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Control of Fleece Rot and Lumpy Wool by Vaccination: Feasibility Review and Options

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Abstract

Fleece rot and lumpy wool develop following prolonged wetting of sheep when bacterial proliferation in wool and on skin induce exudation of serum proteins onto the skin surface, and damage to wool follicles and fibres. These processes create an attractive environment for blowflies to lay eggs, leading to body strike. Current reliance on insecticides for prevention and treatment of body strike and breech strike is being increasingly challenged by development of insecticide resistance. This review examines the large body of past research on the bacterial causes of fleece rot and lumpy wool, the genetics of sheep susceptibility and resistance, the characteristics of the resulting immune defence reactions, and attempts to control fleece rot and lumpy wool by vaccination. Limitations associated with the technologies available at the time for these past vaccination studies and knowledge gaps are identified. With increasing adoption of genetic selection for breech strike resistance and new breech modification technologies, the *relative* importance of body strike as a cause of disease and death of sheep may increase in coming years. Due to the limited geographic distribution and the availability of management solutions for controlling lumpy wool, it is suggested that fleece rot is the more important target for a new vaccine. Development of a vaccine requires identification of 1) relevant antigens, 2) a protective immune defence mechanism, 3) a clinical measure of vaccine efficacy, and 4) a strategy for vaccine use that is practical for producers while addressing epidemiological characteristics of the disease in the field. Detailed strategies to address these challenges in developing a new vaccine to reduce the incidence of fleece rot and associated body strike are described.

1. Executive summary and recommendations

Fleece rot and lumpy wool are the result of bacterial infections on the skin and in the fleece of sheep that induce exudative dermatitis, wool damage, and predispose sheep to fly strike on the body. A large quantity of research has examined causes, the inheritance of sheep susceptibility and resistance, characteristics of the resulting innate and adaptive immune responses, and the potential of vaccines for controlling these diseases. This report assessed past research and recent technological advances that together create new opportunities for the development of immunological strategies to control fleece rot and lumpy wool through vaccination, with potential beneficial consequences for reducing breech strike and body strike. The report also assessed the commercialisation opportunities for fleece rot and lumpy wool vaccines.

Skin provides a defensive barrier against microbial invasion. Like other body surfaces, it is populated by microbiota which co-exist in a symbiotic host-microbe ecology that can be disrupted by adverse environmental or host-related conditions. Disruption of the skin barrier function, typically by sustained wetting due to rain, is characterised by a sequential cascade of events involving inflammation, bacterial proliferation, and serum exudation. During the inflammatory dermatitis, odorant attractants for gravid female blowflies are produced directly by metabolic activities of bacteria and following degradation of wool and skin by bacterial enzymes, which lead to increased risk of blowfly strike. Although differences exist between fleece rot and lumpy wool in these processes, the prevention of these diseases in sheep by vaccines is anticipated to generate the additional benefit of decreasing the incidence of fly strike. This outcome will help to offset the increasing incidence of insecticide resistance in blowflies.

1.1 Fleece rot

Sustained wetting of sheep for several days leads to proliferation of bacteria on fleece. Conventional bacterial culture methods reveal a dominance of *Pseudomonas species* including *P. aeruginosa*, *P. maltophilia*, *P. putida*, and *P. stutzeri* as well as *Bacillus subtilis* and much lower numbers of other bacterial species. An underlying premise in past studies was that bacterial prevalence equates to significance as a cause of disease. *In vivo* application of *P. aeruginosa* to clean fleece sites on healthy sheep that were then subjected to continual wetting developed fleece rot thereby supporting a causal role for *P. aeruginosa*. *P. maltophilia* is also found on breech skin during urine stain. Enzymes and other exoproducts, including exotoxin A and phospholipase C, damage wool follicles and wool fibres while pyocyanin and other pigmented bacterial products discolour wool. In severe cases, serum exudate forms a crust in wool which is less pronounced and of a less scab-like character in fleece rot than in lumpy wool. Fleece rot usually resolves spontaneously when wool and skin dry after the period of sustained wetting. The bacterial infection in fleece rot is non-invasive; nonetheless, sheep develop antibodies to bacterial exoproducts during a typical case of fleece rot. The contribution of these antibodies to the resolution of fleece rot is unknown. Fleece rot prevalence is very strongly influenced by the occurrence of adverse wet weather events and can affect a large proportion of some flocks in extreme outbreaks.

Susceptibility of sheep to fleece rot is influenced by heritable (genetic) factors that are associated with body conformation, wool and skin characteristics and perhaps also some immune functions. The sporadic expression of fleece rot limits potential for direct selection of sheep for resistance and has focussed attention on indirect selection for favourable wool characteristics. Divergent genetic selection lines established by NSW DPI at Trangie have revealed differences in wool characteristics and immune functions that may contribute to resistance. Resistant sheep generate higher levels of antibody to *Pseudomonas* antigens during infection and

following experimental vaccination. Nonetheless, acquisition of natural immunity to fleece rot from infection in the field appears to be relatively low, and the mechanisms of acquired (partial) immunity have not been confirmed.

Experimental vaccines were studied by several groups in the 1980s and early 1990s and two patents filed. Vaccination studies did not comprehensively examine a wide range of bacterial isolates, however in one study a single *P. aeruginosa* isolate protected sheep against experimental challenge with three *P. aeruginosa* isolates. The "Burrell" patent specified a multivalent bacterin vaccine containing killed bacterial cells and culture filtrate from *Pseudomonas spp aeruginosa*, *maltophilia*, *stutzeri* and *putida*. In a field trial of the vaccine, there was reduced incidence of fleece rot and body strike in vaccinated sheep. The mode of action of the vaccine was claimed to be antibody directed against bacterial exoproducts providing protection against fleece rot and indirectly, from body strike. The duration of antibody response to vaccination was not reported. Studies by Chin and colleagues at NSW DPI found phospholipase C (a likely virulence factor) secreted from *P. aeruginosa* to be highly immunogenic and led to lodging of a vaccine patent. However, field trials of the vaccine were not reported.

1.1.1 Limitations of past fleece rot studies

Despite the demonstration more than three decades ago that an experimental vaccine using an antigen derived from the soluble material (exoproducts) secreted from specific *Pseudomonas* species could partially protect sheep from fleece rot and fly strike, the licencing of the vaccine to an Australian biotechnology company did not progress through a complete commercialisation phase. Historical technical limitations and commercial factors contributing to this failure (summarised below) include poorly defined antigens, a poorly identified mechanism of immune defence, poor characterization of causal bacterial dynamics, and unclear cost / benefit structure of the commercial environment. Since that time, there have been transformative technological developments and radical commercialisation changes that now create new opportunities for vaccine production. Four specific limitations of past vaccine efforts are detailed below. These are inferences drawn from informed comments, relevant scientific publications and current scientific knowledge. These limitations provide guidance and focus for future efforts.

(i) ***Poorly defined antigens.*** In the previous studies, the vaccine antigen derived from a specific *Pseudomonas* species was complex, poorly defined and may not have been reproducibly produced. The antigen is the core of a vaccine. The *Pseudomonas* antigen that was used in the vaccine was derived from soluble material secreted by *Pseudomonas* grown in culture. The rationale behind the design of the vaccine was that components in the soluble material may be essential for the infectivity of the *Pseudomonas* species in fleece rot and therefore antibody-mediated neutralisation of these components by the immune system would prevent bacterial proliferation and therefore the development of fleece rot. It is now known that the secreted components from bacterial culture may be difficult to reproducibly generate in the laboratory and they may be substantially different from what occurs in a field infection. The complexity and stability of these components may have limited vaccine efficacy. A more precisely defined antigenic fraction of the secreted material or a specific component(s) could decrease vaccine complexity, enhance stability and possibly improve vaccine efficacy. There are currently considerable new technologies that could hasten the identification of specific vaccine antigens.

(ii) ***Poorly characterised mechanism of immune defence.*** It is likely that the mechanism of action of the vaccine was mediated by the presence, in exudate on the skin, of specific antibodies raised against crucial infectivity factors present in the secreted material from *P. aeruginosa* culture. Thus, one key determinant of efficacy of the

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vaccine was likely to be the quantity of antibody in the exudate, which is a function of 1) the vaccine adjuvant (an agent in the vaccine that helps to produce a stronger immunological response against the vaccine antigen), 2) the leakage of antibody from blood into the serous exudate, and 3) the stability of the antibody in the exudate. Identification of the immune defence mechanisms conferring protection provides clarity to vaccine design. For instance, vaccine technology can now markedly increase the quantity of antibody generated. (The patents describing the vaccine also alluded to versions of the vaccine that additionally included extracts of whole *P. aeruginosa* bacteria, which may, in their own right, have adjuvant activity, however no results were presented.)

(iii) ***Identity of causal bacteria.*** Multiple *Pseudomonas* species are implicated in the generation of fleece rot although *P. aeruginosa* is the dominant species. In addition, each species has substantial strain diversity. The extent of the importance of this bacterial diversity in the past vaccine is unknown. The vaccine may not have protected sheep from all of these strains and *Pseudomonas* species due to conformational variants in the vaccine antigens. There have now been massive technological changes in multiple areas that allow precise identification of the diversity of bacterial species and strains in a natural complex mixture and improvements in the utility of this information in vaccine design. Identification of causal bacteria will help clarify why acquisition of natural immunity to fleece rot infections appears to be poor. For instance, bacterial strain variation between episodes of fleece rot could result in immunity acquired from an earlier infection providing little protection during subsequent infections. A vaccine encompassing relevant antigenic heterogeneity could protect against this bacterial diversity.

(iv) ***Unclear cost / benefit of commercial trials.*** Commercialisation of the past vaccine may have been compromised by the extent of vaccine efficacy in field trials, the cost and complexity of vaccine manufacture, and the presumed need for broad efficacy against the *Pseudomonas* species and strains that were likely to be encountered in field infections. The main aim of the vaccine was to indirectly diminish fly strike risk but there was only limited demonstration of this capability in field trials. It was also unknown whether the vaccine decreased both body strike and breech strike or just the former, and whether bacterial diversity adversely affected vaccine efficacy. Another limitation may have been the market size at the time. This was likely relatively small, as the sheep industries at that time were not subject to the current export market pressures associated with control of fly strike. A significant issue at the time was also the structural and financial difficulties of the commercial partner.

1.2   Lumpy wool

Lumpy wool is an infectious exudative dermatitis of sheep skin characterized by crusting and matting of wool resulting in lower wool value and lower yield per animal and increased risk of blowfly strike. The incidence is highly variable and primarily dependent on a wet and warm period of weather. The causative bacterial agent is *Dermatophilus congolensis,* which is spread between sheep by contact transmission. The bacterium can infect many animal species but particularly livestock and in rare instances, humans. *D. congolensis* has a complex life cycle with one stage, the hyphae, able to penetrate the outer layer of skin. Repeated infection and natural clearance of the bacterium occur in sheep. Currently, lumpy wool is managed by a combination of standard hygiene practices, isolation of affected sheep, self-healing of sheep, and treatment of severely infected animals with antibiotics. There is little or no information available about *D. congolensis* strain variation or antibiotic resistance.

There is some evidence for naturally acquired immunity as older sheep are less susceptible. However, this effect could also be due to wool follicle and immune system maturation. Experimental vaccinations of sheep with antigens derived from specific *D. congolensis* life stages produced antibodies to these antigens in sheep sera and in washings of the skin from areas of the fleece affected by lumpy wool. However, it is unclear whether these antibodies were protective. Vaccination of sheep with antigens from the (poorly defined) filamentous life stage of *D. congolensis* resulted in fewer and less severe incidences of lumpy wool in some experimental trials. The inconsistent results were attributed to strain variation of *D. congolensis* as the isolate used for isolation of antigens in the vaccine was different from the challenge isolate.

1.2.1 Limitations of past lumpy wool studies

The experimental results from vaccination trials suggest that some protection of sheep from lumpy wool can be induced. However, there is a need for more precise identification of the protective antigens used in the vaccine and elucidation of the mechanism that underlies the effects of the vaccine on lumpy wool incidence and severity. A major issue limiting vaccine efficacy is likely to be *D. congolensis* strain variation, the extent of which is currently unknown. The use of antibiotics to control *D. congolensis* in severely affected sheep may be a strong factor influencing the range of strain variation in the field. Moreover, there may be strains of *D. congolensis* resistant to antibiotics that should be monitored from the perspectives of livestock and human health. New technologies are well suited to answering these issues.

The currently used measures to control lumpy wool, which include management practices, self-healing of sheep and antibiotic treatment in severe instances, in combination with the limited geographical occurrence of the disease, indicate that the market size for a stand-alone vaccine is relatively small. One possibility is that a vaccine preventing lumpy wool in sheep could be incorporated into a fleece rot vaccine and/or a blowfly strike vaccine.

1.3   Skin immune responses

The results of several investigations of fleece rot and lumpy wool focus attention on antibody in skin as the mode of vaccine protection, especially for fleece rot. Immunoglobulins (antibodies) are present in skin washings, and small quantities of the antibody isotypes IgA and IgM are actively transported into skin secretions by the polymeric Ig receptor. No mechanism for active transport of the IgG1 and IgG2 isotypes onto the skin surface of sheep has been identified. IgGs are more effective than IgA and IgM at neutralizing bacterial exoproducts. It has been shown that local antibody production can be induced in the skin of sheep, however the importance of local production to effective antibody-mediated vaccine protection is unknown. Furthermore, induction of local antibody production throughout the whole of the fleece-bearing skin of sheep may not be feasible for practical reasons. Conventional vaccination technologies administering relevant antigens subcutaneously with a potent adjuvant to provoke high levels of IgG antibodies are likely to be appropriate for vaccine induced protection.

1.4   Lessons from human biomedical research

In human medicine*, P. aeruginosa* is a very serious cause of morbidity and mortality in cystic fibrosis, burns patients and many other infections of mucosal and skin surfaces. Multidrug resistance makes control of *P. aeruginosa* infection with antibiotics extremely difficult in humans. The mechanics of immune defence in the lungs of cystic fibrosis patients make burns infections a more relevant source of information than cystic fibrosis for application to fleece rot research. Extensive human vaccine studies over many years have failed to produce commercial vaccines although there are several candidate antigens that are untested or incompletely tested.

