction in number of larvae or total larval biomass recorded.

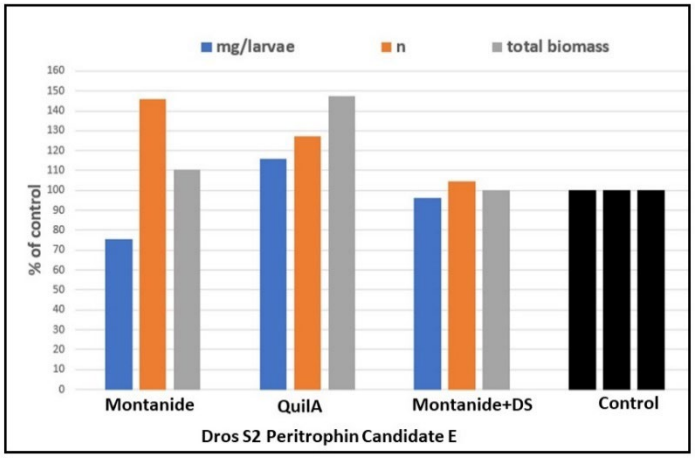


Figure 25: *In vivo* larval growth bioassay assessment after 24 hours of sera from animals vaccinated with Drosophila S2 insect cell produced Peritrophin Candidate E. Results shown are group mean values (n= 4 sheep), for larval weight, number of larvae survived and total larval biomass measured after 48 hours growth on sheep. Results are normalised as a percentage of the Control adjuvant only animal group.

Future work will investigate the glycosylation profile of Peritrophin Candidate E produced recombinantly in Dros S2 cells and compare with the same recombinant protein produced in the lepidopteran insect cells and the

native protein produced by blowfly larvae. This may help explain the differences in performance of the same antigen obtained from the different systems. This line of investigation will help inform future strategies in recombinant antigen engineering and production to enhance efficacy of antigens in flystrike vaccine development.

'Type R' recombinant multi-antigen vaccination trials

Initial Trial

A multi-recombinant antigen vaccine designated 'Type R', was developed and tested in sheep. The initial formulation tested included two Peritrophin-Mucin candidates (A and C), two Peritrophin candidates (E and F) and a Mucin candidate (D). The initial trial used a formulation strategy whereby soluble shellfish chitin (chitosan) was combined with the recombinant antigens and then formulated with Montanide ISA 61VG adjuvant. This vaccine was administrated to sheep over three doses, 4 weeks apart and a Control group given chitosan and adjuvant only. A good immune response was measured in the chitosan + antigens + adjuvant group with peak titre achieved after a single vaccine injection. There was no significant immune response registered for the chitosan + adjuvant only group (Figure 26).

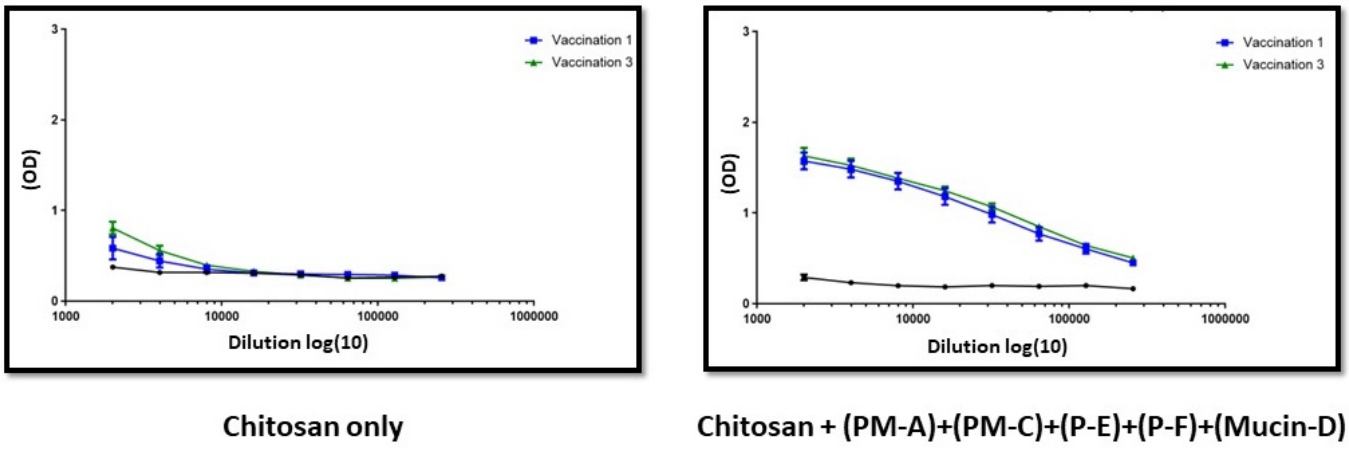


Figure 26: Mean ELISA results of a sheep group (4 sheep per group) vaccinated with 'Type R' multiple (n=5) insect cell produced candidate antigens 1. Black = pre-vaccination sera, Blue = post 1st vaccination, Green = post third and final boost vaccination. Vaccine formulation included addition of chitosan.

In vitro larval feeding and growth assessment of larvae fed serum collected from the sheep vaccinated with the 'Type R' multi-antigen vaccine displayed a significant and substantial reduction in growth compared to the control group of sheep that only received chitosan and adjuvant. A mean larval weight reduction of ~70% was recorded with a reduction of ~40% in viable larvae (Figure 27). Overall larval biomass, a function of larval weight and numbers surviving, also showed a substantial reduction in the chitosan plus 'Type R' multi-antigen vaccinated sheep sera in *in vitro* assays. In contrast, the Chitosan + Adjuvant group showed no significant differences when likewise compared to the Control adjuvant only group. Combining recombinant protein antigens with soluble (reacetylated) chitosan, may result in strong binding or coalescing of the protein with the chitosan leading to antigen conformation reflecting that encountered in the native state in the larval PM. This formulation and conformation may represent a key step to significantly improving an effective immune response from these antigens.

A) B)

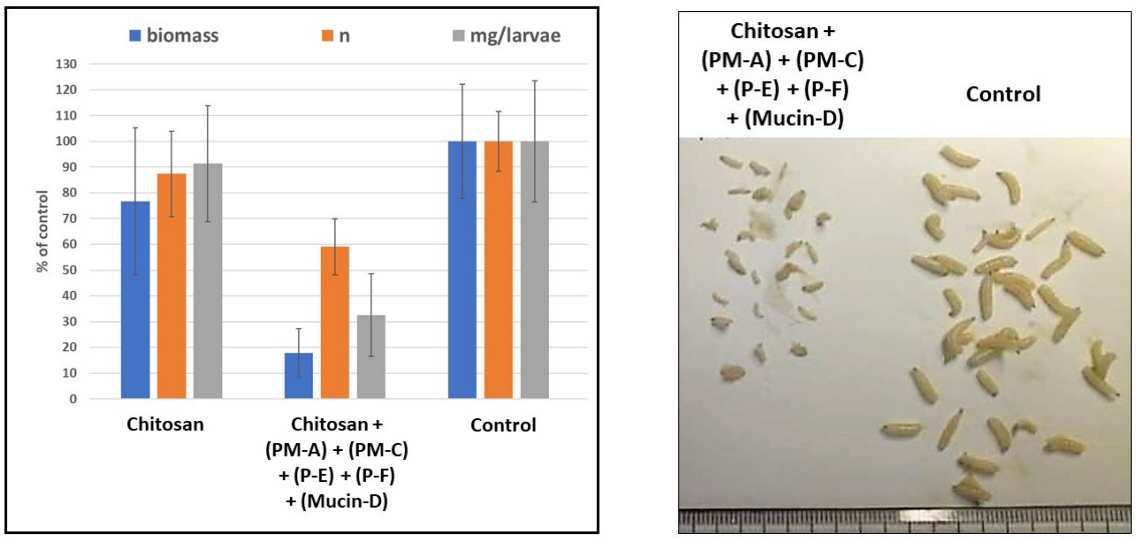


Figure 27: *In vitro* larval growth bioassay assessment after 72 hours of sera from animals vaccinated with 'Type R' recombinant multi-antigen vaccine formulated with chitosan. **(A)** *L*arval growth bioassay assessment. Results shown are group mean values (n= 4 sheep, 5 replicates each) for larval weight measured after 72 hours growth. Results are normalised as a percentage of the Control adjuvant only vaccinated animal group. A Chitosan + adjuvant only group is also shown as a comparative control. **(B)** Image of larvae recovered from an *in vitro* feeding bioassay after 72 hours feeding on sheep sera from sheep vaccinated with 'Type R' vaccine.

Type 'R' scale up independent and combined antigens

Type R Individual antigen assessment

The six 'Type R' antigens, designated Ag1 to Ag6 as described in Table 2 were formulated individually with Montanide ISA61VG adjuvant plus chitosan and administered IM three times at 4 weekly intervals. All candidate proteins were produced individually as recombinant proteins in lepidopteran cell lines. The trial groups were made up of 4 sheep per each single antigen plus a control group. Blood was collected and sera isolated prior each vaccination and at 4 weeks post final dose.

Table 2: List of Insect Cell recombinant antigens being produced at scale to allow recombinant multivalent antigen and single antigen sheep vaccination trials to be undertaken.

Antigen #	Common name
Ag1	Peritrophin Mucin A
Ag2	Peritrophin Mucin C
Ag3	Peritrophin E
Ag4	Peritrophin F
Ag5	Mucin D
Ag6	Peritrophin Mucin B

All antigens produced a good immunological response in all the vaccinated sheep with the titre achieving maximal level within the first two doses (Figure 28). Titre was assessed with blood/sera collected immediately before each of the three injections carried out at 4 weeks apart and then 4 weeks post the third injection.

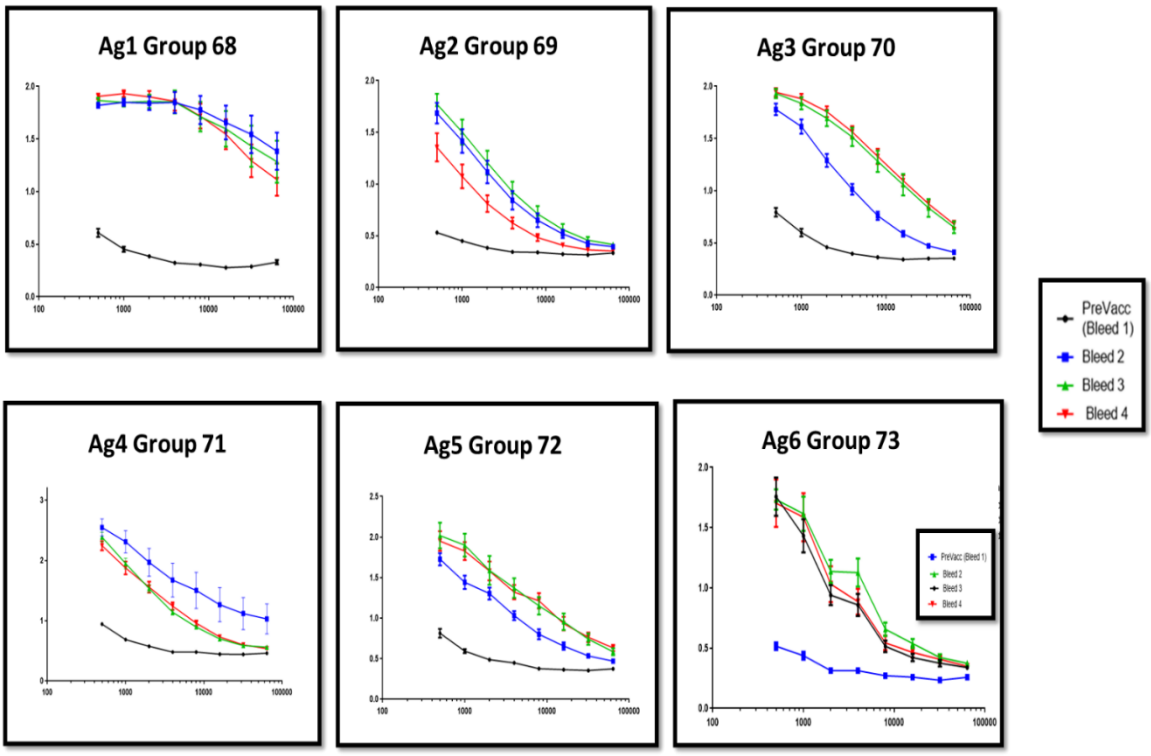


Figure 28: Antibody titre analysis by ELISA for Trial Groups 68-73 comprising the individual recombinant protein antigens of the 'Type R' vaccine.