Keratinocytes (which are the principal cells comprising the skin epidermis) are a source of antimicrobial peptides such as the defensins. The gene expression profile of the epidermis, like many other tissues, undergoes epigenetic changes during an episode of strong activation, such as during *Pseudomonas* colonisation, leading to a sustained capacity for enhanced reactivity during subsequent disease challenge episodes. The contribution of such "acquired non-immunological cutaneous defence memory" to resistance to bacterial dermatitis has yet to be examined in sheep.

1.5   Technical challenges for vaccine development

Development of a new vaccine involves at least four major stages that need to be undertaken re-iteratively: 1) identify appropriate antigens; 2) identify an immune mechanism for protection; 3) establish a clinical measure of vaccine efficacy, and 4) develop a strategy for vaccine use that is practical for producers while addressing epidemiological characteristics of the disease in the field. Based on disease incidence and geographical range there is a stronger rationale for renewed research on a fleece rot vaccine than a lumpy wool vaccine.

Stage 1 for a fleece rot vaccine needs to address current unknowns in skin and fleece rot microbial ecology and serotype diversity within bacterial species. It also needs to address the heterogeneity and differences of pathogenic exoproducts produced by fleece rot bacteria when cultured in the laboratory and during the natural infection process that limit current knowledge of appropriate antigenic targets.

For Stage 2, evidence suggests that circulating levels of the IgG antibody may provide an effective mechanism for immune protection against fleece rot and associated fly strike. To validate this evidence, protective antibody titres and duration of protection need to be established. An important question is whether sufficient antibody can be present on normal skin to confer protection, or whether antibody can be delivered in the serous exudate in early stages of a fleece rot infection to limit wool damage and discolouration, the attraction of blowflies and disease progression. The results obtained from previous vaccine trials suggest this is achievable. If a multivalent vaccine is required (in order to protect against a diversity of bacterial serotypes and exoproducts), the risk that antigenic competition may limit the magnitude of antibody responses to some antigens would need to be examined. Conventional adjuvants currently used in commercial sheep vaccines with strong activity in promoting IgG antibody responses are likely to be adequate.

Stage 3, developing a clinical measure of vaccine efficacy, could be challenging. Is reduction in wool pigmentation, crusting and fibre damage (aka fleece rot) a sufficient goal to justify development of a new vaccine, or are reduction in body strike and potentially also breech strike necessary criteria of vaccine efficacy? Either goal requires a disease challenge model for assessing efficacy. The contemporary environment for ethical use of animals in experiments could limit the use of artificial disease challenges, for instance in a wetting shed. In-principle approval of disease challenge experiments by an Animal Ethics Committee could be a valuable

preliminary step in developing a new vaccine research program. Studies employing natural field challenge would be feasible but would provide a much slower means for assessing vaccine efficacy. *In vitro* models, for instance of antibody effects on bacterial function and on inhibition of attraction of gravid female blowflies to bacteria-generated odorants, could play an important role in fast-tracking development. However, their limitations need to be recognised.

Stage 4 requires consideration of the practical strategies required for successful use and adoption of new vaccines in the field, and could include:

- A stand-alone fleece rot vaccine with a prime boost administration regimen in lambs followed by annual boost at the beginning of seasonal period of risk in environments with a seasonal risk profile (e.g., Mediterranean), plus strategic boost administration before Bureau of Meteorology forecast periods of greatly enhanced risk due to major wet weather events;

- A fleece rot vaccine combined with a multivalent clostridial vaccine administered in the typical clostridial vaccine regimen;

- A fleece rot vaccine combined with a potential flystrike vaccine administered prior to onset of peak flystrike season (early Spring) coinciding with sheep care, including crutching and drenching;

- A combined fleece rot and lumpy wool vaccine administered as per the first dot point.

1.6   Potential benefits of a new vaccine

A contemporary analysis of the benefits of a new vaccine would need to address not only the economic costs of the vaccine used by producers but also make an assessment of the evolving commercial risks and public perceptions of the industry that may arise from the absence of action on this front. Increasing adoption of genetic solutions to breech strike and new methods for breech modification are likely to reduce the occurrence of breech strike. In this scenario, the *relative* importance of body strike as a cause of fly-related disease and death in sheep is likely to increase. This outcome may be further compounded by the increasing incidence of blowfly resistance to the insecticides used for control of blowfly strike. Thus, fleece rot and body strike may gain more prominence as diseases of commercial significance and ethical concern. This review focuses primarily on the technical feasibility of a new vaccine and does not provide a detailed assessment of the values matrix underpinning the commercial and ethical environments for vaccine development.

Nonetheless, at *prima facie* value, an effective fleece rot vaccine has the potential to reduce costs to industry from fleece rot and body strike, as well as the possibility of reducing susceptibility to some (probably yet to be identified) underlying microbial factors contributing to breech strike. A vaccine would be an adjunct to direct and indirect genetic selection of sheep for resistance to fleece rot and current husbandry practices, rather than a substitute. Furthermore, a fleece rot vaccine has the potential to strongly bolster the animal welfare credentials of the Australian sheep and wool industries, both directly and indirectly, thereby minimising market risks to the wool and sheep meat industries.

1.7   Recommendations

1. *Undertake an investigation of microbial ecology and pathogenesis of dermatitis in order to identify appropriate vaccine antigens*
    a. Comprehensive microbiome analysis of microbial ecology in fleece rot, lumpy wool and the breech of sheep susceptible to breech strike. A microbiome analysis will greatly assist the identification of bacterial isolates required for *in vitro* or challenge efficacy trials and antigenic targets for vaccine development.
    b. Compile a list of possible vaccine antigens by undertaking comprehensive bioinformatics analyses of publicly available *Pseudomonas aeruginosa* and *Dermatophilus congolensis* genome sequences. Identify all secreted proteins, all known infectivity and pathogenicity protein factors, and known antibiotics resistance genes. Cross-reference information obtained with strains of *P. aeruginosa* and *D. congolensis* identified by experimental microbiome analysis of fleece rot and lumpy wool. From the list of antigens, identify strain-specific and species-specific structural variants.
    c. Identify non-protein secreted products from *Pseudomonas aeruginosa* and *Dermatophilus congolensis*. The secreted microbial products will include a subset of infectivity and pathogenesis factors, which are prime candidates for inclusion in respective vaccines. Mass spectrometry can experimentally identify the secreted factors. Structural differences in non-protein antigens for different bacterial strains will need to be determined.
    d. Finger-print *Pseudomonas aeruginosa* strain specific genetic differences in fleece rot samples. The approach will determine *Pseudomonas aeruginosa* population structures (strains) in fleece rot samples, antibiotic resistance profiles and similarities of population structures with *Pseudomonas* populations that infect humans. Strain variation will be important from the perspective of fleece rot vaccine design.

2. *Identify mechanism(s) of immune-mediated protection induced by vaccination.*
    a. Use candidate antigens from results of microbial analyses, and establish a primary goal of vaccination: to control fleece rot, lumpy wool and body strike, or body strike and some breech strike.
    b. Develop *in vivo* models to assess mode of vaccine action for use in stage 3.

3. *Develop in vitro correlates of immune-mediated protection*
    a. Use *in vitro* immune function correlates of protection (e.g. antibody mediated neutralisation of bacterial exoproduct activity) to inform adjuvant choice and vaccine delivery route and regimen.
    b. Use an *in vitro* model to examine potential duration of action in the field.
    c. If fleece rot vaccine shares a common mode of immune-mediated protection (e.g. antibody in skin secretions and exudates) with the Blowfly Vaccine Project (AWI Project ON-00619), integrate the two projects.

4. *Undertake vaccination efficacy trials and develop field strategies for use of the vaccine*
    a. Establish clinical efficacy in the field against primary and secondary vaccine indications: i.e., to control fleece rot, lumpy wool and body strike, or body strike and some breech strike as per 2a).
    b. Determine duration of vaccine action.
    c. Determine whether the fleece rot vaccine can be combined/formulated with other current sheep vaccines.
    d. Develop protocols for vaccine use that accommodate the influence of management practices, sheep age, duration of action, and climatic variables on practicality and efficacy of vaccination.

2. Scope and aims of review

The scope and aims of the review are as outlined by AWI and detailed below:

This project is to engage a team of experts in vaccine development, immune response, molecular biology, microbial ecology and genomics, led by CSIRO with engagement from external consultants and researchers, to undertake a comprehensive feasibility review of literature addressing fleece rot, lumpy wool and similar bacterial conditions that contribute to the incidence of flystrike, and to provide recommendations on future research strategies to target their development through a vaccine approach.

A key outcome of the review will be the identification of opportunities and challenges for the design of potential research projects that will utilise these above technologies to develop an effective commercially viable control strategy for fleece rot, lumpy wool and similar bacterial conditions in sheep with an aim to reduce the incidence of flystrike.
This review will:
  ● *use scientific literature and patents to assess historical and current knowledge and what has been achieved in relation to a vaccine against fleece rot*
  ● *identify gaps in knowledge*
  ● *identify challenges and opportunities, especially opportunities enabled by new technological developments*
  ● *make recommendations*

3. Process

A scientific panel of people with broad and complementary knowledge of blowfly strike, fleece rot, lumpy wool, sheep physiology, disease, genetics, vaccinology, immunology, genomics and microbiology was assembled to provide input into the review. The panel members have substantial numbers of peer-reviewed scientific publications in the area of the review as well as knowledge of relevant past scientific and commercialization efforts. Members of the panel have also been leaders in the development and application of new technologies that have radically changed scientific discovery strategies over the last two decades. The details of their expertise are listed in **Appendix 1.** The group met on February 28, 2020 at the University of Queensland to plan the review. Communication was subsequently maintained by email, WebEx meetings and, in the initial phase, commentary in Google Docs.

To identify relevant literature and knowledge, searches were performed of the PubMed database with various combinations (using AND, OR, NOT operators) of the following terms: [fleece rot], [blowfly strike], [sheep], [lumpy wool], [dermatophilosis], [pseudomonas], [dermatophilus], [vaccine], [wool], [livestock]. In a similar manner, searches were also performed on internal CSIRO databases, Web of Life databases, Australian and World patent databases, AWI project progress reports, industry position reports, and PhD theses. Relevant scientific reviews often led to additional sources of historical information. Searches were also performed on publicly accessible databases to identify the status of genome sequences for bacterial species relevant to fleece rot and lumpy wool.

Members of the panel contacted people who had direct scientific and commercial knowledge of relevant vaccine efforts undertaken about three decades ago in Australia. They provided insight into the reasons why vaccine commercialisation did not take place at that time.

A set of key points is often listed at the end of a section of the review to summarise information. The scientific literature did not always provide a consensus view. This position is noted in the report but, where possible, the panel interpreted available information based on their expertise and the quality of available information. Gaps in knowledge were also noted. Each section of the review also contains specific comments from its authors.

The review recommendations were formulated using the available knowledge, insight and expertise of the panel, in conjunction with industry feedback.

4. Fleece rot

4.1   Background

The first reports of fleece rot in Australia occurred in the second half of the 19th Century when pastoralists reported a green discoloration as a fault in wool. The condition, termed water stain or weather stain was reproduced by Stuart (1894) by transferring bacteria isolated from affected wool to the skin of healthy sheep. Seddon and McGrath (1929) recognized that sheep varied in susceptibility to this condition, and that moisture in the fleece was important for disease development, which was accompanied by an inflammatory reaction in skin (dermatitis) (Bull, 1931) (**Figure 1**). A summary of control points for fleece rot is shown in **Figure 2**. Following the emergence of fly strike, caused by *Lucilia cuprina,* as an important disease of sheep in the first few decades of the 20th Century, Seddon and his colleagues undertook intensive research and established a link between wool characteristics and body conformations that favoured prolonged wetting of the fleece, resulting in a bacterial dermatitis (fleece rot) and susceptibility to infestation by blowfly larvae (Gilruth *et al.*, 1933).
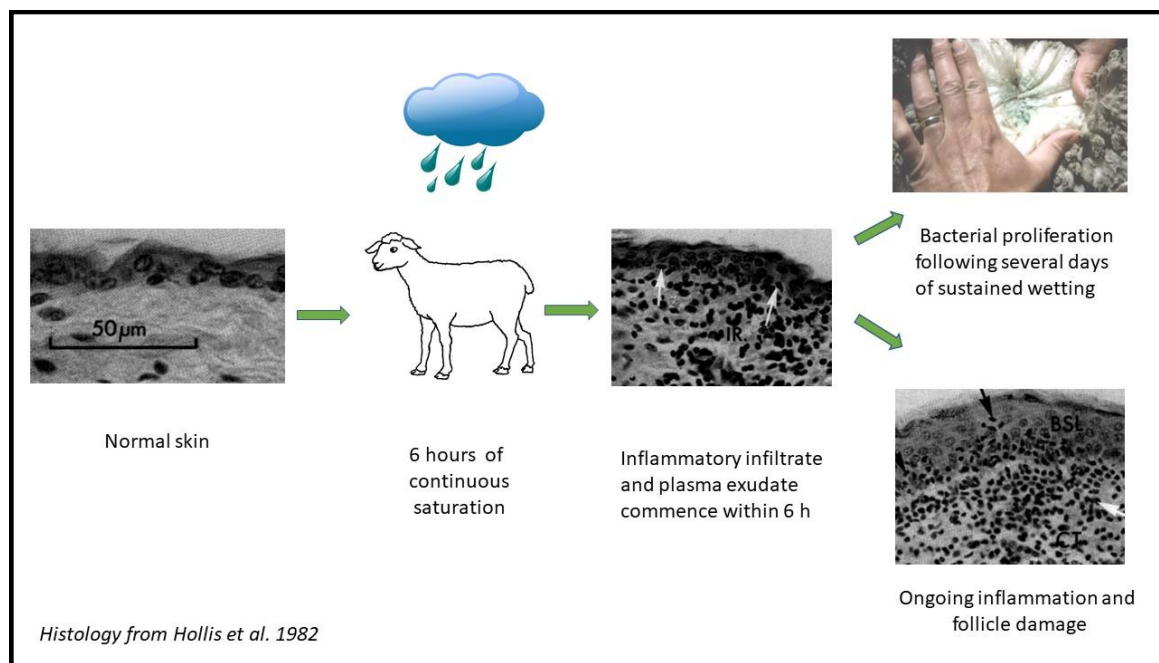

Figure 1. Inflammation in sheep skin following prolonged wetting.

4.2   Prevalence

Field studies on the microbial aetiology, progression of infection, host defence reactions and genetics of resistance to fleece rot have been hampered by the substantial variation in prevalence and severity of the disease from year to year due to variation in rainfall level. Seasonal variation in prevalence has been studied at Trangie, NSW (32° 01' S, 147° 59' E), in fine and medium woolled Merinos. In 1976, 1977 and 1978, prevalence was 37, 23 and 8%, respectively (Atkins and McGuirk, 1979), and over the subsequent seventeen year period the average prevalence for fleece rot based on 3,339 records in yearling sheep was 23.5% (Mortimer *et al.*, 1998). Similar variability was seen at Longford NSW (30° 20' S, 151° 27' E) where average prevalence of fleece rot in 6,213 fine and medium woolled Merino lambs at 10 months of age in the years ,1991 to 1996 was 26.7%, and ranged from 0.46% in 1991 to 75.2% in 1996 (Li *et al.*, 1999). Prevalence of fleece rot in one Merino flock and two Corriedale flocks in the South Island of New Zealand ranged from 65 to 100% in a two year interval (Keown and Reid, 1997).

Young sheep are more susceptible than older animals to both fleece rot and dermatophilosis (Atkins and McGuirk, 1979; Burrell *et al.*, 1992; Edwards *et al.*, 1985; Hayman, 1955). This might be due to effects of age on physical characteristics of wool and skin such as staple structure and the durability or thickness of the lipid barrier (Hayman, 1955), or could reflect the requirement of young sheep to be exposed to the infectious agents before they can express a degree of immunity as older animals (Chin and Watts, 1991). Effective acquired immunity may also require exposure of sheep to a succession of different bacterial strains. Immaturity of the immune system of young sheep and their sensitivity to stressors following weaning are also likely to contribute to the greater susceptibility of young animals (Colditz *et al.*, 1996b).

4.3   Pathogenesis

Early studies on the pathogenesis of fleece rot established that wetting for several days without an opportunity to dry out leads to loss of waxes and a decrease in the hydrophobic (protective) properties of the waxes in wool as the free sterol content (including cholesterol, lanosterol and dihydrolanosterol) of the waxes increases (Hay and Mills, 1982). Bacterial enzymes, especially phospholipase C from *P. aeruginosa* are thought to contribute to these changes (Chin and Watts, 1988; Goodrich and Lipson, 1978; James *et al.*, 1984b). Breakdown of the skin wax layer following artificial wetting is associated with an increase in abundance of *P. aeruginosa* (Merritt and Watts, 1978a, 1978b), and chemical disruption of the sebaceous layer with petroleum ether can greatly increase the incidence of fleece rot (James and Warren, 1979). The detergent action of potassium salts in suint (natural wool grease) may contribute to the disruption of the wax layer (Freney, 1940) and is in accord with the association between suint content of wool present before wetting and susceptibility to fleece rot (James *et al.*, 1984b).

Dermatitis and development of a serous exudation commence within 6 hours of wetting of healthy skin (Chapman *et al.*, 1984; summarized in **Figure 1**) and with prolonged wetting there can be disruption of normal follicular structures (Nay and Watts, 1977). Human and mouse studies have shown that keratinocytes contain the pro-inflammatory cytokine interleukin-1α (Kupper *et al.*, 1986) and produce other inflammatory cytokines including interleukin-8 during skin trauma (Larsen *et al.*, 1989) that may contribute to cutaneous inflammation seen following prolonged hydration of skin in sheep. Exudation of serum (Watts and Merritt, 1981b) and

infiltration of the dermis and epidermis by neutrophils and discoloration of the fleece occur without invasion of the epidermis by bacteria (Burrell *et al*., 1982a).

In addition to neutrophils (Burrell *et al*., 1982a), the dermatitis caused by fleece rot is heavily infiltrated by other leukocyte classes. Following the application of a staple of wool soaked in an overnight culture of *P. aeruginosa* to skin there was a significant increase in the number of CD1+ leukocytes and CD4+ T lymphocytes in dermal biopsies taken at 6 hours (IG Colditz, KG Altmann and DL Watson, unpublished observations). Numbers of CD8+ T lymphocytes and gamma delta + T lymphocytes were significantly elevated by 24 hours. The bacterial infection did not induce changes in cell populations present in efferent lymph draining the infection site indicating that the reaction in this experimental model was very localised. When broad areas of an animal are affected by fleece rot there is likely to be a stronger systemic effect on leukocyte dynamics.

4.4   Microbial aetiology of fleece rot

Studies employing conventional aerobic bacterial culture methods have identified a number of bacterial species on the skin, fleece and fleece rot lesions of sheep that could initiate and/or sustain fleece rot (Belschner, 1937; Burrell and MacDiarmid, 1984; London and Griffith, 1984a, 1984b; Lyness *et al*., 1994; MacDiarmid and Burrell, 1992; Seddon and McGrath, 1929; Stuart, 1894). Based on the limited microbiological data from these studies, the bacterial agent considered causative of fleece rot is *P. aeruginosa;* however, other fleece bacteria probably play a significant role (Chin and Watts, 1992; Kingsford and Raadsma, 1997). It is noteworthy that these early studies were limited by the methods available at the time for identification of microbiota.

Pseudomonads are a major opportunistic, ubiquitous genus of bacteria that can be obtained as pure cultures from some established fleece rot lesions. Many *pseudomonads* have been identified from fleece rot infections including *P. aeruginosa, P. maltophilia, P. alcaligenes* and *P. mendocina* (Burrell and MacDiarmid, 1984; Chin *et al*., 1995; Chin and Watts, 1992; London and Griffith, 1984a, 1984b; MacDiarmid and Burrell, 1992). Some controversy persists as to the identity of the bacterial species primarily responsible for induction of fleece rot. Only *P. aeruginosa* is known to produce the green pigment (pyocyanin) observed in some fleece rot lesions (Chin and Watts, 1991; Merrit and Watts, 1978b), while *P. maltophilia* is considered to be responsible for yellowish brown discolouration. In a field survey, Kingsford and Raadsma (1997) were able to detect *P. aeruginosa* in only 14% of 646 sheep affected with fleece rot. Nonetheless, the presence of *P. aeruginosa* was associated with increased severity of fleece rot and subsequent occurrence of blowfly strike.

Only limited molecular genetic analysis has so far been conducted on the bacteria present during fleece rot and none appears to have been undertaken on dermatophilosis in sheep. Kingsford and Raadsma (1995), using primers designed to amplify a *P. aeruginosa* specific region of the 16S rRNA gene, examined *P. aeruginosa* in fleece washings collected from a flock of 100 sheep. PCR (polymerase chain reaction) results agreed with bacteriological isolation in 89% of fleece samples tested, 2% of samples contained organic PCR inhibitors in the fleece washings, and 3% were below the sensitivity of detection. Use of nested PCR did not increase the sensitivity of detection compared with single round PCR. Other bacterial species present in the samples were not identified. The PCR test used measured only the presence or absence of the bacterium i.e., the test was not quantitative and therefore did not comment on the extent of the *P. aeruginosa* infection in the samples.

More recently, Dixon *et al.* (2007) sequenced 16S rRNA genes to identify bacteria present prior to and during onset of fleece rot in the fleece rot resistant and susceptible selection sheep lines housed at the Trangie Agricultural Research Station (DPI, Trangie, NSW). Several novel bacteria associated with fleece rot were reported. The bacterial composition appeared to differ between fleece rot resistant and susceptible animals, with at least four highly represented bacteria present in the susceptible line animals and absent from the resistant line animals. The study concluded that although *P. aeruginosa* has been clearly associated with fleece rot severity in sheep, there may be other agents responsible for predisposing sheep to, or protecting sheep from, the disease. The prevalence of *Pseudomonas* species in fleece rot samples investigated by Dixon *et al.* (2007) seemingly contrasts with the results from Kingsford and Raadsma (1995) described above, however, this is likely due to methodological and experimental design differences. Microbial ecology of the breech in the presence and absence of dags or urine stain does not appear to have been studied in detail to date. Consequently, the importance of odours from bacteria recognised as (or potentially) important in fleece rot for attraction of gravid flies to the breech is unresolved.

4.5 Diversity of bacteria implicated in fleece rot

One potential approach to prevent fleece rot is to vaccinate sheep against the specific bacteria initiating and sustaining this infectious disease. Experimental fleece rot vaccines have been investigated; however, no commercial vaccine has been produced (Burrell, 1985; Burrell and MacDiarmid, 1986; Burrell and MacDiarmid, 1988; Dai, 1997; Schiller *et al.*, 1981a; Schiller *et al.*, 1981b). One technical reason for the lack of success of a commercial vaccine is that multiple bacterial species, and strains of the most prevalent species, *P. aeruginosa*, could be involved in initiating and sustaining fleece rot. This bacterial diversity may complicate vaccine design and manufacture, and ultimately vaccine efficacy. Moreover, the spectrum of bacterial diversity could also change due to geography, local environment, weather, sheep genetics, fleece type, and local management practices. Hence, a vaccine may need to be designed to contain antigens reflecting the specific bacterial diversity underpinning the generation of fleece rot i.e., the bacteria involved in initiating and sustaining fleece rot. Alternatively, the vaccine antigen could be one that is conserved across all strains likely to be encountered in the field.

4.5.1   Approaches used to measure bacterial diversity in fleece rot

Bacterial diversity in fleece rot has been investigated using a variety of approaches including: (i) bacterial infectivity and pathogenicity characteristics in sheep (Stuart, 1894); (ii) growth medium-specific laboratory culture of fleece rot bacteria in association with the characterisation of morphological properties of bacterial colonies, bacterial growth characteristics and profiling of specific secreted enzymes (Watts and Merritt, 1981a, 1981b; MacDiarmid and Burrell, 1986; London and Griffith, 1984a, 1984b; Burrell and MacDiarmid, 1984); (iii) DNA profiling (Norris *et al.*, 2008; Dixon *et al.*, 2007; Kingsford and Raadsma, 1997); (iv) serology (MacDiarmid and Burrell, 1986; Dai, 1997; Burrell and MacDiarmid, 1984), and; (v) the ability of specific vaccines to protect sheep from some fleece rot bacterial species or strains but not others, including serological differences (Burrell and MacDiarmid, 1988; Burrell and MacDiarmid, 1986; Dai, 1997).

4.5.2 Fleece rot bacteria

Healthy fleece contains a diversity of bacterial species (Lyness *et al.*, 1994; Watts and Merritt, 1981a, 1981b; Dixon *et al.*, 2007; Norris *et al.*, 2008) that undergo sudden and enormous growth in numbers under conditions of high moisture content of fleece and warm temperatures (Norris *et al.*, 2008; Watts and Merritt, 1981a; Burrell *et al.*, 1982a). Watts and Merritt (1981a, 1981b) calculated that washings from healthy fleece contained about 3,000 bacteria/ml and this number showed a remarkable increase to 700 million/ml in washings from areas affected by fleece rot. Eisemann *et al.* (personal communication) suggested that this spectacular bacterial growth could be caused by bacterial reproduction as well as the reactivation of metabolic activity in many desiccated bacteria.

P. aeruginosa is typically prevalent in samples of fleece affected by fleece rot (Norris *et al.*, 2008; Watts and Merritt, 1981b; Dai, 1997; Burrell *et al.*, 1982a; Burrell and MacDiarmid, 1984). The characteristic green colour of the affected fleece is also consistent with a green pigment (pyocyanin) secreted by *P. aeruginosa* and its absence in other pseudomonads (Chin and Watts, 1992; Norris *et al.*, 2008; MacDiarmid and Burrell, 1986; Burrell, 1990; Dai, 1997; Watts and Merritt, 1981a, 1981b). Notably, pyocyanin has antibacterial (bacteriostatic) activity suggesting that *P. aeruginosa,* under favourable conditions, actively suppresses the growth of other bacterial species to enhance its own survival (Dai, 1997). A related bacterial species, *P. maltophilia,* is considered responsible for a yellowish-brown fleece discolouration less frequently associated with fleece rot (MacDiarmid and Burrell, 1986).

P. aeruginosa is highly motile and causes a broad group of opportunistic infections in humans, including infections of injured skin (especially burns), urinary tract, middle ear, cornea, and lung especially in immunocompromised individuals (Hoggarth *et al.*, 2019; Merakou *et al.*, 2018; Priebe and Goldberg, 2014; Watts and Merritt, 1981b). *P. aeruginosa* uses aerobic respiration for optimal metabolism although it can also respire anaerobically on nitrate or other alternative electron acceptors. This considerable metabolic plasticity is a major reason for the ubiquitous presence of *P. aeruginosa* in humans, animals and their environments. Also underpinning the metabolic versatility of *P. aeruginosa* in causing infections at very different anatomical sites on animals is the "extremely complex genomic diversity" in the population, which includes mobile genetic elements superimposed on genetic diversity (Hoggarth *et al.*, 2019; Lozano *et al.*, 2018; Winstanley and Rumbaugh, 2018; Freschi *et al.*, 2018). In particular, some mobile genetic elements contain the antibiotic resistance gene, beta-lactamase, which makes *P. aeruginosa* a formidable human pathogen. Patients infected with *P. aeruginosa* usually require aggressive and prolonged antibiotic treatments. Similar issues of genetic (strain) variation are likely in the contribution of *P. aeruginosa* to fleece rot in sheep, but have not been investigated. The knowledge gained by studies on *P. aeruginosa* infections in humans will help identify strain variants involved in fleece rot.