*I*n vitro larval feeding assessment was performed for the sheep in the trial groups that received single antigen formulated vaccines. Larval growth was assessed as a function of weight, survival, and larval biomass recovered and normalised to the control group animals that received only chitosan and adjuvant formulated injections (Figure 29). Pre-vaccination serum and post-final vaccination serum were assessed in 24 hr in vitro feeding trials. Results show that there were no significant differences between the antigen trial groups in the pre-vaccination trial whilst the post vaccination sera showed some reduction in larval growth in the vaccine groups. Ag3 and Ag6, i.e. Peritrophin candidate E and Peritrophin-Mucin candidate B respectively displayed the most significant reduction in larval growth in this trial with a 28% and 32% reduction in mean larval weight and 40% and 45% reduction in total larval biomass recovered. This information was used to inform formulation of multi-antigen vaccine formulations with reduced number of recombinant antigens in follow-up vaccine trials. The potential for future commercial development of a recombinant vaccine would be further enhanced with a reduced requirement for antigen inclusion.

A) B)

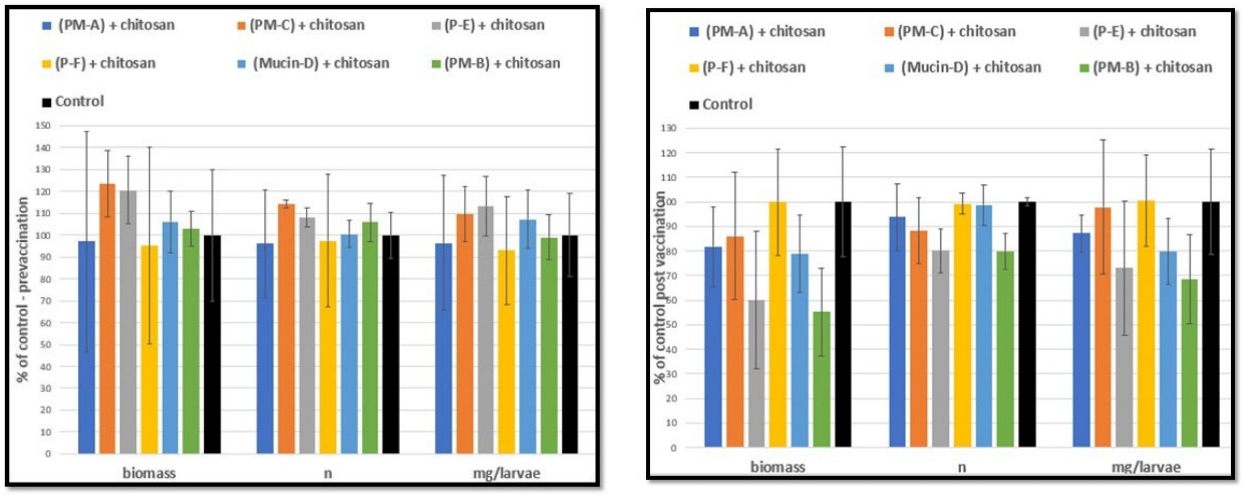


Figure 29: *In vitro* larval feeding bioassay assessment of **A)** Pre-vaccination sera and **B)** Post-vaccination sera of sheep vaccinated with individual insect cell recombinant protein antigens Ag1-Ag6 plus chitosan.

Comparison of post- versus pre-Vaccination sera *in vitro* feeding trial data showed an effect of vaccination with each recombinant antigen plus chitosan in reducing larval weights and total biomass with negligible effect on total number of larvae recovered. It must be noted that there was also an apparent effect observed in the control group but not generally to the same degree as the vaccinate trial groups. The reason for this reduction in the Control group is not clear but maybe due to general increase of lysed blood cell components such as haemoglobin in the post-vaccination serum resulting from bulk blood collection technique immediately post euthanasia of the animals. The increased heme levels may slow larval growth. The trend however indicates a significant effect on larval growth of the vaccination using the recombinant antigens (Figure 30).

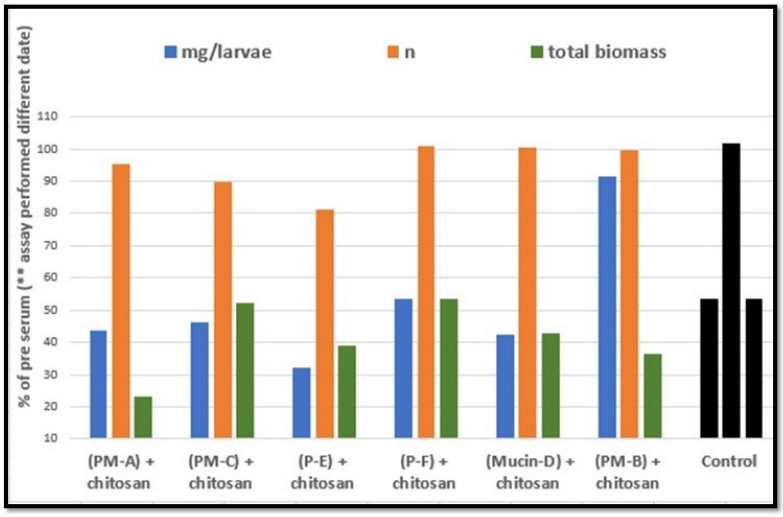


Figure 30: In vitro larval feeding bioassay assessment sera of sheep vaccinated with individual insect cell recombinant protein antigens Ag1-Ag6 plus chitosan as normalised to Pre-vaccination data.

'Type R' Dual Antigen Recombinant Vaccine Assessment

Based on preliminary information and results from the single antigen 'Type R' vaccine trials, we moved to selecting a trimmed down version of recombinant antigens for formulating and progressing the multi-valent 'Type R' recombinant vaccine as a bi-valent antigen derivative. Two recombinant protein antigens, Ag3 and Ag6, were combined and formulated with several different adjuvants, and administered either intra-muscular (IM) or sub-cutaneous (SC). The response by the trial sheep to this vaccination regime was assessed by ELISA and *in vitro* larval feeding assays. Six sheep constituted each trial group. Table 3 summarises the Trial Group formulations and injection sites. The best performing formulation was progressed to an *in vivo* on-sheep larval implant trial and is described in the subsequent section of this report.

Table 3: Outline of bi-valent 'Type R' vaccine trial investigating two key recombinant antigens. IM; intra-muscular, SC; sub-cutaneous injection route.

Trial Group	Vaccine	Depot	Adjuvant
75	Dual Recombinant Ag3 (Peritrophin E) + Ag6 (Peritrophin Mucin B) + Chitosan	IM	Montanide ISA 61VG
76	As above (75)	SC	Montanide ISA 61VG
77	As above (75)	IM	Montanide ISA 61VG+QuilA
78	As above (75)	SC	Montanide ISA 61VG+QuilA
79	As above (75)	IM	Mont ISA61VG + Dextran Sulfate 500K
80	As above (75)	SC	Mont ISA61VG + Dextran Sulfate 500K
87	Control – No Antigen	IM/SC	Montanide ISA 61VG+QuilA
88	Control – No Antigen	IM/SC	QuilA
89	Control – No Antigen	IM/SC	Mont ISA61VG + Dextran Sulfate 500K

The bivalent 'Type R' vaccine was formulated using chitosan and several adjuvants independently to assess potential optimisation of immune response with respect to vaccine formulation. The benchmark adjuvant used thus far in the Flystrike Vaccine project has been Montanide ISA61VG and for this trial we also investigated a combination adjuvant approach including 1) Montanide ISA61VG alone, 2) Montanide ISA61VG plus QuilA, 3) Montanide ISA61VG plus Dextran Sulphate 500K. A significant immunological response was recorded for each vaccine formulation and for both IM and SC depot of injection (Figure 31). As has been routinely observed, peak antibody titre was achieved in most cases 4 weeks after initial vaccination or by 4 weeks post second vaccination.

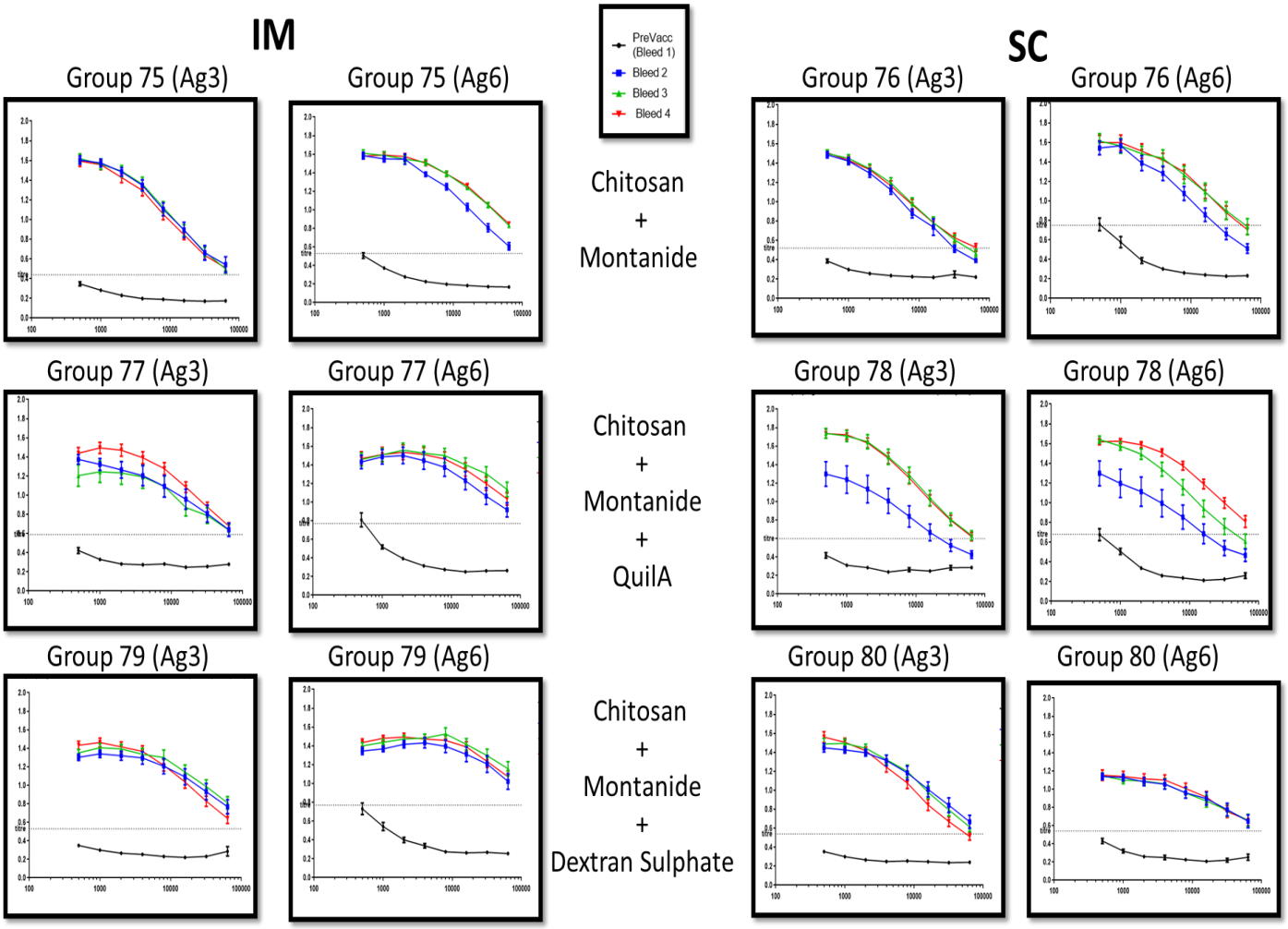


Figure 31: ELISA assessment of antibody titres for bivalent 'Type R' vaccine groups. Antibody titres raised to each antigen was independently assessed and denoted as Ag3 or Ag6 for the bi-valent vaccine.