Several investigations used conventional aerobic bacterial culture methods to grow *P. aeruginosa* taken from washing samples from sheep skin, fleece and fleece rot (Seddon and McGrath, 1929; Burrell and MacDiarmid, 1984; London and Griffith, 1984a, 1984b; MacDiarmid and Burrell, 1992; Lyness *et al.*, 1994; Watts and Merritt, 1981b). Moreover, fleece rot was induced in fleece samples in the laboratory by application of *P. aeruginosa* to the fleece samples *in vitro* and prevented when an antibacterial compound (merthiolate) was first applied to the fleece before the application of *P. aeruginosa* (Watts and Merritt, 1981b; Merritt and Watts, 1978a). Thus, this study proved that fleece rot is a result of bacterial action and *P. aeruginosa* can initiate and sustain fleece rot. Using the same laboratory assay, it was demonstrated that adult *L. cuprina* showed a 6-fold greater preference for potential ovipositing in wool samples growing *P. aeruginosa* compared to another bacterial species, *Bacillus*

subtilis, which is commonly found in healthy fleece (Watts and Merritt, 1981a, 1981b; Merritt and Watts, 1978a). This result was consistent with the increased risk of blowfly strike in fleece rot-affected areas populated by a prevalence of *P. aeruginosa*. In a field survey of 100 sheep, Kingsford and Raadsma (1995) detected *P. aeruginosa* by laboratory bacterial culture of 30 samples, of which 25 were also positive for *P. aeruginosa* detected using a highly sensitive 16S rRNA assay that identifies *P. aeruginosa* by DNA sequence in one small region of its genome. The analysis was not quantitative and thus the relative abundance of *P. aeruginosa* in these samples was not determined, nor any association with fleece rot. However, the result is consistent with the view that *P. aeruginosa* is an opportunistic pathogen normally present in low numbers in healthy fleece. Collectively, these multiple investigations generally agree that *P. aeruginosa* is the main cause of fleece rot. However, experimental data formally proving that *P. aeruginosa* causes fleece rot (i.e., the ability to initiate and sustain fleece rot), rather than capitalising on the favourable growth conditions generated by fleece rot, are still limited (Norris *et al*., 2008).

Some investigators suggested possible contributory roles to fleece rot and attractiveness to blowflies from other bacterial species, including pseudomonad species besides *P. aeruginosa* (Kingsford and Raadsma, 1997; Dixon *et al*., 2007; London and Griffith, 1984a, 1984b; Lyness *et al*., 1994; Jansen and Hayes, 1987; Burrell and MacDiarmid, 1988; Emmens and Murray, 1983). Kingsford and Raadsma (1997) surveyed 1,568 sheep and identified fleece rot in 646 sheep. Of the affected sheep, only 14% were positive for *P. aeruginosa* using a 16S rRNA assay, thereby implying the involvement of other bacterial species in the generation of fleece rot. There was, however, a strong association of the presence of *P. aeruginosa* in these 14% of samples with the severity of fleece rot. An earlier publication by Kingsford and Raadsma (1995) showed that the 16S rRNA assay was highly correlated (89% agreement) with *P. aeruginosa* identification by culture methods. Using samples from healthy skin, healthy wool and fleece rot sites, Dixon *et al.* (2007) used 16S rRNA sequence profiles to identify predominant bacterial populations representing eight major bacterial orders. Thus, considerable bacterial diversity was associated with the samples. The investigators examined the dynamics of bacterial changes occurring during the transition from healthy sheep skin and fleece to fleece rot in populations of sheep that were genetically resistant or susceptible to fleece rot. Four anonymous bacterial populations were identified that were differentially associated with the development of fleece rot in the resistant and susceptible sheep. The fleece rot samples acquired after sheep wetting contained abundant populations of pseudomonads. The study concluded that although *P. aeruginosa* had been associated with fleece rot severity, there could be other bacterial species responsible for predisposing or protecting sheep from fleece rot.

Dai (1997) identified nine bacterial species associated with fleece rot and strong serological responses in sheep injected with outer membrane proteins from *P. aeruginosa*. Importantly, serological differences were demonstrated in sheep bred for resistance and susceptibility to fleece rot when they were vaccinated with *P. aeruginosa* outer membrane proteins, and there were generally greater antibody responses in the resistant sheep (Chin and Watts, 1991; Gogolewski *et al*., 1996). Dai (1997) also noted that the predominant phenotypic form of *P. aeruginosa* present in fleece rot is the mucoid strain, which secretes copious quantities of an extracellular glycocalyx (alginate) that prevents dehydration. Mucoid *P. aeruginosa* infections in humans are particularly difficult to treat as th*e* alginate physically hinders normal host defence mechanisms and is a barrier to diffusion of combinations of antibiotics (Döring and Pier, 2008; Merakou *et al*., 2018; Sharma *et al*., 2011). For human diseases like cystic fibrosis, non-mucoid *P. aeruginosa* strains often initiate infection but then are supplanted by a mucoid *P. aeruginosa* strain, which is much more difficult to eradicate and often leads to chronic infection.

In summary, the results from all these investigations reinforce the general conclusions that *P. aeruginosa* is normally present in low numbers in fleece from healthy sheep and it opportunistically proliferates in the wet and warm conditions associated with the development of fleece rot. The massive increase in the number and dominance of *P. aeruginosa* in fleece rot samples compared to healthy fleece directly implicates this bacterial species as the microbial agent initiating and sustaining fleece rot development. Notably, *P. aeruginosa* strain variation in these investigations was not specifically sampled due to technological limitations. Some investigations concluded that other bacterial species may also contribute to the generation of fleece rot.

4.5.3 Bacterial diversity and impacts on experimental vaccines

Burrell (1985) vaccinated sheep with soluble proteins produced by one isolate of *P. aeruginosa* grown in culture. The vaccine elicited protection against three challenge isolates (serotypes) of *P. aeruginosa*. This result demonstrated that the vaccine could protect sheep from fleece rot and was efficacious against at least three different strains of *P. aeruginosa*. Australian and New Zealand patents (now expired) for this research (Burrell and MacDiarmid, 1986; Burrell and MacDiarmid, 1988) claimed that a vaccine to protect sheep from fleece rot could include soluble antigens and outer membrane antigens from *P. aeruginosa* alone or in combination with similar antigens from *P. putida. P. stutzeri or P. maltophilia*. Supporting evidence for the range of patent claims was not provided. The vaccine was licenced, but not commercialised, by Biotechnology Australia Ltd (Sydney). The reasons for lack of commercialisation of the vaccine three decades ago are outlined in Section 7.4 and include *P. aeruginosa* strain variation.

Schiller *et al*. (1981a, 1981b) vaccinated sheep with seven strains of *P. aeruginosa* and demonstrated that sheep immunoglobulins containing highly specific antibody titres could protect mice from experimental challenge with *P. aeruginosa.* The study was a feasibility investigation for the generation of a vaccine for protection of human burns patients from *P. aeruginosa* infection, however the research was not further progressed.

4.5.4 *P. aeruginosa* population structure

The genetic structure of *P. aeruginosa* populations may have implications for the design of a vaccine to protect sheep from fleece rot. These population structures have been intensively investigated in the human and veterinary contexts from the perspective of tracing acquired antibiotic resistance, serotypes and DNA variations (Serrano *et al*., 2017). However, similar investigations have not been performed for *P. aeruginosa* in fleece rot. *P. aeruginosa* causes a variety of opportunistic, and often nosocomial infections in multiple human tissues (Lyczak *et al*., 2000), where it causes significant human morbidity and mortality (Talbot *et al*., 2006). The bacterium is also responsible for many types of infections in veterinary medicine (Serrano *et al*., 2017). *P. aeruginosa* naturally has innate resistance to many antibiotics but can also acquire resistance to antibiotics through a variety of mechanisms including chromosomal mutations and the acquisition, through horizontal gene transfer, of autologous genetic (DNA) elements harbouring genes that confer antibiotic resistance (Serrano *et al*., 2017; Silby *et al*., 2011).

Serrano *et al*. (2017) concluded that the genetic structure of the *P. aeruginosa* population was the same in human health and various veterinary environments i.e., there was no evidence for clonal expansions of genetic

variants characterising different ecological niches (e.g. specific disease types, tissues, or animal species). This unusual characteristic of the population likely reflects a large and versatile *P. aeruginosa* genome that enables high adaptability of the bacteria to different environments because of its intrinsic metabolic versatility. If confirmed in fleece rot, these *P. aeruginosa* population characteristics may need to be taken into account in the design of the antigens or *P. aeruginosa* serotypes included in the vaccine to ensure its broad efficacy. Thus, it may be an early priority to perform a *Pseudomonas* fingerprint analysis for genetic and serological variation in diverse fleece rot samples (sites on sheep, geography, climatic zones, sheep breeds and wool type) and at different stages of fleece rot development. One advantage of a potentially stable *P. aeruginosa* strain population structure is that a multivalent vaccine incorporating antigens representing this population structure would be expected to maintain efficacy in different locations, seasons, and from year to year. There is also a low possibility that such a vaccine could have wider use for livestock and human disease control.

Using metagenome *in silico* analyses of *P. aeruginosa* from human infections, it is possible to identify serotype groups or strains with differing pathogenicity (Serrano *et al.*, 2017). This type of approach could be very beneficial for the identification of *P. aeruginosa* strains important in fleece rot. The genome sequences from 1,311 *P. aeruginosa* isolates involved in causing various human infections, have now been determined and they provide valuable and enabling information about strain diversity and the gene contents of strains (Freschi *et al.*, 2018). The pan-genome of *P. aeruginosa* (i.e., the core set of genes common to all strains) contained 665 genes but this number only represented 1% of the gene content of the entire pan-genome. Thus, there is considerable genetic diversity in the *P. aeruginosa* population. Core genes were as expected, enriched for housekeeping functions. However, core genes were also enriched for genes with unknown functions. About one third of the core genes are still poorly characterized and these represent a valuable and unexplored resource. The core genes are likely essential for normal biological functioning of *P. aeruginosa.* A high priority for future investigation is the identification of core genes encoding secreted proteins, which can be identified using bioinformatics. These secreted proteins may be virulence factors and therefore priority antigens to test in a fleece rot vaccine. Moreover, as these secreted proteins would be derived from the conserved core group of genes, they are more likely to show minimal strain-specific sequence variation. This feature could maintain vaccine efficacy when challenged by multiple strains of *P. aeruginosa.* Core gene sets were also used to infer phylogeny and evolutionary relationships to define strain and species boundaries. This approach identified five major groupings of the 1,311 genomes. The analysis also revealed 3,010 plasmids (autonomous replicating DNA independent from the core genome) or plasmid fragments in the population indicating a strong influence of horizontal gene transfer. A range between 5-12% of these plasmids contained antibiotic resistance genes or virulence genes. The latter may enhance adhesion to tissue surfaces, damage tissue to enhance bacterial dispersal and increase nutrient availability, and enhance bacterial survival. Antibody-mediated suppression of the functions of the encoded virulence proteins is a high priority strategy for vaccine design. Collectively, the information infers that *P. aeruginosa* is highly versatile and a formidable pathogen.

4.5.5   *P. aeruginosa* strain variation - conclusion

Notwithstanding the current deficits in our knowledge of the bacteria involved in fleece rot, the available evidence consistently implicates *P. aeruginosa* as the primary cause and has demonstrated a strong association of the abundance of *P. aeruginosa* with the severity of fleece rot. This conclusion is also supported by the knowledge that in humans opportunistic *P. aeruginosa* infections are implicated as the cause of a range of

diseases that affect mucosal layers and damaged skin (Hoggarth *et al*., 2019; Merakou *et al*., 2018; Priebe and Goldberg, 2014; Watts and Merritt, 1981b). A key to the success of a vaccination strategy for control of fleece rot is knowledge of the strain diversity associated with *P. aeruginosa* and how strain diversity affects the infectivity and pathogenicity of fleece rot challenge and the immunogenicity and protection of a vaccine containing components from multiple *P. aeruginosa* strains. Ideally, the best antigen for inclusion in a vaccine would be one that had a constant structure in all *P. aeruginosa* strains and induced a protective immune response against all strain challenges. Alternatively, a vaccine could contain all of the strain-specific structural variants of a protective antigen. In the latter circumstance, it will be important to assess antigenic competition in a vaccine containing antigens from multiple *P. aeruginosa* strains as the combined antigens may not faithfully replicate the vaccine efficacy of each antigen when tested alone.

4.6   Genetic resistance of sheep to fleece rot

The early observations by Seddon *et al*., (1931a, 1931b) on phenotypic and genetic variation of sheep in susceptibility to fleece rot led to a substantial research effort to better characterize resistance traits for use in sheep breeding programs. Hayman (1953) confirmed that resistance to fleece rot was heritable. As is commonly seen with genetic parameters, estimates of heritability of resistance vary between genotypes of sheep, and between environments, with values from 0.13 to 0.41 reported in the literature (James *et al.,* 1984a; Li *et al*., 1999; McGuirk and Atkins, 1984; Raadsma *et al*., 1989; Raadsma and Rogan, 1987). Estimates of the heritability of severity of fleece rot infection range from 0 to 0.52. The occurrence of environmental conditions conducive to expression of the disease can be sporadic, thus there has been considerable interest in the identification of correlated (indicator) traits that would enable indirect selection of sheep for resistance. Most studies have examined traits measured on sheep or the fleece at or around annual shearing.

The traits examined fall into three areas: (i) structural faults of the sheep; (ii) physical characteristics of wool such as colour, structure and fibre diameter; and, (iii) chemical characteristics of wool including wax and suint that affect wettability of the fleece. Some conformation characteristics of the withers and breech, and skin wrinkle were recognized as undesirable in the 1920s and 1930s (Belschner, 1937); however, not all studies support this conclusion (Hayman, 1953; Hayman, 1955; Raadsma, 1993; Raadsma *et al.*, 1987). White, bright greasy wool colour, a trait that is often highly heritable, is usually associated with resistance to fleece rot (Belschner, 1937; Hayman, 1953; Holdaway and Mulhearn, 1934; James *et al.*, 1987; James *et al*., 1984b; Li *et al*., 1999; Paynter, 1961). In Peppin Merinos studied at Trangie, the association of fleece rot with greasy wool colour was not strong (McGuirk and Atkins, 1984; Raadsma and Wilkinson, 1990). Unfavourable relationships between fleece rot incidence and some wool quality characteristics including greasy wool colour and staple structure and character have also been noted (Li *et al*., 1999). Raadsma and Wilkinson (1990) found that objectively measured colour and colour development following incubation of wool samples for five days in a moist atmosphere at 40°C were more highly genetically correlated with resistance to fleece rot than subjectively assessed greasy wool colour. Amongst other physical characteristics of wool, variability of fibre diameter (usually expressed as the coefficient of variation of fibre diameter) is highly heritable, easily measured and genetically correlated with resistance to fleece rot (James and Ponzoni, 1992; Raadsma, 1993). Lower fibre diameter has been associated with resistance in most studies (James and Ponzoni, 1992), with some exceptions (Lipson *et al*., 1982). Staple length has a negligible to moderately negative genetic correlation with severity of fleece rot (Raadsma, 1993; Li *et al.,* 1999). Factors reducing wettability of the fleece include higher wax content,

lower suint content, lower suint insoluble nitrogen content and lower pH (Dowling *et al.*, 2006; Hayman, 1953; James *et al.*, 1984b; Lipson *et al.*, 1982; Pascoe, 1982; Paynter, 1961). On the basis of the associations between wool traits and fleece rot, and using typical wool values and costs of management, Cottle (1996) estimated that indirect selection for resistance to fleece rot through selection for correlated traits could result in a net financial benefit to the producer. Together, these results indicate that there is potential for indirect selection for resistance to fleece rot, although better characterisation of genetic correlations with production traits may be required (Mortimer, 2001). More recently, immune competence has been identified as a potential trait for indirect selection and is discussed in more detail below. The interactions between these collective traits and seasonal variation may need further investigation.

In view of the limitations imposed on field studies by seasonal variation in expression of the condition, experimental conditions for inducing fleece rot in large numbers of sheep were developed by NSW Department of Primary Industries at Trangie, by housing animals indoors and artificially wetting them via overhead sprinklers (McGuirk *et al.*, 1978). Animals were scored for natural fleece rot and blowfly strike and for these two conditions induced by artificial wetting were used to establish divergent selection lines. The resistant and susceptible selection lines have undergone continuous selection for 26 years on natural and artificial expressions of fleece rot and body strike and during this time wool characteristics and physiological responses have been extensively studied. Following 17 years of selection, annual divergence in prevalence between the lines from the population mean of 23.5% for natural fleece rot has been 2.8% and from the population mean of 5.8% for natural flystrike has been 0.4% (Mortimer *et al.*, 1998). These changes are in accord with the high genetic correlation between the traits reported by Raadsma *et al.* (1989).

AWI funded research in Armidale and W.A. on breech conformation traits associated with breech strike recorded little association between breech strike and body strike. In the Armidale flock, the incidence of body strike in weaners was 4% whereas breech strike incidence was 18% (Smith, 2016). Body strike heritability was estimated at 0.16 and was poorly correlated both phenotypically and genetically with breech strike (0.08 and 0.00, respectively) (Smith, 2016). Phenotypic correlations between body strike and fleece traits were all low to negligible (range -0.09 to 0.12). Genetic correlations between body strike and fleece traits were also negligible except for fleece rot (0.56), fibre curvature (-0.41) and assessed wool character (0.28) (Smith, 2010). Fleece rot was not correlated phenotypically or genetically with breech strike (0.02 and 0.07, respectively) (Smith, 2016).

Regarding breech strike, in both the W.A. and Armidale flocks there were no strong genetic correlations with fleece traits. Interestingly, the fleece trait with strongest genetic correlation was coefficient of variation of fibre diameter (CVD), but the sign was reversed in the two flocks (-0.27 in the WA flock and 0.31). Thus, there was no consistent effect. Greasy wool colour was not correlated with breech strike in either the Armidale or W.A. flocks, which is in contrast to the literature cited above which describes a genetic relationship between greasy wool colour and fleece rot/body strike.

Key points on quantitative genetics of resistance to fleece rot

- Direct and indirect genetic selection provide opportunities to reduce susceptibility to body strike

- Susceptibility to breech strike shows little genetic or phenotypic association with body strike, or with wool characteristics associated with susceptibility to body strike

- Bacterial mechanisms contributing to susceptibility to fly strike may differ between breech and body; however, contribution of bacterial activity in the breech to breech strike is largely an unknown

4.7 Molecular genetic studies of resistance to fleece rot

Genetic markers associated with resistance to fleece rot can potentially be used in breeding programs to accelerate selective breeding for resistant sheep. One investigation identified genetic variants linked with the differential expression of ovine genes in skin from sheep that were resistant or susceptible to fleece rot during experimentally induced fleece rot (Smith *et al.*, 2010). Two genetic markers in the gene *Fatty Acid Binding Protein 4* (*FABP4*) were identified although they only explained a small extent (2-6.8%) of total genetic variation for the fleece rot severity trait and have not been independently confirmed. *FABP4* encodes a protein typically expressed in large quantities in adipocytes and at lower levels in macrophages. The protein functions as an intracellular transporter of large fatty acids to cellular organelles and has strong roles in regulating lipid metabolism (Furuhashi *et al.*, 2019; Trojnar *et al.*, 2019). Some of the fatty acids transported by this protein are important intracellular signalling molecules. In a nonclassical manner, the FABP4 protein is also released from adipocytes and macrophages and acts as a potent proinflammatory signalling protein, a function consistent with the inflammation observed in skin tissues affected by fleece rot. Notably, the level of FABP4 protein in blood was increased in people with the skin inflammatory condition, psoriasis (Baran *et al.*, 2017). Moreover, it was recently demonstrated that *FABP4* genetic variants were potentially associated with susceptibility to flystrike in sheep (Burrows, 2018). The small percentage of the genetic variation for fleece rot severity explained by the ovine *FABP4* DNA polymorphisms is insufficient for practical use in breeding programs. More extensive genome-wide genetic association studies are required to provide a practical tool for selective breeding.

4.8 Immunological mechanisms of resistance to fleece rot

The Trangie selection lines have provided a valuable resource for studying immunological mechanisms that might contribute to resistance both to fleece rot and flystrike (reviewed by Colditz *et al.*, 2001). Plasma leakage into skin following intradermal injection of permeability agents (including histamine and bradykinin) tended to be greater in the susceptible line of sheep (Colditz *et al.*, 1992b). In response to the intradermal injection of mediators of neutrophilic inflammation including endotoxin from *P. aeruginosa,* no differences between lines were apparent in the intensity of the neutrophil infiltrate in skin; however, gamma delta + T cells and eosinophils were more prevalent in the skin of susceptible sheep (Colditz *et al.*, 1994). Mast (IgE+) cells were found to be more prevalent in the skin of sheep from the resistant line (Colditz *et al.*, 1994; Nesa, 1994). In support of these findings was the observation in an unrelated population of sheep that mast cells were more prevalent in the skin of sheep with no history of fleece rot than in flock-mates with a record of fleece rot (Colditz *et al.*, 1994). (Mast cells release vasoactive peptides like histamine when activated, for instance when pathogen-derived antigens bind to IgE expressed on the mast cell surface, which then causes plasma leakage from blood vessels into skin and surrounding tissues). A DNA restriction fragment length polymorphism of the *IgE* gene has been found between resistant and susceptible sheep (Engwerda *et al.*, 1998) and may contribute to differences in mast cell function between the lines. Lymphocyte phenotypes in blood did not differ between ewes from the two Trangie selection lines (Colditz *et al.*, 1996a); however, rams from the resistant line had a higher number of

CD5+ lymphocytes in blood in one of two cohorts studied (McColl *et al.*, 1997). Following intravenous challenge of sheep with endotoxin from *P. aeruginosa,* there were higher counts of neutrophils and monocytes in blood from the resistant line (Colditz *et al.*, 2001). Neutrophils are the predominant leukocyte in skin and in the accompanying seropurulent (a mixture of serum and pus) exudate in fleece rot lesions. By the time neutrophils have accumulated in exudates they are usually effete or dead cells and therefore have little or no capacity to kill bacteria. As bacteria do not penetrate skin during fleece rot, neutrophils within the dermis are not able to interact with bacteria. Together, these results suggest that the cellular component of the inflammatory response to fleece rot is likely to play a very limited direct role in controlling infection.

More compelling evidence of a role for host defence in influencing infection is seen in the antibody responses to fleece rot and experimental *P. aeruginosa* infections. Following intradermal injection of *P. aeruginosa* antigens, antibody responses are generally greater in resistant sheep (Chin and Watts, 1991; Gogolewski *et al.*, 1996). In addition, sheep from the resistant line develop higher titres of antibody to *P. aeruginosa* when live cultures of the bacterium are applied to the surface of wetted skin (Chin and Watts, 1991). Further evidence of the potential for sheep to be sensitized to bacteria during dermatitis is provided by the observation of an increased number of plasma cells in skin during resolution of dermatophilosis (Ellis *et al.*, 1987) and by the presence of antibody to *D. congolensis* antigens in skin washings during experimental dermatophilosis (Sutherland *et al.*, 1987); however, no clear relationship between antibody responses to *D. congolensis* antigens during field or experimental infections and resistance to reinfection has been identified (Ambrose *et al.*, 1999).

4.9   Acquired resistance to fleece rot

Some infectious diseases, especially some viral diseases, induce an adaptive immune response during infection that contributes to the clearance of infection and provides strong protection against reinfection for many years. In other instances, animals can become sensitised to pathogens during infection but the acquired immune reactivity may fail to control the infection. During experimentally induced fleece rot, sheep develop antibodies to *P. aeruginosa* and several other skin microbes (Chin and Watts, 1992). Antibodies in blood serum increase over the two to six weeks following induction of fleece rot (Chin and Watts, 1992), and in doing so follow the typical dynamics of development of antibody responses exhibited by the adaptive immune system. As noted above, antibody responses to experimental vaccination with *P. aeruginosa* tend to be higher in the Trangie resistance line of sheep. These results suggest that antibody to the skin pathogens present in field cases may contribute to resistance to fleece rot. Nonetheless, there is a need for additional and direct proof that a highly specific antibody can be identified in fleece rot lesions and that the antibody is not rapidly degraded. Passive immunisation of sheep with antibody of known specificity could provide important information about the availability and stability of antibodies in fleece rot exudate.

4.10   Resolution of fleece rot lesions

At annual shearing, bands of discoloration in wool indicate the occurrence of episodes of fleece rot in the preceding year. In field cases, fleece rot usually resolves in days or weeks. Nonetheless, few studies appear to have specifically addressed the factors contributing to the resolution of fleece rot lesions. Hollis *et al.* (1982)

noted in a histological study of fleece rot induced by artificial wetting, that inflammatory cells infiltrated the dermis within six hours of wetting. During nine days of wetting there was substantial thickening of the epidermis. Thirteen days after the cessation of wetting, epidermal thickness had returned towards normal and a band of white wool had emerged below wool discoloured by bacteria; however focal dermatitis was still evident in some sites. Visual scores of susceptibility to fleece rot are based on the width of the band (less than or greater than 5 mm) and presence or absence of crusting within the band as a measure of the severity of the case of fleece rot, as described in the AWI/MLA Visual Scoring Guide below. Since wool grows about 1 mm every four or five days, mild cases of fleece rot evident as colouration less than 5 mm in width are likely to have resolved within three weeks. Many of the weather events inducing fleece rot are likely to last less than three weeks in which timeframe moisture content of wool could return to normal thereby permitting microbial ecology to restore normal skin flora. This theoretical scenario is in accord with wetting and drying rates of the fleece being factors contributing to fleece rot susceptibility (Raadsma, 1989). As an alternative mechanism of resolution, the antibody titres to fleece rot pathogens begin to increase in serum at around two weeks and could be contributing to resolution of lesions within this same timeframe.

Fleece rot (FLROT)

Age: Over 6 months and after a significant fleece rot challenge.

When: Anytime, provided a minimum of 5 months of wool growth and preferably after a significant fleece rot challenge and before dust is deposited in the effected wool.

Fleece rot is caused by high humidity leading to multiplication of fleece rot bacterial at skin level and as a result causes staining and in more severe cases exudate from the skin. Fleece rot score refers to the width of the band in the wool of stain and/or crusting that result from fleece rot bacteria. Stained bands can be yellow, green, red-orange, pink-violet, blue, brown or grey in colour. Crusting results from skin exudate deposited in the wool and when fresh or the fleece

is wet, it may be soft however still gives the feel of extra staple thickness.

Note: fleece rot should not be confused with Dermatitis (or 'lumpy wool'), which tends to form columns of hard lumps along the staple.

How to score: Open the fleece at a minimum of **three sites** along the full length of the backline and look for evidence of bands of stain and crusting. The highest score across the sites is recorded as the score.

Rule of thumb: A sheep with Score 1 has no staining or crusting. A sheep with Score 5 has a band/s of 'crusting' greater than 5 millimetres wide, with or without stain.

Score 1:
No band of stain or crusting.

Score 2:
Band of stain <10mm wide. No crusting.

Score 3:
Band of stain >10mm wide. No crusting.

Score 4:
Band of crusting <5mm wide, with or without stain.

Score 5:
Band of crusting >5mm wide, with or without stain.

From AWI/MLA Visual Sheep Scores Guide (Australian Wool Innovation Limited, 2019).

4.11　Vaccines against fleece rot

A large research effort was undertaken in the 1980s and 1990s to develop vaccines against *P. aeruginosa.* Bacterins (dead bacteria or their components used in a vaccine) prepared from *P. aeruginosa* were able to reduce the severity of fleece rot in pen and field experiments and also to reduce the incidence of body strike (Burrell, 1985; Burrell, 1990; Burrell *et al*., 1982b); however when the serotype of *P. aeruginosa* present in the field differed from that used in the vaccine there was little protection. Following the observation that sheep developed antibodies to surface antigens of *P. aeruginosa* during experimental infections (Chin *et al*., 1995), Chin *et al*. (1996) examined the potential of outer membrane proteins of the bacteria as vaccine candidates. The antigens, especially phospholipase C, were highly immunogenic and a patent was filed; however, vaccine efficacy trials have not been reported in the scientific literature. In the main vaccine field trial arising from the work of Burrell and colleagues (Burrell, 1985), field challenge resulting from climatic conditions conducive to fleece rot occurred 8 to 12 weeks after vaccination. Longer term studies on the duration of protection have not been reported. Antibody titres to vaccination are likely to have been near peak levels during this period. Exudation of antibody against bacterial exoproducts onto skin was claimed in the patent to be the mechanism of protection conferred by vaccination. The patent provided for inclusion of bacterial culture filtrate and/or whole bacterial cells from *P. maltophilia*, *P. aeruginosa, P. stutzeri* and *P. putida* (Burrell and MacDiarmid, 1986; 1988).