The bivalent vaccine formulated with chitosan and Montanide adjuvant resulted in a 15% to 27% reduction in mean larval weight and 5% to 28% in total larval biomass (IM and SC applied respectively) when compared to the Montanide only vaccine group assessed in *in vitro* larval feeding bioassays (Figure 32). No reduction in larval numbers were recorded. There was no reduction in larval growth for the Montanide and Dextran Sulfate 500K or Montanide and QuilA formulated vaccine groups (result not shown).

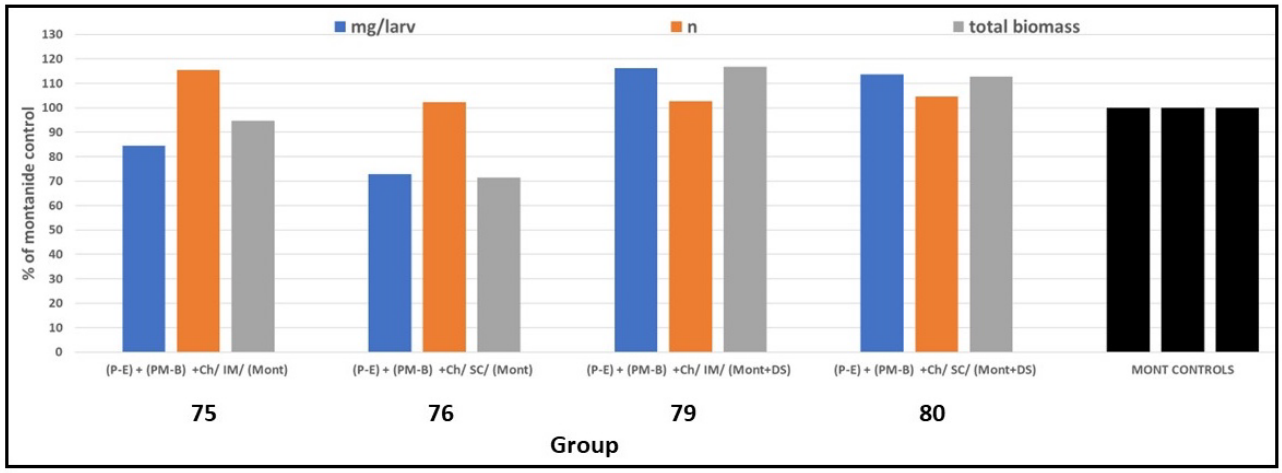


Figure 32*: In vitro* larval feeding and growth assessment of the bi-valent antigen vaccine groups formulated with either Montanide or Montanide + Dextran Sulfate 500K, administered IM or SC.

In vivo on sheep larval implant assessment of larval growth was performed on Group 79, Peritrophin candidate E and Peritrophin-Mucin candidate B bi-valent antigen Montanide and Dextran Sulfate formulated vaccine. The group consisting of 6 sheep did not return any significant effect on larval growth (Figure 33). The *in vitro* testing was performed after the *in vivo* and similarly did not demonstrate any significant larval growth inhibition effects. In hindsight, either Group 75 or 76 should have been chosen for testing, but this was not possible due to timing and resource constraints in performing *in vivo* testing. The results demonstrate an apparent failure of the bivalent recombinant vaccine formulation to significantly affect larval growth at this point in time.

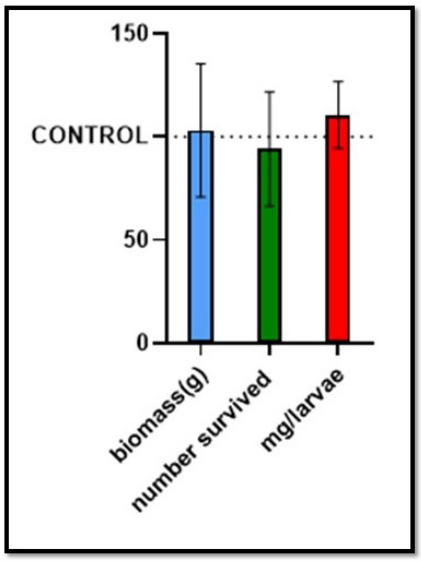


Figure 33: In vivo larval feeding and growth assessment of bi-valent antigen vaccine group 79 formulated with Montanide + Dextran Sulfate 500K, administered IM.

‘Type N’ native antigen vaccination trials

Initial Trial

Peritrophic matrix produced by sheep blowfly larvae in the laboratory was collected and formulated into a ‘Type N’ native protein antigen vaccine and tested in sheep for immune response. Initial trialling of the native protein antigen was tested as an antigen plus Montanide adjuvant only, or antigen plus chitosan plus Montanide adjuvant formulation. This approach mirrored the ‘Type R’ recombinant antigen investigations that were undertaken in parallel. Three injections IM, each 4 weeks apart vaccine administration protocol was followed, after which the sera from the vaccinated animals was assayed for induced antibody titre response and effect on larval growth using *in vitro* larval feeding and growth assays. ELISA analysis showed an enhanced antibody titre in the vaccine formulation that combined chitosan with the native protein antigen. Surprisingly the native antigen only plus adjuvant formulation did not induce a high antibody response in this initial trial (Figure 34).

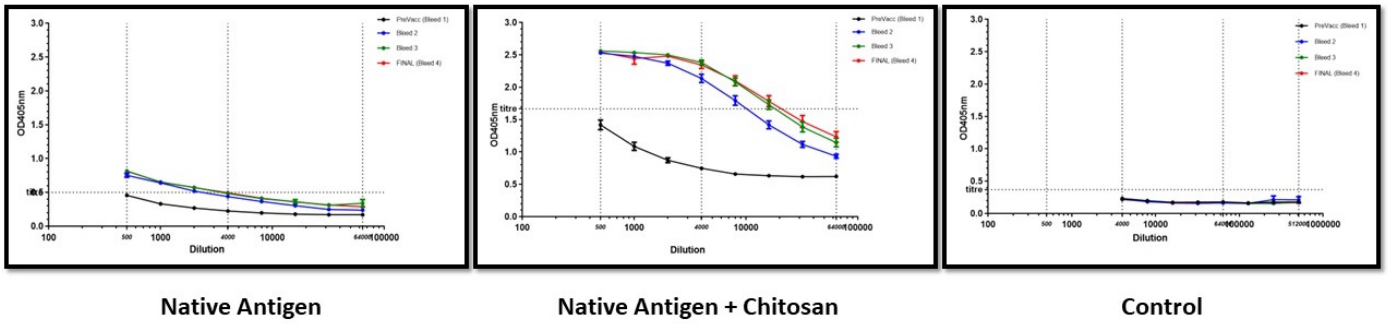


Figure 34: ELISA assessment of antibody titres for ‘Type N’ native antigen vaccine groups.

Assessment of sheep sera obtained from the vaccinated animals was performed using *in vitro* larval feeding and growth assays. There was a good response observed to native antigen plus chitosan formulation with Montanide adjuvant with a significant reduction in larval growth, biomass and survival of larvae fed on the immune sera after 48 hours growth. Mean larval weight, number survived, and total biomass recovered was reduced by ~65%, 60% and 80% respectively for larvae grown on native antigen vaccinated sera compared to the control no antigen control sera fed larvae (Figure 35A). Figure 35B shows a sample of larvae collected from the *in vitro* bioassay after 72 hours feeding demonstrating the marked effect on growth of the larvae fed on Type ‘N’ vaccine produced sheep serum. These results support further exploration of native antigens in vaccine development and as a benchmarking tool for recombinant vaccine antigen vaccine development. Enhancing efficacy of a native antigen ‘Type N’ vaccine formulation and optimising production of native antigen may also provide an option for future vaccine development as a viable alternative to engineering and production of a ‘Type R’ recombinant vaccine approach. No *in vivo* on-sheep testing was performed with this initial trial ‘Type N’ vaccine, but was undertaken in follow-up experiments.

A) B)

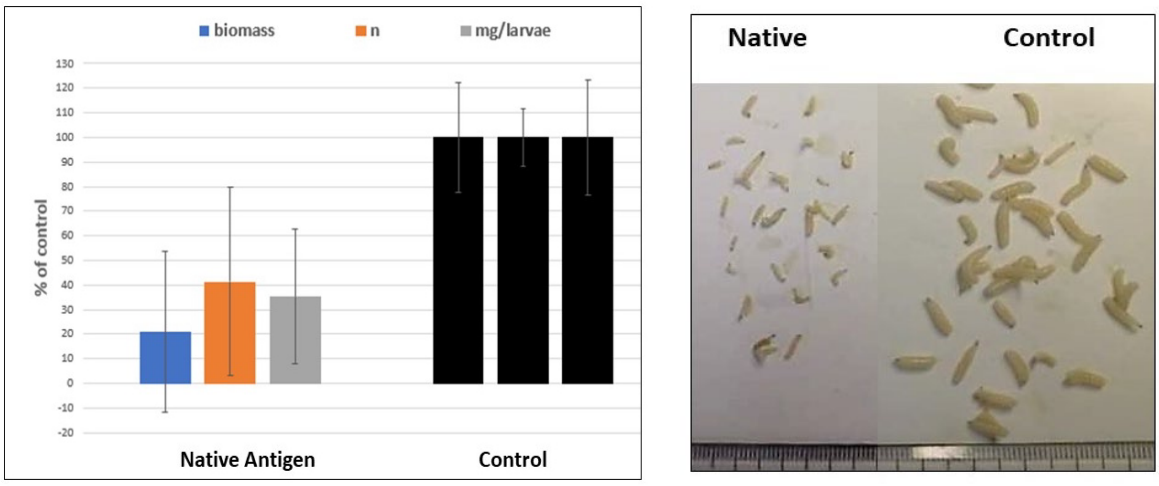


Figure 35*: In vitro* larval feeding bioassay assessment of Type 'N' native antigen vaccine. **(A)** *In vitro* larval feeding and growth assessment of the Type 'N' native antigen vaccine formulated with chitosan and Montanide administered IM. **(B)** Image of larvae recovered from an *in vitro* feeding bioassay after 72 hours feeding on sheep sera from sheep vaccinated with 'Type N' vaccine.