Key points on pathogenesis and host response to fleece rot

- Expression of fleece rot is highly dependent on weather conditions. Instances of high prevalence, even if they occur at a low level of severity, suggest that in adverse environmental conditions, natural resistance to fleece rot associated with physical characteristics of wool and skin may be overwhelmed and pathogenic bacteria flourish;

- Fleece rot pathogens do not penetrate the epidermis;

- Fleece rot lesions can include a complex mix of bacterial species;

- Sheep can become sensitized and produce antibodies to fleece rot pathogens during an episode of fleece rot;

- Prolonged skin hydration is necessary for sensitization to fleece rot pathogens;

- Immunoglobulins present in plasma leak onto the skin surface during dermatitis;

- Dermatitis induced by prolonged wetting of the skin can precede fleece rot;

- Fleece rot pathogens exacerbate dermatitis caused by prolonged skin wetting;

- Antibody titres to fleece rot pathogens rise late within a typical (3 week) case of fleece rot;

- High antibody titres are associated with resistance to fleece rot;

- In the absence of antibody to fleece rot pathogens, greater exudation of plasma proteins onto the skin surface is associated with susceptibility to fleece rot;

- There is little evidence for innate or adaptive *cellular* immune responses contributing to protection against fleece rot pathogens.

These findings indicate that there is promise that effective vaccines generating antibodies against fleece rot pathogens may be able to reduce occurrence and severity of fleece rot and the associated risk of fly strike. Vaccines can influence several levels of host control over infectious diseases. They can eliminate infection (for instance by promoting phagocytosis and killing of bacteria), they can inactivate the bacterial factors that induce host pathology (for instance exotoxin A and phospholipase C of *P. aeruginosa*), or they can reduce production of bacterial products that contribute to disease ecology (for instance, so called quorum sensing signals released by bacteria that modify swarming behaviour and expression of virulence factors). In the context of fleece rot, vaccines could assist the host to limit bacterial proliferation, to limit activity of bacterial products that induce exudative dermatitis, or to limit activity of bacterial factors (odorants) that attract blowflies to the sheep. Important questions raised by these opportunities include:

i.   which bacterial species and bacterial virulence factors are necessary for inclusion in a vaccine?;
ii. can antibody titres in skin be elevated prior to the development of wetting-induced dermatitis to prevent proliferation of fleece rot pathogens?;
iii. is a delay in the presence of high titres of antibodies on the skin surface until their arrival as a component of the serous exudate that accompanies dermatitis compatible with effective control of skin microbiota by vaccination?

The evidence reviewed above suggests that antibody in skin and in skin exudates plays a pivotal role in influencing the development and progression of bacterial dermatitis. The capacity for immune responses to be induced locally in skin by vaccination and for antibodies to be delivered to the skin surface is addressed in the next section.

4.12   Immune responses in skin of sheep and their potential to modulate skin microbiota

The antibody classes IgA and IgM have strong activity in agglutinating bacteria on epithelial surfaces to prevent their invasion, whereas IgG1 and IgG2 inactivate bacterial toxins and promote phagocytosis and killing of bacteria by leukocytes. These immunoglobulins are present in washings from normal skin of sheep and cattle (Lloyd *et al*., 1979). In ruminant gut and mammary tissues, IgG1 is transported across epithelia by the receptor termed FcRn, which binds the Fc portion of the IgG1 molecule, while IgA and IgM are transported across epithelia by the poly FcR receptor. From studies of antibody responses in skin washings following intradermal vaccination with *D. congolensis* in cattle, Lloyd *et al*. (1987) concluded that selective transport of IgA and IgM occurred in skin by a local secretory process (suggesting that FcR may be present in epithelial cells lining sebaceous glands or sweat glands to mediate transport of IgA and IgM into glandular secretions), whereas IgG1 and IgG2 arrived at the skin surface by a transudative process that does not involve active receptor mediated transport. Colditz *et al.* (1992a) confirmed the absence of a mechanism for active receptor-mediated transport of IgG1 across skin in sheep. Following the observation of elevated numbers of plasma cells in skin of sheep recovering from dermatophilosis (Ellis *et al*., 1987) and the presence of antibody to *D. congolensis* antigens in skin washings (Lloyd and Jenkinson, 1981; Lloyd *et al*., 1987; Sutherland *et al*., 1987), methods were examined for inducing local antibody production in skin of sheep (Colditz and Watson, 1993; Colditz *et al*., 2002). Using recombinant peritrophic membrane antigens from *L. cuprina* larvae as experimental antigens, sheep were primed systemically then boosted by intradermal injection of antigen plus the adjuvant matrix ISCOMs. Three

weeks after boosting, interstitial fluid was harvested by the method of Watson *et al.* (1992) from the vaccination sites and from control skin sites receiving matrix ISCOMs alone without antigen, on the same animals. Within-animal comparison of antibody titres in interstitial fluid indicated that local intradermal immunisation had elevated local antibody concentrations in skin (Colditz *et al.*, 2002). The isotype of the locally produced antibody was not determined.

Protein antigen incorporated in ISCOMs can induce a systemic antibody response when painted onto intact skin in sheep (Colditz and Watson, 1993). In a similar fashion, bacterial ADP-riboslyating exotoxins (BARE) such as cholera toxin can induce systemic immune responses when applied to the intact surface of normal skin in a range of species including sheep (Chen *et al.*, 2000; Chen et al., 2002; Cope and Colditz, 2000) and cattle (Hammond *et al.*, 2000; Morrow *et al.*, 2001). This vaccine delivery method has been termed transcutaneous immunisation (TCI). BARE also act as adjuvants for the induction of immune responses to antigens co-administered by TCI (Glenn *et al.*, 1998). An important feature of this vaccine delivery method is the role of skin hydration in facilitating the uptake of the epicutaneously applied antigens and BARE adjuvant. Exotoxin A from *P. aeruginosa* is a BARE and has been found to induce systemic immune responses when applied to intact skin in mice (Glenn *et al.*, 1999). Ovine isolates of *P. aeruginosa* produce exotoxin A (Burrell and MacDiarmid, 1984; London and Griffith*,* 1984a, 1984b) so it is likely that the strong immunogenicity of *P. aeruginosa* reported in experimental infections and natural fleece rot may be due in part to the capacity of exotoxin A to enhance responses of the host to other pseudomonad antigens. The hydration of skin and subsequent breakdown of the epidermal barrier are features of fleece rot that would also facilitate sensitization of the host.

While local deposition of antigen in skin, for example by intradermal injection with a device like the Skintraction™ injector, can induce local antibody production at the site of vaccination (Colditz and Watson, 1993) as well as systemic antibody responses (Colditz and Paull, 2010), local injection over the whole of the animal body by a transdermal injector is impractical. The transcutaneous immunisation route was designed for systemic vaccination via the transcutaneous route, principally to avoid needle phobia in humans, rather than as a method for whole-of-skin local immunisation. The major drawback for commercial application of TCI in sheep is the requirement for skin hydration. While natural infection with *P. aeruginosa* during fleece rot may mimic TCI in terms of its mode of action for inducing a systemic immune response, failure of natural infection to induce protective immunity suggests that transcutaneous vaccination with a live attenuated *P. aeruginosa* vaccine might not be feasible, again in part for the need for skin hydration that might itself induce fleece rot.

Despite the potential to induce local production of IgA and IgM in skin, the above considerations lead to the conclusion that systemic vaccination by a conventional route such as subcutaneous injection with a formulation designed to promote high titres of IgG1 and IgG2 class antibodies to skin pathogens is the most promising approach for vaccine development. The compatibility of this strategy with potential inclusion of a fleece rot vaccine into existing vaccines for control of other diseases, such as the 5-in-1 vaccine, is unclear. The importance of systemic host immune competence to vaccination responses is discussed in the next section.

Key points on immune responses in skin

- Local production of IgA and IgM antibodies in skin occurs in some types of dermatitis;

- Local production of antibodies in skin can be induced by vaccination;

- Systemic production of IgG antibodies can be induced when antigens are applied to hydrated skin in the presence of bacterially derived adjuvants termed BAREs;

- Exotoxin A is a BARE that is expressed by *P. aeruginosa* and its expression may augment the systemic immune response of sheep to *P. aeruginosa* antigens during fleece rot;

- Induction of local antibody production across the whole skin surface as a control strategy for bacterial dermatitis is impractical.

4.13 Harnessing antibody responses by vaccination to modify skin microbiota

The strength of the systemic antibody response to vaccination is influenced by several factors, particularly:
- Genetics of the sheep;
- Phenotypic status of the sheep;
- Antigens within the vaccine;
- Adjuvant in the vaccine;
- Immunophysiology of antibodies.

4.13.1. Influence of genetics of sheep on immune responses

It has been recognised for several decades that individual sheep vary in the strength of their antibody response to vaccination and that a portion of the variation is heritable (Berggren-Thomas *et al*., 1987; Nguyen, 1984; Raadsma *et al*., 1996). Genes are considered to influence two aspects of the immune responsiveness: (i) specific responsiveness to individual antigens, and; (ii) general responsiveness expressed broadly to many different antigens (Biozzi *et al*., 1971; Raadsma *et al*., 1996). In general terms, specific responsiveness is most strongly influenced by genes associated with the Major Histocompatibility Complex (MHC), whereas general responsiveness is an additive genetic effect influenced by many genes (Glass, 2004). These two aspects of immune responsiveness were studied in detail by Raadsma and colleagues (Raadsma *et al*., 1996; Raadsma *et al*., 1999) in sheep vaccinated with a commercial footrot vaccine and a 5-in-1 clostridial vaccine. The heritability of antibody titres varied from 0.22 and 0.66 for 9 antigens from *Dichelobacter nodosus* while heritability estimates for antigens from *C. tetani* and *C. chauvoei* were 0.12 and 0.24, respectively. Genetic correlations between antibody responses to various pairs of antigens ranged from -0.08 to +0.41. The authors concluded that there was little prospect for selection of sheep with an enhanced capacity to mount strong antibody responses to *all* vaccine antigens.

The Trangie sheep selection lines for resistance and susceptibility to fleece rot and fly strike support this conclusion. Whereas the resistance line expresses higher antibody responses to injection of *Pseudomonas* antigens than the susceptible line as noted above (Chin and Watts, 1991), antibody responses to *L. cuprina* antigens did not differ between lines following artificial infection with larvae. Similarly, antibody responses to the *C. tetani* component of the 5-in-1 vaccine did not differ between selection lines in the CSIRO *Haemonchus* or *Trichostrongylus* selection flocks (Colditz *et al*., 1996b). Thus, immunological differences between selection lines (not discussed here) that are known to contribute to resistance to specific disease did not confer differential immune responsiveness to vaccines unrelated to the specific disease resistance under selection.

Notwithstanding these results and similar findings in other species suggesting that selection for antibody production to vaccination may not be a realistic goal, several studies have examined the potential to selectively breed dairy cattle (Hernandez *et al.*, 2006), beef cattle (Hine *et al*., 2019), pigs (Mallard *et al.*, 1992; Wilkie and Mallard, 1999) and sheep (Hine *et al*., 2017) for a trait termed general immune responsiveness (or general immune competence). In these studies, general immune responsiveness has been based on a combination of antibody and cell mediated immune response measures. High immune responsiveness is favourably associated with temperament and growth rate during weaning in beef cattle, growth rate in pigs, and a reduction of several diseases in dairy cattle. Estimated breeding values for general immune competence are available for beef cattle and dairy cattle for use by Australian producers.

A recent study explored associations between general immune competence, stress-responsiveness and temperament and important health and production traits in 2,613 lambs and 945 adult ewes in the MLA resource flock (Hine *et al*., 2017). General immune competence was moderately unfavourably correlated with the temperament trait and flight time, and moderately positively correlated with haptoglobin responses to the combined effects of vaccination and management-induced stress. Favourable genetic correlations between general immune competence, internal parasite resistance and several carcase characteristic traits including tenderness and intramuscular fat were observed in lambs. Although unfavourable genetic correlations were observed between general immune competence and certain wool traits such as fibre diameter and yield, favourable correlations were observed for other wool traits such as staple strength. The study demonstrated that there is potential to select sheep for *general* immune competence.

An important conceptual difference lies between studies such Raadsma *et al.* (1996, 1999) on antibody responsiveness to vaccination and studies on general immune competence as a trait balanced for antibody and cell mediated activities of the immune system. Antibody and cell mediated responses are often antagonistic and selection simultaneously for both is thought to select for a general reactivity of the immune system that overrides this antagonism. Furthermore, phenotyping for general immune competence is undertaken in the context of stressors such as weaning and repeated handling over a short period of time. Stressors are well recognized to compromise immune function (Dantzer and Kelley, 1989). Hence, general immune competence measured in the face of stressors is thought to provide a measure of resilience to the day to day fluctuations experienced within the production environment that compromise immune function, health, production and welfare (Berghof *et al*., 2018; Colditz and Hine, 2016; Lung *et al*., 2019). In this respect, resilience addresses a diversity of physiological, behavioural and psychological factors experienced in the production environment that modulate performance of the immune system. Refinements to methods for measuring resilience are currently under investigation (Berghof *et al*., 2019; Elgersma *et al*., 2018; Poppe *et al*., 2020). Immune competence as a key component of resilience may have implications for the efficacy of vaccines to help control disease. Studies underway in the Merino Lifetime Productivity AWI project are examining associations between general immune competence, fleece rot and other disease and production outcomes in sheep. Further studies are needed to examine the influence of selection for general immune competence on efficacy of vaccines to protect against disease.

Selection of farm animals over many decades for increased productivity has led to a decreased capacity to cope with day to day fluctuations in the production environment and to increased susceptibility to disease (Rauw *et al.,* 1998). For example, in sheep, selection for clean fleece weight is associated with reduced immune function and increased susceptibility to internal parasite infections (Masters and Ferguson, 2019). Selection for production modifies metabolic activities in target tissues such as muscle or the wool follicle, which leads to a

change in partitioning of metabolic resources (e.g., protein and energy) between tissues. For the immune system, the penalty of reduced priority for use of resources may be especially apparent during the heightened demand associated with strong immune activation by pathogens (Colditz, 2008). The inclusion of immune competence as a "production" trait in the breeding objective provides the opportunity to improve the priority of the immune system for metabolic resources.

Key points on genetics of immune responses in sheep

- Low prospect for selection of sheep with an enhanced genetic capacity to mount strong antibody responses to *all* vaccine antigens.

- Within an animal, the genetic influence on antibody responses varies between the different antigenic components of a vaccine. As a consequence, the relative titres of antibodies to various antigens within a vaccine will differ between sheep, and animals that respond poorly to some of the antigens in a vaccine are likely to be found in every population. Vaccines are therefore unlikely to protect all animals in a flock.