Native Protein Antigen Vaccine ('Type N') adjuvant, dose and longevity study

The 'Type N' native antigen vaccine was further investigated to explore vaccination delivery depot, utility of additional and combined adjuvants and the longevity of the immune response. This was undertaken to investigate if these would assist in any way to promote immunological response in the vaccinated sheep and how long it lasted for. Table 4 describes what was performed in this trial.

Table 4: Trial outline describing individual group testing regime of the Type N vaccine.

Trial Group	Vaccine Type	Antigen (mg)	Injection Depot	Adjuvant
81	Type N	5	IM	Mont ISA61VG + QuilA
82	Type N	5	SC	Mont ISA61VG + QuilA
83	Type N	5	IM	QuilA
84	Type N	5	SC	QuilA
85	Type N	5	IM	Mont ISA61VG + Dextran Sulphate
86	Type N	5	SC	Mont ISA61VG + Dextran Sulphate
87	Control	0	IM and SC	Mont ISA61VG + QuilA
88	Control	0	IM and SC	QuilA
89	Control	0	IM and SC	Mont ISA 61VG+ Dextran Sulphate

Injection depot and antigen dose

Six trial groups (81-86) consisting of 6 sheep each were vaccinated with 5mg each of Type N antigen. It is noted that these early preparations of native antigen contained a significant degree of bacterial protein contamination from the protein culture which contributed to the protein content. This contaminating factor is now controlled for and eliminated from native antigen preparations and investigations going forward are using a more refined

and larval targeted native antigen preparation. In this trial, the total antigen preparation was freeze dried, resuspended in Phosphate Buffered Saline (PBS) and quantified using a Bradford Colorimetric Assessment (BCA) protocol. Sheep received either intra-muscular (IM) or sub-cutaneous (SC) injections of the vaccine as per standard vaccination practice. Intradermal (ID) vaccinations were not performed as this was not a practical approach using the current formulation. It is noted however with current progress of dermal delivery vaccines that there is merit in future investigations to investigate some form of dermal delivery, i.e. trans or intra-dermal to promote a specific skin induced immunological response that may promote enhanced protection at the skin surface interface between host and parasite. Each sheep for the adjuvant and depot delivery study received 3 injections of the vaccine in total with a single injection administered in the selected depot at 4 week intervals over the 12 week period of the trial. Sheep serum was collected immediately preceding each injection and at conclusion of the 12 weeks, i.e. being 4 weeks post the final injection. The serum was used to assess antibody titres raised from the vaccinations. Larval feeding *in vitro* bioassays were performed on sera collected at the start and completion of the trial.

Adjuvant assessment

Adjuvants perform a function of amplifying and helping prolong the immunological response to the antigen. This project has primarily used Seppic Montanide ISA61VG, a non-animal-based vegetable water-in-oil emulsion adjuvant. We trialled two additional adjuvants, QuilA and Dextran Sulphate in conjunction with the Montanide adjuvant to assess their effect on inducing an immune response to the Type 'N' vaccine formulation. QuilA is an aqueous plant based saponin vaccine adjuvant that has been used in a variety of veterinary vaccines. This adjuvant is used with good effect in BarberVax™, a purified native antigen vaccine that is used commercially for control of Barber's pole Worm, *Trichostrongylus spp*. Hence it was assessed for potential use in formulation of the 'Type N' flystrike vaccine. Dextran Sulphate (500,000) is a high molecular weight polymer that has been studied for its ability to induce an enhanced immunological response when used in some experimental vaccine formulations. It was shown by previous CSIRO research to synergise the immunological response against a crude blowfly antigen when combined with Freund's Incomplete, a mineral oil adjuvant. The concept of combining Dextran Sulphate with Montanide ISA61VG in formulating the 'Type N' vaccine was tested in this trial to see whether it also improved immunological response to the vaccine.

Immunological response ELISA assessment

Blood was collected and serum isolated immediately prior each vaccination injection and at 4 weeks post final injection. The immunological response to the vaccine antigen was measured using ELISA at each time point for each animal. Figure 36 shows a graphical representation of the mean antibody titre for animals in the group using a dilution course analysis. The results demonstrate that each vaccination group raised a high antibody titre to the vaccine antigen. The titre analysis demonstrated that peak titre was generally achieved after the second vaccination administration (bleed 3 serum) as observed in numerous other vaccine trials that we performed.

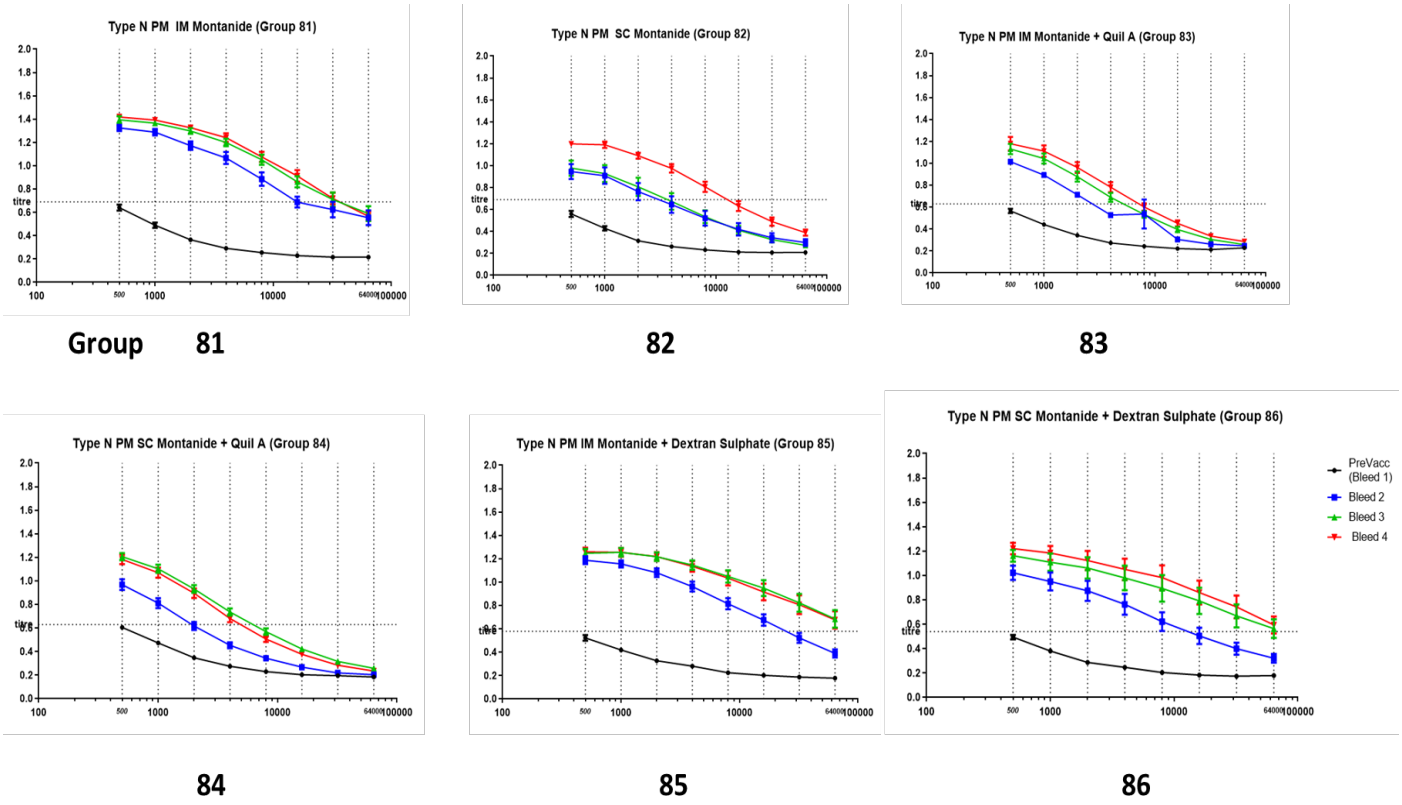


Figure 36: Antibody titre raised to 'Type N' vaccine antigen Groups 81 – 86 over the trial time-course. Bleed 1 (pre vaccination), Bleed 2 (1st injection response), Bleed 3 (2nd injection response), Bleed 4 (3rd injection boost response).

In vitro assessment of Trial 12 'Type N' vaccine.

An *in vitro* larval feeding and growth bioassay assessment using a synthetic skin matrix and sera collected from the vaccinated sheep, was used to assess 'Type N' vaccine induced antibodies effect on the growth of blowfly larvae. Larvae were allowed to feed on the sera and skin matrix over a 48-hour period from neonate to early/mid third instar. Larvae were then collected, counted, and weighed to determine effects on growth. The results were normalised to the same growth parameters with respect to the same animal's pre-vaccination serum and immunological state. The results for all the animals in the nine trial groups, including the three control groups is shown in Figure 37. The results demonstrated a marked reduction of ~40-60% across the growth parameters measured in Group 85, 'Type N' antigen formulated with Montanide ISA61VG and Dextran Sulphate, administered IM and 20-45% for Group 86 with the same formulation administered SC. A ~10-30% reduction in larval growth parameters was observed for Group 81, 'Type N' antigen formulated with Montanide ISA61VG only, administered IM. The other groups showed marginal to no effects. It should be noted that Control Group 89, Montanide 61VG and Dextran Sulphate adjuvant only, demonstrated a ~10-30% reduction in larval growth which was an unsuspected and unexplained. This result, whilst encouraging for the prototype 'Type N' vaccine, did not achieve the same degree of effect as demonstrated for this vaccine reported in the initial trial where up to a 75% reduction in larval growth parameters were demonstrated. We propose that this was likely due to batch effects of the native antigen preparation and bacterial contamination and degradation of the native antigen which has subsequently been controlled through refined sterile culture procedures.

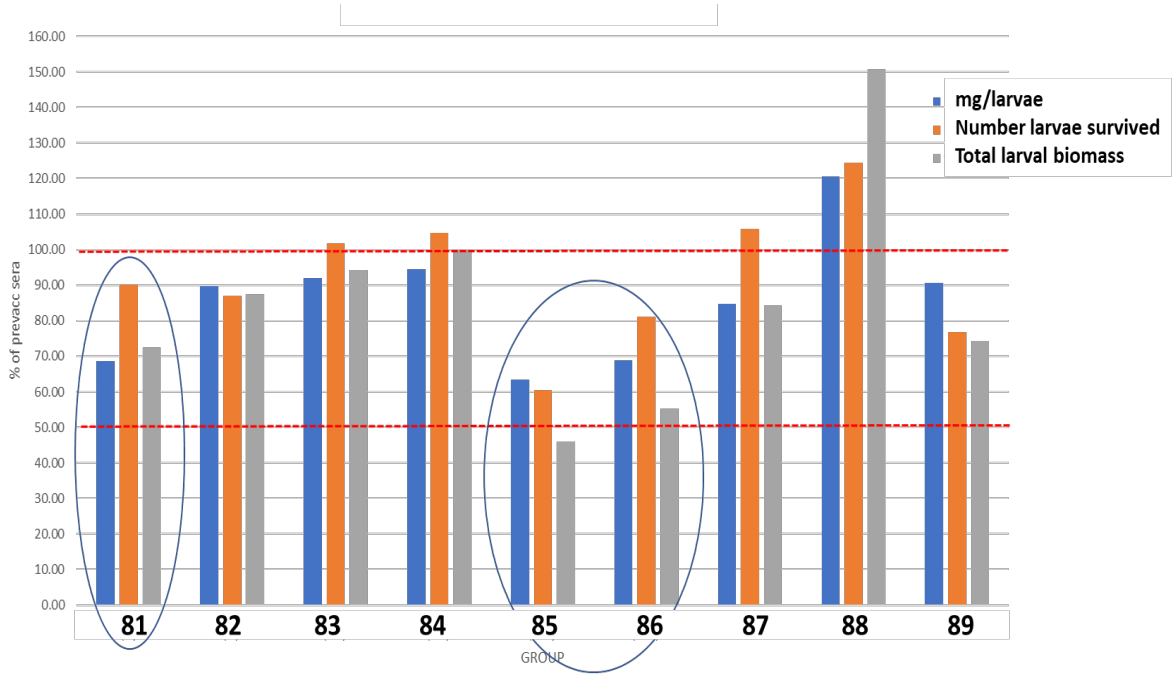


Figure 37: Trial in vitro larval feeding bioassay assessment for 'Type N' vaccine antigen Groups 81 – 86 and respective Controls, Groups 87-89. Results are normalised to the pre-vaccination sera results for the respective groups.

Based on the *in vitro* assessment of the six Type 'N' vaccine trial groups 81-86, three groups, 81, 85 and 86 were tested further using *in vivo* on sheep larval implant testing along with the control groups 87, 88 and 89. Larval growth *in vivo* was assessed after 48 hours growth on sheep at the implant site. Results were normalised to the larval growth measured on the Control animals. Group 81, Montanide only Type 'N' formulated vaccine vaccinated animals demonstrated a marginal~10-20% reduction in the growth parameters measured whilst Groups 85 and 86 displayed no response to the vaccine *in vivo* (Figure 38).

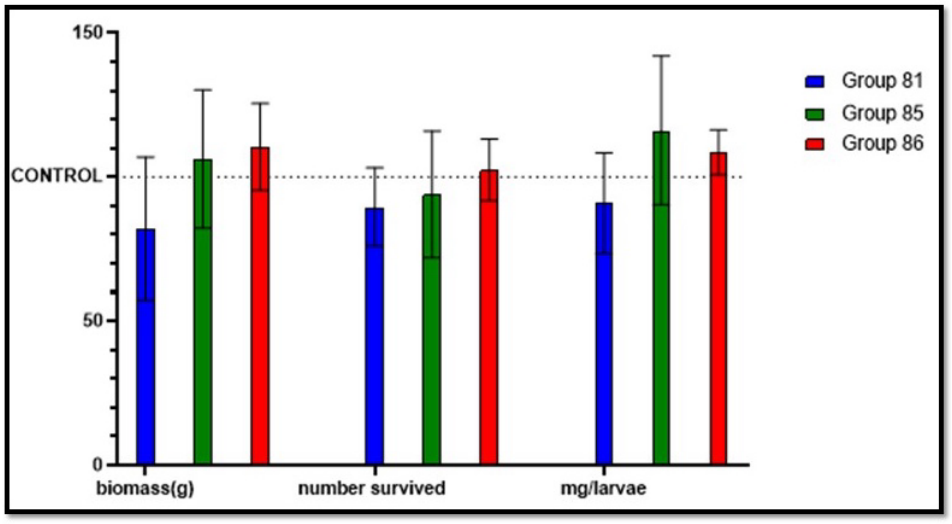


Figure 38*: In vivo* larval feeding and growth assessment of 'Type N' antigen vaccine groups 81, 85 and 86.

Native Protein Antigen Vaccine ('Type N') Dose and Longevity Effects

We investigated the 'Type N' native vaccine with respect to the effect of dose and depot of vaccine delivery on the longevity of the immune response in sheep with respect to antibody titre and *in vitro* larval growth effects over an 18-month period (Figure 39). The two best performing trial groups, Group 52 and 55 along with Control

Group 64 were selected for an annual- boost vaccination at 11 months post first vaccination for ongoing monitoring and assessment.

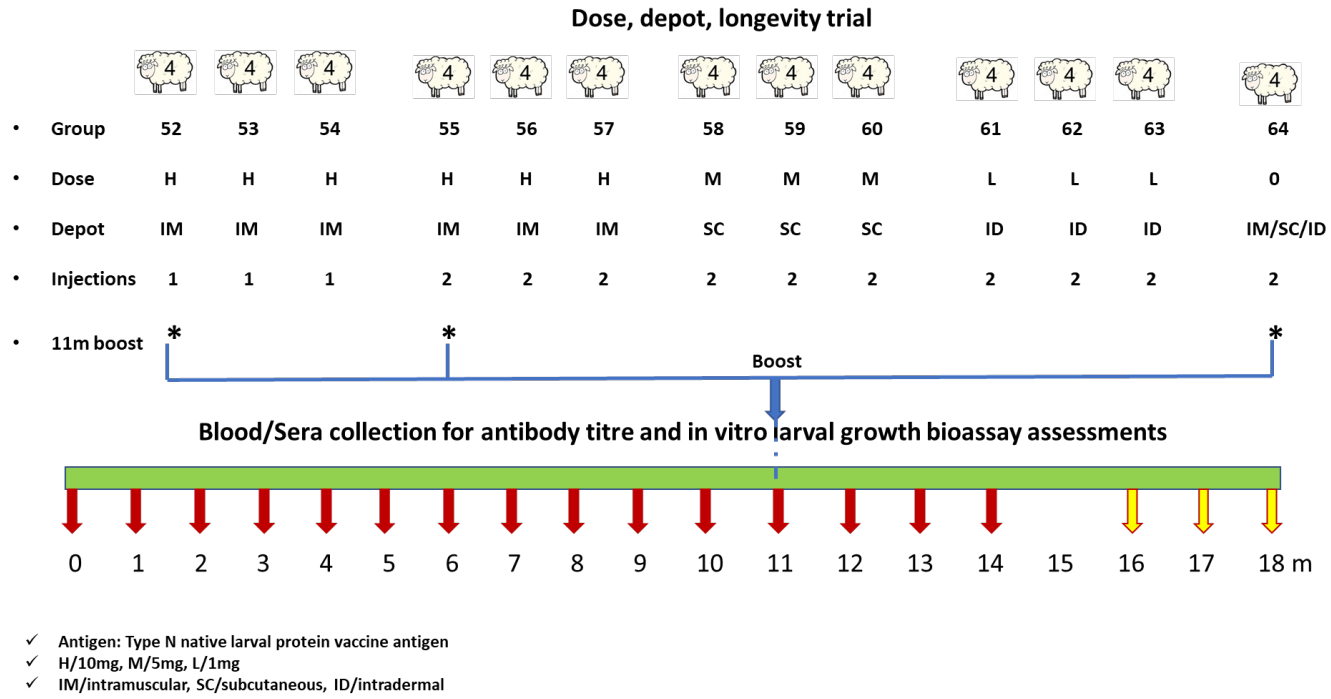


Figure 39: Illustration of Trialplan for testing dose, depot, and longevity parameters of "Type N' prototype vaccine.

ELISA analysis of the 13 different vaccine groups was undertaken (Figure 40). The ELISA results up to the 6months after the initial vaccination showed the following for the respective trial groups:

Group 52, 1 dose, 10mg (H), IM: Moderate immune response not registered until 2months after vaccination peaking at 5 months and maintained till 6months.

Group 53, 1 dose, 10mg (H), SC: Poor to very low immune response observed over entire 7month study.

Group 54, 1 dose, 10mg (H), ID: Moderate immune response not registered until 2months after vaccination and peaking at 5months. Antibody titre declining after 5months.

Summary Group 52-54: IM and ID single dose vaccine performed equivalently whilst SC single dose performed poorly in elucidating an immune response.

Group 55, 2 dose, 10mg (H), IM: Moderate immune response not registered until 2months after vaccination peaking at 5 months and falling gradually thereafter till 7months.

Group 56, 2 dose, 10mg (H), SC: Moderate immune response not registered until 2months after vaccination peaking at 5 months and falling gradually thereafter till 7months.

Group 57, 1 dose, 10mg (H), ID: Moderate immune response not registered until 2months after vaccination peaking at 5 months and falling gradually thereafter till 7months.

Summary Group 55-57: All displayed moderate immune response with no discernible differences between depot of injection.

Group 58, 2 dose, 5mg (M), IM: Moderate immune response not registered until 2months after vaccination peaking at 5 months and falling gradually thereafter till 7months.

Group 59, 2 dose, 5mg (M), SC: Low to moderate immune response not registered until 2months after vaccination peaking at 5 months and falling gradually thereafter till 7months.

Group 60, 2 dose, 5mg (M), ID: Low to moderate variable immune response not registered until 2months after vaccination peaking at 5 months and falling gradually thereafter till 7months.

Summary Group 58-60: IM response appeared most consistent.

Group 61, 2 dose, 1mg (L), IM: Low to moderate immune response not registered until 2months after vaccination peaking at 5 months and falling gradually thereafter till 7months.

Group 62, 2 dose, 1mg (M), SC: Poor to low immune response not registered until 2months after vaccination peaking at 5 months and falling gradually thereafter till 7months.

Group 63, 2 dose, 1mg (M), ID: Low to moderate variable immune response not registered until 2months after vaccination peaking at 5 months and falling gradually thereafter till 7months.

Summary Group 61-63: IM and ID response appeared most consistent.

Group 64, 2dose, no antigen, adjuvant only, IM+SC+ID: No immune response as expected for no antigen control group.

Overall assessment from an antibody titre perspective is that IM injection appeared to perform most robustly and elucidate the best antibody titres with ID performing similarly and SC consistently the lowest performing schedule. The higher antigen doses of 5 or 10mg performed better than the low dose 1mg with a 2-dose regime providing better antibody titre generation. Results demonstrated that generally the antibody titre for the 'Type N' vaccine was maintained for up to 5 months with gradual regression by month 6.