- Resilience, which can be identified in part by the strength of immune responses to vaccination, is a measure of the capacity of animals to maintain health and production in the face of day to day challenges such as disease, routine husbandry practices, and fluctuations in weather.

- Resilience helps draw attention to the fact that adaptive immune responses are not the sole means by which vaccines can improve disease resistance (Netea *et al.*, 2020).

- Resilience may enhance the capacity of commercial vaccines to protect sheep against disease.

4.13.2. Effect of the phenotypic status of the sheep on antibody responses

Antibody responses to vaccination are weaker in lambs, rams, during late pregnancy, during a number of diseases, when animals are in poor body condition and when animals are stressed. While fleece rot is usually associated with periods of wet weather lasting a week or more, it is unclear whether immune functions are compromised during the course of weather events of this nature (Dwyer and Bornett, 2004).

4.13.3. Effect of the antigenic composition of a vaccine on antibody responses

In addition to genetic factors of the host influencing the strength of the antibody response to vaccination, the antigens present within a vaccine can interact in a process termed antigenic competition that modifies relative antibody responses to each antigen. This issue has been particularly important in the development of footrot vaccines comprised of multiple serotypes of closely related fimbrial antigens. Importantly, Raadsma *et al.* (1996) noted that heritability of the antibody responsiveness to footrot vaccine antigens was not correlated to mean antibody titre. Thus, just because an antigen can induce a strong antibody response does not mean that antibody responses to that antigen will have a high heritability. This suggests that non-genetic factors such as competition between antigens for recognition by the immune system can influence antibody responses (Finney *et al.*, 2018). The phenomenon has importance for development of multivalent vaccines against bacterial strains

with similar (but not cross-reactive) immunological identity (e.g., the footrot vaccine (Hunt *et al.*, 1995)). Antigenic competition occurs when different antigens are mixed and inoculated together. Each antigen can induce an immunologic response when inoculated alone, however, when inoculated with other antigens there is a suppressed immunological response to one or more of the antigens. This response can confound vaccine efficacy that aims to protect a host from all bacterial strains represented in the vaccine.

4.13.4. Immunophysiology of antibodies

The generation and decay of antibody titres following vaccination are influenced by the vaccine formulation, the half-life of the immunoglobulin isotypes contributing to antibody and possibly by loss of antibody into sinks such as colostrum during lactogenesis in late pregnancy, the gut lumen during some parasite infections, and due to enhanced catabolism in animals in poor body condition. In a typical adaptive immune response, antibody titres rise for 4 to 8 weeks following vaccination and subsequently decay in accord with the half-life of each immunoglobulin isotype that contributes to the titre. The half-life of IgG1 in sheep, measured by the decay of antibody reactivity in serum following passive immunisation, is between 12 and 24 days (Watson, 1992). Similar values are reported for other species (Hedegaard and Heegaard, 2016). Following vaccination, plasma cells can continue to produce antibody for extended periods of time, thus the decline of antibody titres following vaccination is slower than the decline following passive immunisation and the titre can be increased again by boosting injections.

Importantly, antigenic stimulation of the host by disease pathogens during the course of a disease can have a substantial impact on the kinetics of antibody titres. (This is the usual way by which antibody responses to disease pathogens are induced). Vaccines that rely on antibody to protect animals against pathogens that contain antigens that are normally "hidden" from the host immune surveillance system during natural infections pose substantial difficulties for vaccine development (Willadsen *et al.*, 1993). Examples are provided by the TickGard™ vaccine for cattle and the BarberVax™ vaccine for sheep. In animals primed and boosted with BarberVax™, egg counts remained suppressed at 6 weeks but not at 8 weeks following boosting (David Smith, personal communication). Protection provided by this vaccine is thought to be mediated by IgG antibodies to gut antigens of *Haemonchus*, which are hidden from the sheep's immune system. In the absence of boosting, efficacy of this vaccine requires frequent revaccination. Intervals up to 6 weeks between boosts are recommended in a vaccination program that is tailored to the age of the sheep and the epidemiology of the parasite (http://barbervax.com.au/how-to). The titre of antibody required to provide protection against disease can differ by orders of magnitude between pathogens. Thus, in contrast to the rapid decline over several weeks in protection provided by BarberVax™, protective concentrations of antibody can be present in plasma for years following tetanus vaccination in the absence of (known) boosting by exposure to the pathogen in the interim.

Studies on fleece rot indicate that animals can become sensitized to several skin pathogen antigens in the absence of vaccination. This suggests that natural boosting to vaccine antigens might occur in the field and thus reduce the frequency at which boosting is required.

4.13.5. Vaccine adjuvants

Antigens, adjuvants, other formulation components (termed excipients) and the route of vaccine administration all influence the immune response to vaccination. Adjuvants can influence the strength of the immune response as well as the bias between cell mediated and antibody mediated responses, and the immunoglobulin isotypes of antibodies. The effect of an adjuvant is not always consistent across different antigens. As a consequence, a very common requirement in vaccine development is the need to screen a range of adjuvants for their effect on the immune responses that are targeted by the vaccine. Thus, adjuvants have been screened for their capacity to stimulate IgG antibody to experimental fly strike vaccine antigens (East *et al*., 1992), TickGard[TM] and BarberVax[TM] (David Smith, personal communication). In all three instances, Quil-A or the water-in-oil adjuvant Montanide ISA50V2 proved to be the most effective candidate adjuvants examined that were also commercially acceptable and these would be strong candidates for inclusion in a new vaccine against skin microbiota. There have also been many technological developments in vaccine adjuvants over the last 15 years that provide improved options for generating strong and relevant immune responses (Young, 2019; Reed *et al.*, 2013). Notably, an adjuvant termed Montanide ISA 61VG is a new generation water-in-oil emulsion adjuvant developed and recommended for sheep vaccination promoting cellular immune and IgG responses. While adjuvants have the capacity to enhance immune responses, they do not overcome the limitations imposed by age or genetic factors on immune responsiveness in sheep (Watson *et al*., 1994).

Key points on harnessing antibody responses to vaccination to control skin microbiota

- A portion of the flock may respond poorly to some antigens in any vaccine;

- Genetic selection for general immune competence may be more effective than genetic selection for antibody responsiveness as a strategy to improve protection from disease in vaccinated sheep;

- Impact of weather stress on natural and acquired antibody responses to skin pathogens is unknown;

- Natural boosting by exposure to antigens from skin pathogens could stimulate a longer duration of protection for a putative skin pathogen vaccine than is seen for vaccines like BarberVax[TM] and TickGard[TM] that contain "hidden" antigens;

- During development of a new vaccine to control skin microbiota, it is likely that a panel of adjuvants would need to be screened;

- Antigenic competition is a potential challenge to development of a vaccine that contains antigens of close immunological identity;

- Within the current knowledge base for sheep vaccinology, molecular modelling is likely to have little power to predict antigenic competition in sheep. Animal studies will be needed to establish the importance of the phenomenon for future vaccines. (It is noteworthy that antigenic competition does not appear to be a problem for multivalent clostridial vaccines used in sheep).
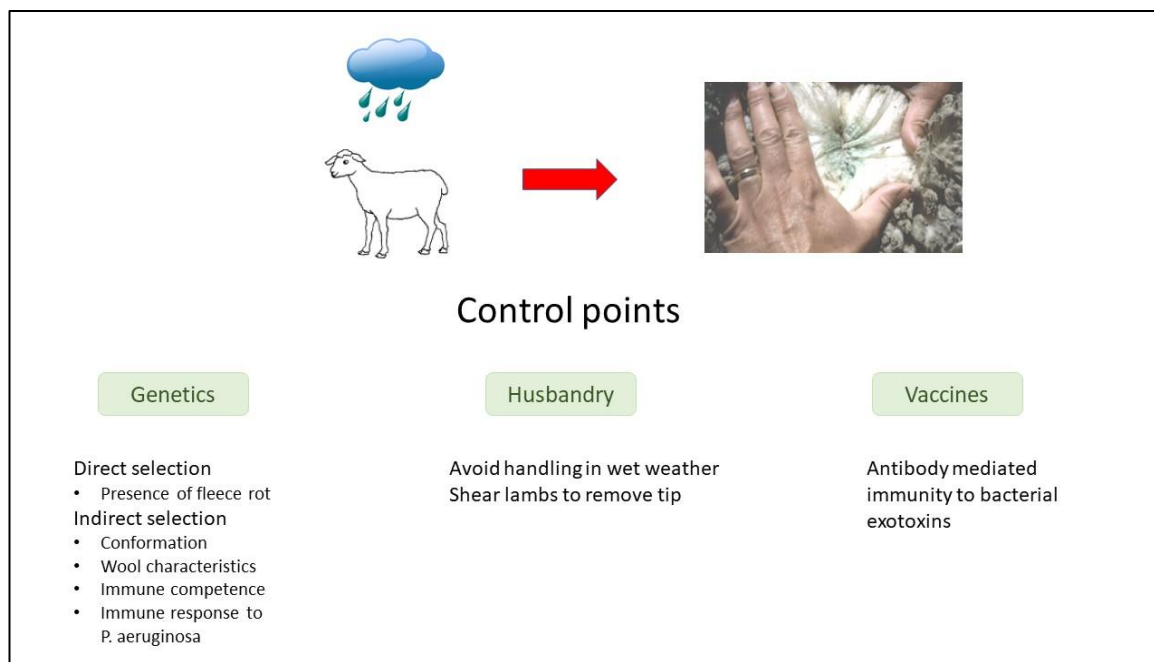
Figure 2. Control points for fleece rot

5.0   Lumpy wool

5.1   Background

To determine the potential of a vaccine for controlling a disease, there is a strong need to identify and understand a broad array of factors that may be important in determining vaccine efficacy. These factors include the environment, lifecycle of the disease agent, genetics of the infected animal, strain variations of the infective agent, identification of protective immune responses in the affected animal, vaccine design and inoculation strategy, and an array of commercial factors. The following text provides information and our conclusions about the importance of these factors for the control of lumpy wool outbreaks in sheep.

Lumpy wool is an exudative dermatitis of sheep skin characterized by crusting and matting of wool (Bull, 1929; Seddon, 1927; Norris *et al.*, 2008; Gardiner, 1971; Roberts, 1965; Roberts, 1966; Ellis *et al.*, 1993; Sheep CRC, 2020; NSW Industry and Investment, 2010; Moriello, accessed 2020). Lesions occur over the dorsal mid-line and spread laterally and ventrally (Berry and Watt, 2017; Radostits *et al.*, 2006). Infections of sheep can be acute, chronic, or sporadic. The major source of body infection is from chronically infected sheep with active lesions typically on the ears and face (Berry and Watt, 2017). The causative microbial agent is *Dermatophilus congolensis,* which is a facultative anaerobic actinomycete spread in sheep by contact transmission (Bull, 1929; Zaria, 1993; Moriello, accessed 2020; Roberts, 1965; Leoni *et al.*, 1993). The evidence for a causal involvement of the bacterium in generating lumpy wool includes: (i) a strong association of the presence of this bacterial species with lumpy wool (Bull, 1929; Roberts, 1965; Moriello, accessed 2020; Ellis *et al.*, 1993; Leoni *et al.*, 1993; Awad *et al.*, 2007); (ii) experimental infections of sheep skin with *D. congolensis* resulted in lumpy wool (references cited in **Table 1**); (iii) antibodies specific to this bacterial species were induced in sheep with lumpy wool (references cited in **Table 1**); and (iv) experimental vaccines containing components of *D. congolensis*

showed some evidence of protection of sheep from lumpy wool (references cited in **Table 1**). *D. congolensis* also causes a world-wide distribution of infectious dermatitis in a broad group of livestock animals including sheep, cattle, horses, goats, camels as well as nonruminant animals including dogs, cats and rabbits (Faris and Hollis, 2013; Marsella, 2016; Ambrose, 1996a; Ambrose, 1996b; references cited in **Table 2**). In the absence of adequate hygiene practices, humans can also be infected (Kaminski and Suter, 1976; Faris and Hollis, 2013; Zaria, 1993).

After initial infection of the skin by *D. congolensis*, a hard scab is formed, which then lifts from the skin as the fleece grows to produce localized, crusted, hard pyramidal masses of often discoloured wool (Bull, 1929; Gardiner, 1971; Roberts, 1966; Moriello, accessed 2020; Norris *et al.*, 2008). These areas of affected wool become an impediment to shearing. Lumpy wool is also called dermatophilosis and sometimes erroneously called mycotic dermatitis (*D. congolensis* is a bacterium and not a fungus). Wetting of fleece for an extended time encourages spread of the infection and thus sporadic outbreaks of lumpy wool are associated with seasonal rain (Berry and Watt, 2017; Gardiner, 1971; Zaria, 1993; Marsella, 2016; NSW Industry and Investment, 2010). Lumpy wool is thought to be predominantly spread within a flock by contact transmission between infected and uninfected sheep although there is a suggestion of transmission via sheep dips that have no or inadequate antibacterial agents (Gardiner, 1971). It is noteworthy that bovine dermatophilosis is spread by tick infestations (Ambrose, 1996b). There is speculation that dermatophilosis in sheep could be spread by blowflies and lice, although there is no direct evidence (Gardiner, 1971; Awad *et al.*, 2007).

D. congolensis has a complex life cycle involving two morphological forms, filamentous hyphae, and motile zoospores (Moriello, accessed 2020; Ambrose, 1996a; Ambrose, 1996b; Marsella, 2016). Hyphae are composed of filaments coated by a mucoid capsule that develop into coccoid cells and these then mature into flagellated zoospores, the infective and motile agent. Persistent wetting of the sheep skin disperses the protective waxy layer on the skin and softens the skin making it vulnerable to zoospore infection from other animals. These conditions promote *D. congolensis* replication and repeated invasion of the epidermis by hyphae resulting in a reactive infiltration of the tissue by immune cells, particularly neutrophils. The consequent inflammation of the infected tissue generates an exudate on the skin surface (Roberts, 1966; Ellis *et al.*, 1989). The exudate maintains hydration on the skin surface near wool follicles and may provide additional nutrition to the bacteria thereby promoting a further cycle of *D. congolensis* proliferation (Ellis *et al.*, 1987; Gardiner, 1971; Ambrose, 1996). Roberts suggested that infection occurs in lambs before the skin wax layer was properly formed or as a result of wet weather that compromises skin barrier function (Roberts, 1965; Roberts, 1966). He also noted that various management practices could facilitate infection.