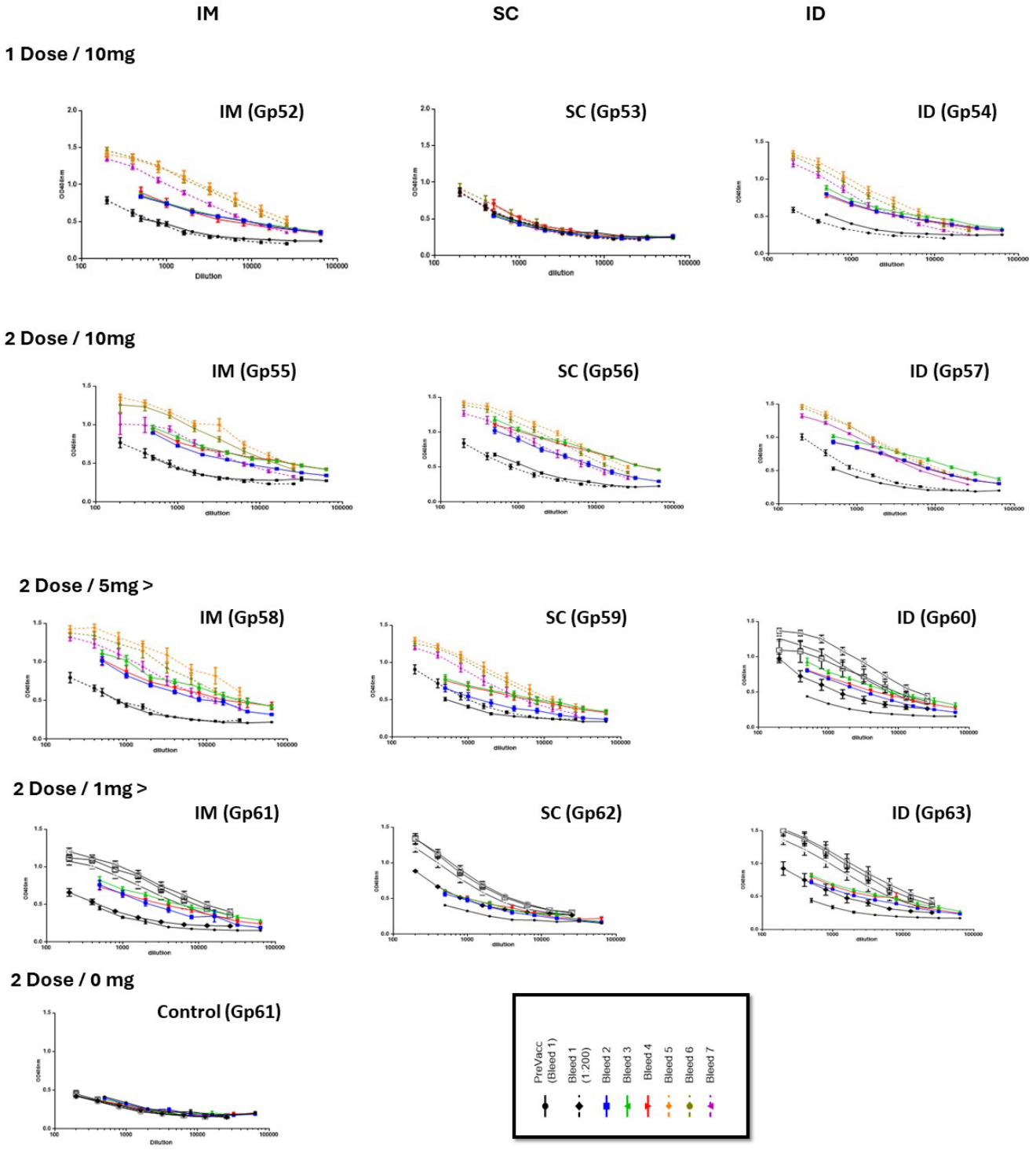


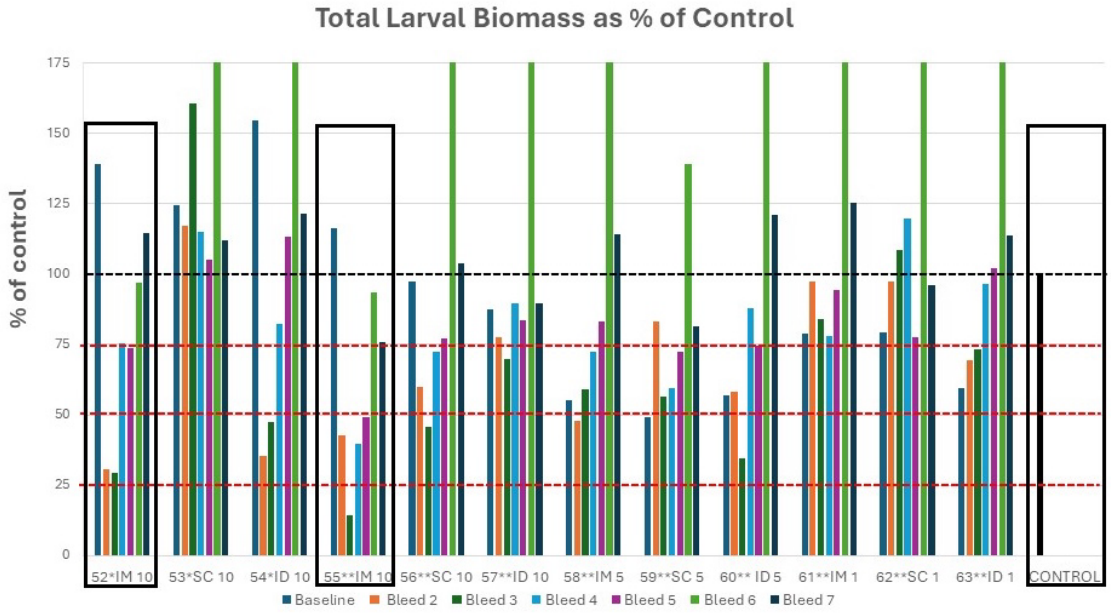
Figure 40: Antibody titre raised to 'Type N' vaccine over the 6-month trial time-course. Bleed 1 (pre vaccination), Bleed 2 (1st injection response), Bleed 3 (2nd injection response), Bleeds 4-7 (post vaccination response). Depot (IM/SC/ID) and dose regime (10/5/1/0mg antigen) of vaccinations are shown.

Native Antigen 'Type N' in vitro longevity

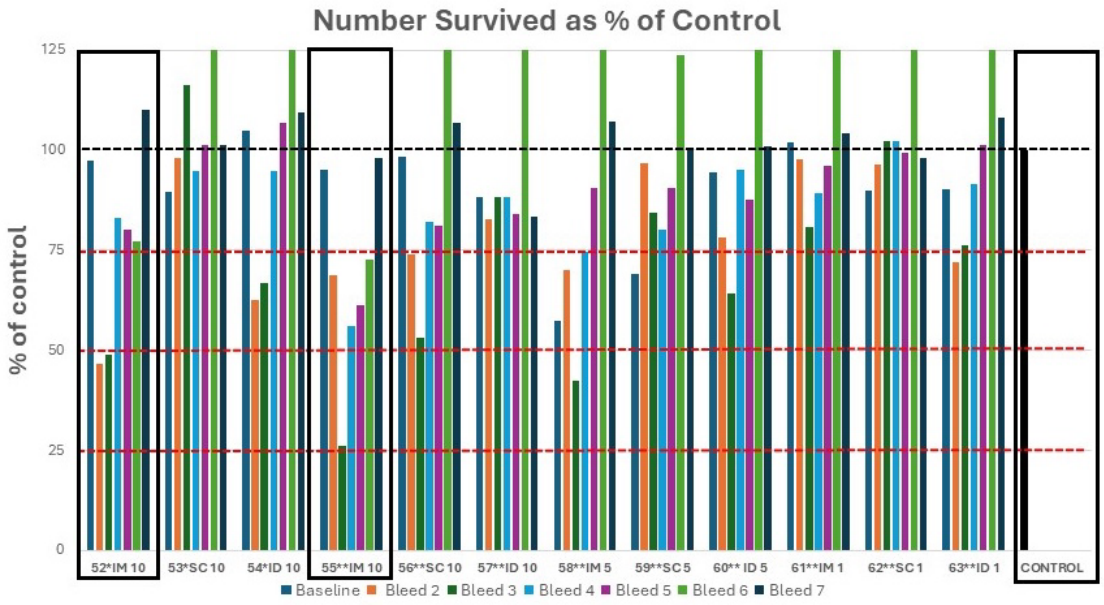
In vitro larval feeding and growth bioassay assessment was performed on the sera from the trial groups for the initial 6-month period following the single or double vaccination performed. Sera was collected each month over this time period and assessed. Assay measurements of growth associated with mean larval weight, larval biomass and larval survival were all normalised to the control group data for these parameters. Results are presented in Figure 41.

In vitro assessment of Trial 8 Type N vaccine Dose and Longevity of Response

A)



B)



C)

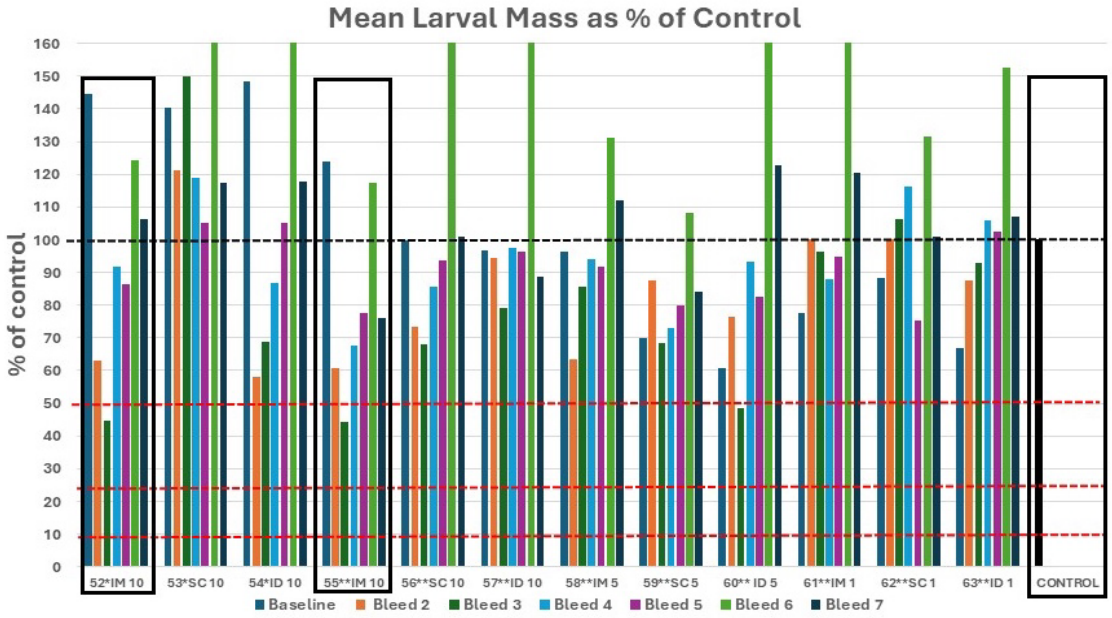


Figure 41: *In vitro* larval feeding and growth bioassay results of Trial 8 groups up to 6 months after initial vaccination of 'Type N' vaccine. Depot (IM/SC/ID) and dose (1 or 2 injections) and antigen quantity regime (10/5/1/0mg antigen) of vaccinations are shown. Results are shown normalised as a percentage of the Control group for each timepoint. Boxed data indicate groups progressed to longer term observation and vaccine boost at 11 months.

Assessment of the prolonged immune response identified Groups 52, IM one dose and 55, IM 2 dose vaccinated as the best performing groups with respect to antibody titre and *in vitro* growth assessment. These two groups and the control group were selected for a prolonged study up to 11 months. At 11 months, Groups 52 and 55 were given a single dose booster vaccination and the groups monitored with ELISAs and *in vitro* feeding assays for a further 6 months. Results show that whilst the peak antibody titre was not achieved until approximately 4 months after the initial vaccination, the most significant effect in growth inhibition of the larvae *in vitro* was observed in the initial 1-2 months of vaccination. This highlights a discord between measured peak antibody titre and most significant *in vitro* growth inhibition effect observed. The extended trial with a boost vaccination at 11 months post initial vaccination for Groups 52 and 55 showed a renewed and elevated boost in antibody titre exceeding that measured after the initial one or two vaccinations 11 months prior. This elevated antibody titre also coincided with a renewed effect on larval growth when tested in *in vitro* larval feeding and growth trials. Whilst growth effect had diminished significantly by month 11, it was quickly reinstated with a single boost vaccination. This data supports the concept where an initial dual vaccination followed by an annual single boost vaccination may be possible and practical for a refined flystrike vaccine formulation in the future.

Antibody assessment at strike site

A significant immune response for the 'Type N' and 'Type R' vaccine was consistently measured and this correlated with *in vitro* inhibition of larval growth. The same however could not be demonstrated for *in vivo* on sheep effects on larval growth. To better understand what was happening with respect to antibody exposure of the larvae at the skin, i.e. host-parasite interaction site, serous exudate present at the skin surface was collected from active larval implant sites on sheep. We used a trial group sheep vaccinated with the Dros S2

produced Peritrophin E antigen where larvae had been implanted and fed for 48 hours. In addition, serous exudate was collected from debrided skin approximately 20-30cm away from the larval implant where there was no larval activity. The serous exudate collected was assayed for the presence of vaccine induced antibodies specific to the Dros S2 Peritrophin E antigen. This was also performed on the Control trial group sheep that received adjuvant only injections. All sheep had received intramuscular injections of the vaccine in the upper muscular region of the hindlegs. Vaccine was administered three separate times at 4 week intervals and assayed 4 weeks post final injection. Figure 42 shows that antigen specific antibodies at the larval implant site and the adjacent non-larval implant were detected in all animals that received various adjuvant formulations of the Dros S2 Peritrophin E antigen. The Control no antigen vaccination group returned a negative background only result. The level of antibody detected at the skin surface was low and corresponded to approximately 1:10 of the antibody titre measured in the circulating blood serum. This demonstrates antibodies can be delivered to larvae feeding on the skin but at levels an order of magnitude less than circulating in the blood. For the vaccine to work effectively, the levels of antibody delivered at the skin surface where the larva feed must be at least 10-fold higher. The critical next steps now are to explore methods of vaccine delivery and formulation that will enhance the antibody levels at the skin surface. This may be achieved by enhancing humoral immune response through IM vaccination or activating immune response mechanisms in the skin through transdermal delivery of the vaccine, better vaccine formulation through use of optimised adjuvants and through antigen optimisation of critical parameters including post translational modifications associated with glycan representation.

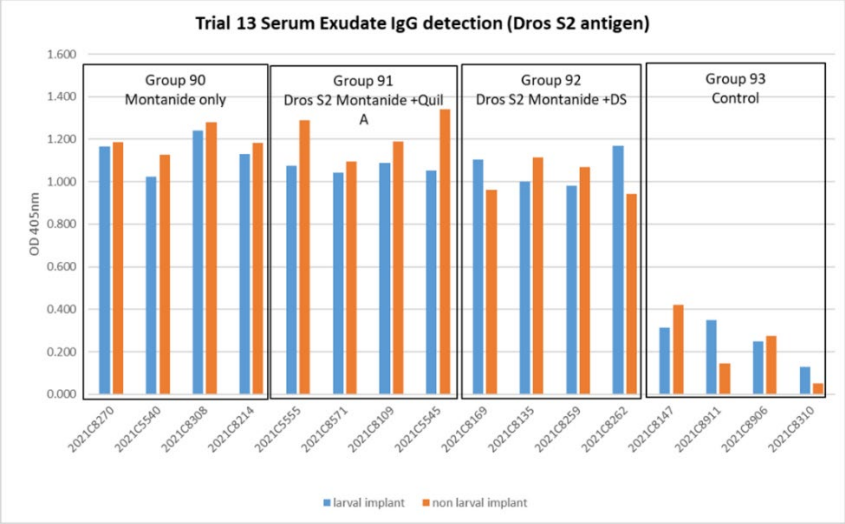


Figure 42: ELISA antibody detection for the presence of Dros S2 Peritrophin E antibodies (IgG) on the sheep skin measured from *in vivo* larval implants and regions where no larval activity was present.

DISCUSSION

Flystrike caused by the sheep blowfly *L. cuprina* continues to be a significant health and wellbeing threat to the sheep industry in Australia, costing more than $320 million annually [5]. With mulesing being in the spotlight for sheep welfare issues and the evolving increase in insecticide resistance, new avenues of research to reduce flystrike are needed.

One way to reduce flystrike occurrence is to reduce susceptibility through the integration of genetics into the Australian sheep wool flock of plain body, reduced body wrinkling and bare breech sheep using genetics and selective breeding [14]. Another way to controlling insect parasites may lie in vaccines. As described in the literature review CSIRO has previously demonstrated that an immune response to certain blowfly larval antigens could be achieved through administration of prototype vaccines resulting in. some degree of efficacy in reducing larval growth. The issue became that the repertoire of potential antigens was quite limited and the technical ability to produce these antigens effectively and at scale was not practical at the time. Recent advances in genome sequencing, DNA engineering, and protein production have allowed for higher throughput and faster vaccine antigen discovery. Here we discuss the research we have recently undertaken to identify additional vaccine antigens, their production and *in vitro* and *in vivo* testing. We conclude by describing the next steps required in developing an effective and commercial vaccine for the sheep industry.

In **objective 1 and 2**, we applied a reverse vaccinology approach whereby candidate protein antigens were identified for prospective vaccine development by exploring the sheep blowfly genome using data we produced by performing transcriptomics on *L. cuprina* across different life stages and from different key tissues including the larval cardia, midgut, and salivary glands. Genes encoding secreted proteins were identified with a focus on their level of gene expression and potential physiological role. A condensed and refined list of prospective proteins were selected for further vaccine antigen development and testing.

Informed by previous research undertaken on flystrike vaccine development, whereby proteins associated with the salivary gland and cardia/anterior midgut secretome were demonstrated to be amenable to vaccine targeting we further pursued these for ongoing expanded investigation. Focus of the current research project was on these tissues as the tough cuticle of the larval carcass and lining of the foregut and hindgut forms a barrier impervious to antibody targeting. The midgut on the other hand does not possess a cuticle/chitin lining but is protected by a semi-permeable membrane structure called the peritrophic matrix that has shown significant promise in antibody targeting. This matrix, produced solely by the cardia and flanking anterior midgut cells has been the central target for our vaccine development.

We identified a subset of 6 specific protein families expressed in neonate larvae at strike establishment, in the cardia/anterior midgut and in salivary glands during early to late larval lifestage growth that we concentrated our selection on for vaccine antigen development. These include the Peritrophins, Peritrophin-Mucins, Chitinases, Serine Proteases and CRiSP/non venom peptide-like proteins. Candidates from each of these protein families were selected, engineered, produced, and tested as recombinant protein antigens in prototype vaccine formulations.

In **objective 3**, we took the selected candidate antigens which possessed signal peptides for secretion and were abundantly expressed, performed protein design, engineering, and produced the recombinant proteins in bacterial and insect cell lines. We predominantly focussed on insect cell line production and engaged with University of Queensland Protein Expression Facility (PEF) to undertake this activity and produce candidate antigens for us to test in prototype vaccine formulations.

The insect cell lines used included lepidopteran cell lines (High Five™ and Sf9) and Drosophila (S2) cells. Bacterial production was not used extensively due to inability of those cells to undertake the requisite post translational modification of the proteins. A majority of candidate protein antigens investigated had complex folding and associated N-linked and in some cases O-linked glycosylation. This made the *E. coli* recombinant protein produced not generally fit for purpose for generation of a relevant and targeted immune response by the sheep. The lepidopteran cell lines were chosen as the principal vehicle for production of recombinant proteins with the potential for downstream commercial and scalable production. These cells possess the requisite post translational machinery to fold and potentially glycosylate proteins and secrete them for harvest and purification. What was not known at the time was whether the relevant glycan structures associated with the sheep blowfly larval proteins were able to be reproduced by these cell lines and is a parameter under current and ongoing investigation.

In **objective 4**, we produced a native antigen cocktail referred to as 'Type N' vaccine formulation derived from the larval PM for benchmarking against the recombinant vaccine performance in sheep vaccine trials. The native antigen cocktail was produced by culturing *L. cuprina* larvae and isolating the extruded PM shed from the gut as a source of the native antigen. We demonstrated that vaccinating with crude PM significantly reduced larval growth and survival in *in vitro* larval feeding and growth bioassays by ~75%. This result was highly encouraging and suggests that ongoing investigation of a native antigen cocktail vaccine is warranted.

In **objective 5**, we tested the efficacy of recombinant candidate antigen vaccine formulations using both *in vitro* and *in vivo* models. The repertoire of vaccine antigens tested returned good immunological responses to the Montanide ISA61VG™ water-in-oil formulation, intra-muscular, three dose regime undertaken in the standard protocol trials. The ELISA analysis of the immune response consistently demonstrated that peak antibody titre was achieved following the second vaccination with the third immunisation having negligible effect on antibody titre increase. After determining an immune response had been raised, we next measured the larval growth and survival using an *in vitro* bioassay method. We found that peritrophins and peritrophin-mucins performed the best with respect to immune response and larval growth inhibition effects. We developed a multi-antigen vaccine ('Type R'), combining several of the peritrophins and peritrophin-mucins antigens into a single vaccine. The 'Type R' multi-antigen vaccine displayed a significant and substantial reduction in growth of up to ~ 75% in *in vitro* trials. An interesting observation was that formulation of the 'Type R' vaccine with chitosan, a soluble form of chitin, resulted in enhanced efficacy of the recombinant antigen vaccine. The mechanism of this may be through additional adjuvant effect of the chitosan or through enhanced conformation presentation of the antigens though their intrinsic role in binding to chitin. This conformation effect has not been able to be proven or resolved to date and chitosan remains an important component in progressive formulation and testing of the vaccine formulations. We found that testing the components of the 'Type R' vaccine as individual or dual antigen vaccines did not produce as an effective larval growth inhibition result as the multi-antigen formulation did. If a commercial vaccine is to be manufactured and for it to be economically viable, it is likely that the fewer vaccine antigens required would be the ideal. Work is needed and is commencing to better characterise and optimise the recombinant antigens with the aim to improve and simplify development of a 'Type R' vaccine in the future.

No significant progress was made with the recombinant vaccine antigens classified as Chitinases or Serine proteases. Whilst immune responses were raised to these recombinant proteins, this translated to only minor effects on larval growth or survival when tested using *in vitro* assays. A ~20% decrease after 48 hours growth for the insect cell produced Chitinase protein and a range of 2-28% for the five *E. coli* produced Serine Protease proteins after 24 hours growth in mean larval weight was observed. Whilst the concept of compromising peritrophic membrane formation and remodelling by knocking out or altering chitinase activity was the

foundation for pursuing this protein, the results from the *in vitro* larval assays suggest that immunological targeting had little to no effect or that the chitinase enzyme was protected from immunological targeting due to its secreted location being on the extra- luminal side of the peritrophic matrix. The Chitinase candidate was hence not investigated further at this stage but could be considered for alternative targeting approaches. The Serine Proteases investigated, being the most highly abundant forms of this multi-gene family, whilst likely amenable to antibody targeting through their secretion into the gut and peritrophic matrix lumen were not progressed. The minor efficacy in larval growth reduction and the potential for compensation in serine protease activity by the other 120+ serine protease family members were the key reasons for this.

The two CRISP proteins tested as insect cell produced recombinant protein antigens produced good immune responses and up to a 50% reduction in larval growth *in vitro*. This family of salivary gland produced proteins of which there are 26 annotated members, remain an interesting class of proteins to better characterise and test in the future.

We also performed vaccine trials using the native antigen cocktail, 'Type N'. While 'Type N' significantly reduced growth when assessed using *in vitro* bioassays, when tested *in vivo* on sheep, only limited reduction in larval growth was observed. Finally, we used the 'Type N' vaccine to test single and twin dose immune titre longevity and larval growth inhibition response in sheep. This was assessed with *in vitro* larval feeding assays and monitored antibody levels with ELISA assays. We found that growth effects on larvae were strongest within the 4-week period after the 1st or 2nd vaccination and slowly diminished over a 6 to11 month period. At month 11, optimal antibody titre and growth inhibition effects *in vitro* was quickly reinstated with a single boost vaccination. These results suggest that an initial 2 dose vaccination followed by a single annual booster vaccination may be possible and practical for a refined flystrike vaccine formulation in the future.

We have found both historically and recently that native proteins either individually purified or as a tissue extract protein cocktail derived from PM generally perform better in producing a protective immune response in sheep than recombinant proteins based on these proteins. We aim to investigate several approaches to expand our understanding of this effect and to better characterise the blowfly larvae PM and the target antigens we have concentrated our efforts to date on. To expand on this work, we will seek to better understand the structure of the PM and quantitate relative abundance and structural modifications using proteomic and glycomic approaches. Another way to expand on this work is to focus on the 'Type N' vaccine production. The 'Type N' vaccine is currently produced using non-serum media, including yeast, milk, and wheat germ to allow cheaper and quicker PM culturing. Producing the PM using 'sheep like' media; sheep serum or liver, is showing promise in producing a more flystrike relevant PM, that should help increase 'Type N' vaccination efficacy.

Previous work in 2001 showed that the inhibitory effect of native peritrophin-95 was associated with antibodies targeting not only the polypeptide but also the glycans [34]. This previous work highlights the importance glycosylation (decorative sugars) has on creating a protective immune response. Since 2001 the analysis of glycosylation through glycomics and glycoproteomics has significantly advanced. We have recently developed an integrated glycomics and glycoproteomics platform and this capability is allowing us to measure glycans present on candidate antigens both from native PM and from their recombinant antigen counterparts. If the glycan structures present on antigens is critical, this will enable us to determine if they are different between natively produced and recombinant antigens and help inform future strategies in recombinant protein antigen production.

There are many factors that contribute to producing a protective immune response in a vaccine, including adjuvants and delivery systems. Here we have predominantly used Montanide ISA61VG as the primary adjuvant for our vaccine development due to its ability to help illicit a good humoral immune response, low cost, and

potential use in livestock vaccines. However new adjuvant technologies are becoming more common in human health which may be translated and have significant benefits in livestock vaccines. One of these technologies is liposomes, which are spherical vesicles that can be used to both transport antigens and create a strong immune response without negatively affecting the host. To expand on this work here, we would utilise a liposome adjuvant and compare it to traditional adjuvants to see liposome technologies can improve the protective immune response. Here we have performed predominantly intramuscular route of delivery of the prototype vaccines with some limited subcutaneous and intradermal delivery. As flystrike is a skin-based issue, with blowflies physical scratching and feeding at the surface of the skin, potentially an optimised dermal delivery approach could be a more effective approach. Dermal patch or gas driven dermal delivery devices are being investigated for vaccine or therapeutic delivery and is an approach that could be explored for flystrike vaccine delivery. The epidermis and dermis have been shown to be effective vaccination sites, rich in antigen-presenting cells [78,79], and shown to be advantageous for dose-sparing and thermostability of vaccines, highlighting that the skin might be an ideal vaccination site [82 77,80,81].

With the continued development of resistance to insecticides and phasing out of some husbandry practices, the key to controlling insect parasites may lie in vaccines. Designing vaccines is multifaceted and extremely difficult but this is an area of scientific endeavour that has seen a resurgence of research activity based on the increasing need for effective vaccines and innovations through development of new technological approaches. We have shown here that recombinant antigen production does not always produce a good protective immune response. A better understanding of how the sheep blowfly *L. cuprina* glycosylates proteins and the development of vaccines that specifically target important glycan structures is critical to producing an effective vaccine against flystrike. In addition, deployment of new adjuvants and delivery methods, such as liposomes and dermal vaccinations, could further improve vaccine efficacy. The future of flystrike and endo- and ecto-parasite vaccines may lie in glycosylation, novel formulation approaches and mucosal or skin vaccine administration, helping innovate livestock vaccine technologies into the 21st century.

IMPACT OF WOOL INDUSTRY – NOW & IN 5 YEARS' TIME

Current estimates place the total economic costs of sheep blowfly control at up to $320 million per annum. The development of a flystrike vaccine if successful will have substantial economic, environmental, and welfare benefits for the sheep wool and meat industry. Current control measures for flystrike are associated with a range of issues that a vaccine will help overcome. These advantages will assist in industry acceptance and market uptake of the vaccine. The substantial annual and ongoing cost to the sheep industry reinforces the need for development of a long lasting and cost-effective vaccine. The vaccine will be of significant interest to all sheep wool and meat graziers and of commercial interest to a VetPharma company. The research efforts to develop a flystrike vaccine are well positioned to make significant market impact, secure a strong IP position, and potentially generate a strong return on investment. The use of insecticides is the mainstay approach to current control of flystrike however insecticides have a limited lifespan once applied and may need to be reapplied multiple times throughout the season to maintain efficacy for controlling flystrike. The ability of sheep blowfly to acquire resistance to insecticides is a key issue and has resulted in an increasing number of insecticides becoming ineffective. In addition, insecticide residues in the environment, wool, and meat, are significant problems associated with their continual use. Mulesing, an effective but painful and distressing procedure to the sheep, whereby skin is surgically removed from the breech and tail region, usually without anaesthetic, is vehemently opposed by animal welfare groups and has resulted in negative marketing issues for the sheep industry. The wool industry had aimed to stop mulesing by 2010 but still has a substantial way to go before the practice can be totally withdrawn from use. Other initiatives, including the use of skin clips to remove wool growing skin around the breech and tail in a less distressing way than mulesing, are used by some of the industry but have not been widely adopted. Breeding of Merino sheep with non-wrinkled skin and bare breeches is being continually pursued by many operators in the industry, but this genetic approach will take many years and will be difficult to achieve complete elimination of wrinkled skin traits in the wool production population. The requirement for new generation flystrike control strategies, and the significant economic value of this market, make the development of a cost-effective efficacious flystrike vaccine an attractive commercial option. A flystrike vaccine will provide whole animal protection, be painless to the animal, eliminate the welfare issues associated with mulesing, will have the potential of being long acting and have no adverse environmental impacts. In addition, vaccines are traditionally long-lived and don't generally suffer resistance problems as is prevalent for insecticides. A flystrike vaccine represents an attractive and critical addition to the sheep industry suite of control strategies for flystrike control in the longer term as other tools become less effective, unable to be used and redundant. The industry needs to maintain focus on developing novel new and acceptable strategies that will effectively control flystrike on sheep. Significant progress has been made in better understanding the biology and genetics of the sheep blowfly through sequencing of the fly's genome and characterisation of the genetic diversity found across Australia where sheep are grazed. This information, together with gene and protein characterisation undertaken at the larval tissue and lifestage level has allowed progress in development of a vaccine to combat this parasite. We have developed a suite of potential candidate antigens in native 'Type N' and recombinant 'Type R' form and demonstrated a high degree of efficacy of some formulations of up to 75% reduction of larval growth in laboratory *in vitro* bioassay assessment. The next stage of the project will be to develop a more comprehensive understanding of these vaccine antigens, enhance their isolation or production to improve their efficacy and fine tune formulation and delivery approaches. There is a key piece of the puzzle relating to recombinant antigen production associated with correct post translational modification (glycosylation specifically) of the cultured proteins that needs to be resolved. We feel that overcoming this roadblock in effective antigen production will greatly assist or solve the problem we have had in optimal recombinant antigen production. This coupled with better formulation and delivery will be conducted over the next 3 years should lead to demonstration of effective *in vivo*, on sheep

efficacy of our prototype vaccine leads. At this stage we envisage moving into small field trials and regulatory testing in combination with industry and animal health company partnering. A commercial vaccine is likely ~7 years away but we will know categorically within the next 3 years whether the approaches taken will lead us to the goal of an effective flystrike vaccine for the Australian sheep wool and meat industry.

CONCLUSION & RECOMMENDATIONS

Flystrike is one of the greatest challenges facing the sheep livestock industry, it is a significant threat to the health and wellbeing of sheep and costs in more than $320 million annually to control [5]. Resistance to insecticide is becoming more and more common and mulesing is becoming less and less accepted. The key to controlling flystrike and other ecto- and endo-parasites may lie in vaccines. Developing vaccines to combat ecto- and endo-parasites is difficult due to the complexity of insects and nematodes. Here we have shown that vaccines derived from extracted *L. cuprina* proteins, and some insect cell recombinant proteins can be used to produce an immune response with varying degrees of efficacy when tested *in vitro*. However, the recombinant antigens produced in standard insect cell lines appear to be sub-optimal individually and do not produce the degree of protective immune response required. We believe a better understanding and replicating how *L. cuprina* glycosylate their peritrophic matrix through larval on-sheep growth phase is critical to producing an effective vaccine against flystrike. This will drive flystrike vaccine development to the crucial next stage. We have also highlighted the need to investigate the use of highly immunogenic adjuvants and novel skin delivery methods to assist in producing the most effective vaccine possible. We feel through the critical support of sheep producers through AWI research funding that substantial gains have been made in the incremental progress to developing a flystrike vaccine. The future of flystrike control and the Australian sheep industry will benefit from continued support of research activities associated with flystrike control in vaccines, glycosylation, and novel technologies, transforming and supporting the industry well into the 21st century.

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LIST OF ABBREVIATIONS AND/OR GLOSSARY

CSIRO	Commonwealth Scientific and Industrial Research Organisation
CSIR	Council for Scientific and Industrial Research
AWI	Australian Wool Innovation
MLA	Meat and Livestock Australia
AGRF	Australian Genome Research Facility
PM	Peritrophic Matrix
NGS	Next Generation sequencing
EU	European Union
Rdl	Resistant to dieldrin
HPV	Human papillomavirus
RPKM	Reads Per Kilobase of transcript per Million mapped reads
CRiSPs	Cysteine Rich Secretory Proteins
PEF	University of Queensland Protein Expression Facility
UoM	University of Melbourne
PBS	Phosphate-Buffered Saline
PVDF	Polyvinylidene Difluoride
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
IMAC	Immobilized Metal Ion Affinity Chromatography
NSW	New South Wales
IgG	Immunoglobulin G
ELISA	Enzyme-Linked Immunosorbent Assay
OD	Optical Density
PM-A	Peritrophin-Mucin A
PM-B	Peritrophin-Mucin B
PM-C	Peritrophin-Mucin C
P-CD	Peritrophin Candidate CD
P-E	Peritrophin E
P-F	Peritrophin F
M-D	Mucin D
BCA	Bradford Colorimetric Assessment

ID	Intradermal
IM	Intra-Muscular
SC	Sub-Cutaneous
PTM	Post-Translational Modifications
TEV	Tobacco Etch Virus
PMF	Peptide Mass Fingerprinting
CBD	Chitin Binding Domain
MGD	Mucin-like Glycosylated Domain
SPR	Surface Plasma Resonance
AEEC	Animal Experimentation and Ethics Committee
AQIS	Australian Quarantine Inspection Service
OGTR	The Office of Gene Transfer Regulator