Ellis *et al.* (1987) concluded from the research of Roberts (Roberts, 1966; Roberts, 1967) that "*resolution of lesions formed by D. congolensis was associated with delayed-type hypersensitivity whereas resistance* (to infection) *was associated with antibodies to somatic* (cellular) *antigens of D. congolensis"*. The latter conclusion is consistent with the development of resistance to infection via naturally acquired immunity. Sheep with lumpy wool are associated with "*lowered production of wool, decreased wool value, culling losses, treatment costs and difficulty shearing*" (Berry and Watt, 2017; Bateup and Edwards 1990; Edwards 1985; Edwards *et al.*, 1985). It is suggested that lumpy wool may be a predisposing factor for fleece rot and both conditions are predisposing factors for blowfly strike (Gherardi *et al.*, 1981; Gherardi *et al,* 1983; NSW Industry and Investment, 2010; Wilkinson, 1979), which has substantial financial, environmental and animal ethics impacts on the wool industry (Norris *et al.*, 2008; Tellam and Bowles, 1997; James *et al.*, 2019). Thus, effective prevention of lumpy wool is a strategy that can potentially decrease the incidence of body strike in sheep. Lumpy wool also reduces the efficacy of pour-on insecticides used for lice control (NSW Industry and Investment, 2010).

Management of lumpy wool on sheep is achieved by monitoring spontaneous self-healing or in the case of severe infections the use of intramuscular injection of sheep with long-lasting antibiotics (Berry and Watt,

2017). The antibiotics used are oxytetracycline or penicillin (Benacellin™) administered by a single intramuscular injection. The use of long-lasting antibiotics ensures that animal handling occurs only once. The efficacy of antibiotic treatment is reported as variable (Berry and Watt, 2017; Norris *et al.*, 2008; Zaria, 1993; Scrivener and Vizard, 1995). Moreover, there is increasing public and human health care disquiet about the use of antibiotics in livestock animals and especially the promotion of antibiotic resistance in unrelated bacteria in the environment and the presence of antibiotic residues in sheep products. Therefore, the availability of antibiotics for control of lumpy wool in the medium to long-term future is uncertain (Norris *et al.*, 2008). Management of dermatophilosis also involves standard human hygiene practices after direct contact with infected animals and farm tools. Additional management practices in use include isolation of infected sheep, the avoidance of penning groups of infected and uninfected sheep, clipping of wool, drying affected sheep, disinfection of lesions, disinfection of equipment, use of dips containing antibacterial additives and zinc sulfate sprays (Sheep CRC, 2020; Gardiner, 1971; NSW Industry and Investment, 2010). No commercial vaccines are currently available for protecting sheep from *D. congolensis* infection despite some limited experimental efforts in the late 1980s and early 1990s.

Key points on background

- *D. congolensis* is the likely cause of lumpy wool;

- *D. congolensis* has a complex life cycle on the skin surface;

- Current management relies on monitored self-healing and antibiotics in severe cases.

5.2   Susceptibility of sheep to *Dermatophilus congolensis*

The susceptibility of sheep to *Dermatophilus congolensis* is complex and likely strongly modified by interacting combinations of immune responsiveness, environmental conditions, age, gender, bacterial strain variation, bacterial life cycle, bacterial pathogenicity factors, protective immune responsiveness in sheep developed after infection, and genetic resistance of sheep to infection. The genetic resistance factor could relate to both the genetics underpinning wool and skin structure, and an effective immune system (Sheep CRC, 2020). Sheep of all ages are susceptible to dermatophilosis; however, the infection rate is higher in younger animals (Berry and Watt, 2017; Norris *et al.*, 2008). The latter observation may reflect changes in wool fibre physical structure with age (particularly lower wax levels in young sheep and wool fibre diameter) (Edwards *et al.*, 1985), maturation of the sheep immune system with age (Watson *et al.*, 1994), and/or acquired immunity (Gardiner, 1971; Norris *et al.*, 2008). Teasing out the relative contributions of these factors to sheep resistance has been difficult. Chronically infected sheep can act as a reservoir of infection in the flock and those sheep that are unresponsive to antibiotic treatment are culled to remove the risk of infection and improve the genetics-based resistance of the flock to dermatophilosis (NSW Industry and Investment, 2010).

Key points on susceptibility of sheep to *Dermatophilus congolensis*

- Susceptibility of sheep to *D. congolensis* is complex and multifactorial.

- Lumpy wool is more likely in younger sheep.

5.3 Genetic susceptibility and resistance of sheep to dermatophilosis

The identification of ovine phenotypic or genetic markers of resistance to dermatophilosis infection may potentially be used in selective breeding programs to progressively enhance resistance to the infection in a population of sheep over multiple generations. The advantages of this approach are that the increased resistance is permanent in the selected population, incremental increases of resistance can be obtained in each generation, and the use of genetic (DNA) markers can accelerate the resistance gain in each generation. In practice, however, the extent of genetic contribution is low and variable (Norris *et al*., 2008). The resistance heritability using experimental infections of sheep was reported as less than 0.14 (Lewer *et al*., 1987) and 0.15 (range 0.05-0.44) (Raadsma *et al.*, 1988). The heritability of the severity of the *D. congolensis* infection in sheep ranged between 0.25 and 0.42, i.e., moderately heritable (Raadsma, 1992). There have been no recent confirmatory measurements of these heritability values. Thus, sheep have a low level of heritable resistance to dermatophilosis but a greater genetic contribution to the regulation of severity of the infection. Apart from the culling of chronically infected sheep unresponsive to antibiotic treatment from breeding programs, there has been no concerted effort to use this information in breeding programs. One prediction may be that selective breeding for resistance will not greatly influence the infection rate in sheep but could affect the scale of the infection on sheep, especially the speed of healing of lesions caused by dermatophilosis.

Genetic variants in the ovine MHC-DQA2 major histocompatibility locus have been associated with resistance to a bacterial dermatitis of the hoof (footrot) caused by the bacterium *Dichelobacter nodosus* and a commercial molecular marker is available for selective breeding for resistance to this disease (Escayg *et al*., 1997; Norris *et al*., 2008). A bovine genetic marker in the major histocompatibility region (boLA-DR/DQ) was linked with high susceptibility of cattle to bovine dermatophilosis. A selective breeding programme using this bovine genetic marker to eliminate affected animals in a breeding program resulted in "*marked reduction in disease* (dermatophilosis) *prevalence*" (Mallard *et al*., 2003). However, the selection of animals based on genetic variation at the major histocompatibility complex may unintentionally adversely impact resistance of animals to unrelated viral, bacterial, or parasitic diseases (Norris *et al*., 2008). Hence, the general value of DNA markers located within the major histocompatibility genetic locus for use in livestock selective breeding programs for disease resistance enhancement is unclear.

The genetic resistance of sheep populations to infectious challenge by *D. congolensis* may be a minor contributor to the total disease variation in the population due to strong environmental influences, like weather, and considerable bacterial strain variation (see Section 5.4). It is also likely that the genetic contribution to disease resistance in the sheep population could be directly modified in a complex interactive manner by the environmental effects and bacterial strain variation. In addition, in most animal models, the genetics of disease resistance is typically polygenic, i.e., genetic resistance is due to many (likely interacting) genetic variants, each of small effect size. In summary, insufficient specific genetic markers are currently available for efficient selective breeding of sheep populations for resistance to lumpy wool. In general, individual genetic markers will not be efficient in selective breeding programs and therefore a whole genome selection strategy will be required for genetic progress in the programs. The molecular tools that enable this approach are now available for sheep (Jiang *et al*., 2014; Naval-Sanchez *et al*., 2018; Al-Mamun *et al*., 2015).

Key points on genetic susceptibility and resistance of sheep to dermatophilosis

- Some genetic resistance of sheep to infection but heritabilities are generally low;

- Strong environmental influences on disease incidence.

5.4   *D. congolensis* strain variation

Microbial strain variation is an evolutionary strategy that increases the survival chances of a microbial species in a variable environment; hence, genetic variation is widely prevalent. Vaccination approaches against microbial diseases are sometimes confounded by microbial strain variation. *D. congolensis* has considerable strain variation (Ellis *et al.*, 1993; Ellis *et al.*, 1991; Gogolewski *et al.*, 1992; Masters *et al.*, 1997a; Masters *et al.*, 1997b). The morphological and chemical properties of thirty isolates of *D. congolensis* that infect sheep from throughout Australia showed substantial variation in haemolytic activity on blood agar, mucoid nature of colonies, motility, flagella density and polarity, restriction enzyme profiles of the bacterial DNA, proteins, carbohydrate content and a range of enzyme activities (Ellis *et al.*, 1993). The ranking of the infectivity of these isolates was associated with isolate haemolytic activity and three enzymatic activities; these activities may be highlighting the effects of virulence factors. The differences in restriction enzyme profiles highlight slight differences in the DNA present in each strain and hence a genetic basis to the different characteristics of the isolates. In many cases in the past, the technologies used to detect strain variation at the DNA level often had low resolving power by current standards i.e., DNA restriction length polymorphisms, SDS-PAGE of polypeptides, immunoblots and PCR of ribosomal RNA. Thus, the extent of *D. congolensis* strain variation may be substantially underestimated.

D. congolensis strain variation is associated with differential pathogenicity in sheep (Ellis *et al.*, 1991). The relative infectivity of each strain is unclear. Strain variation can also induce differential immune responses in sheep after natural infection, differential immune responses in experimentally infected sheep (Ellis *et al.*, 1991), and differential protective immune responses in sheep generated using candidate vaccine antigens from different bacterial strains (Ellis *et al.*, 1991). Similarly, dermatophilosis in cattle is also associated with *D. congolensis* strain variation (Ambrose *et al.*, 1999; Hiraizumi and Tagawa, 2014; Ambrose, 1996b; Ambrose *et al.*, 1997; Larrasa *et al.*, 2004; Larrasa *et al.*, 2002).

Key Points on *D. congolensis* strain *variation*

- Substantial *D. congolensis* strain variation;

- Strain variation associated with incidence and severity of lumpy wool.

5.5   Acquired natural immune resistance to *D. congolensis* in sheep

The presence of acquired natural immunity in an animal to a disease is the hallmark of a protective immune response to an infectious agent. This is highlighted by the ability of the immune system to retain the memory of a previous microbial infection and mount a rapid neutralizing immune response to subsequent infection. This type of response provides a strong feasibility statement for the development of a vaccine that mimics this natural response but without the adverse effects of the disease agent.

Potential naturally acquired immunity could help explain the decreasing prevalence of dermatophilosis with sheep age. However, there is contradictory information about whether dermatophilosis in sheep generates a natural protective immune response to subsequent infections. Moreover, the relevant scientific information is limited. Documentation from the Sheep CRC indicates that most sheep develop immunity to dermatophilosis after 4-6 weeks of infection (Sheep CRC, accessed 2020). Roberts (1966) also described acquired immunity of sheep to dermatophilosis. Dermatophilosis in cattle is thought to generate a naturally acquired immunity (Ambrose *et al.*, 1999).

In another study, healthy unexposed sheep were compared with healthy sheep having a history of chronic *D. congolensis* infection, after a controlled challenge with *D. congolensis* zoospores (Ellis *et al*., 1992). There were more lesions and weaker lymphocyte response to the skin infection in the chronically infected group despite this group having a stronger antibody response to *D. congolensis*. The sheep groups had no differences in fleece characteristics, skin wax and suint (natural grease in wool) concentrations that could account for the differences in susceptibility of these two groups.

Comparison of a group of chronically infected merino sheep without active lesions with a group that has naturally recovered from dermatophilosis after reinfestation challenge with *D. congolensis* zoospores showed similar reinfection rates, severities of lesions, rates of resolution of the disease and abilities of sheep sera to kill zoospores (Ellis *et al*., 1989). Despite these disease response similarities, there were several immunological and inflammatory response differences between the two sheep groups. This information suggests that the latter response differences were irrelevant to the ability of the sheep to control the challenge infections.

Collectively, these studies are difficult to reconcile. Some studies provided evidence of acquired immunity to *D. congolensis*, however another study demonstrated that chronically infected sheep having a weaker protective immune response compared to the unexposed sheep group, and that antibody titre to *D. congolensis* generated by the infected sheep was an indication of a functional humoral immune response, however, it was unrelated to protection from disease. The presence of a strong and specific antibody response to *D. congolensis* is important as it demonstrates that the infection is activating the immune system even though the infectious agent is only present in the surface layers of the skin i.e., the immune surveillance system in the sheep is recognising *D. congolensis* as a potential threat and mounting a humoral (antibody-mediated) defence. The lack of antibody-mediated protection could be a consequence of raising a humoral immune response to the infectious zoospores rather than the invasive hyphae life-stage present in the epidermis, which is likely responsible for generating skin inflammation and disease pathology. In addition, there could be insufficient antibody response to specific and crucial *D. congolensis* antigens at the site of the infection. Thus, the protective role of antibodies to *D. congolensis* antigenic components is unclear. Another possibility is that sheep acquire natural immunity only after natural infection with a succession of *D. congolensis* strains.

Key Points on acquired natural immune resistance to *D. congolensis* in sheep

- Natural infection of sheep with *D. congolensis* generates specific antibodies even though *D. congolensis* is only present on the skin surface and within the outer external layer of the skin i.e., the sheep immune surveillance system responds to *D. congolensis* infection on skin.

- There is inconsistent evidence that repeated infection of sheep with *D. congolensis* generates a protective immune response.

5.6   Antibody to *D. congolensis* on sheep skin

Specific antibody responses to three *D. congolensis* antigens (flagella, filament and soluble antigen) from different life-stages of *D. congolensis* were investigated in sera and skin surface washings from sheep experimentally infected with three temporally separated inoculations of *D. congolensis* (Sutherland, *et al*., 1987). The serology demonstrated that there were strong and rapid (7-21 days) antibody responses in sheep sera to each of these antigens. Antibody was also present in skin washings but it was detected later (28-42 days) than in sera and was more variable. This investigation demonstrated that *D. congolensis* life-stage specific antigens can induce a strong and specific antibody response in the sera of sheep *and* on the surface of sheep skin. The latter is likely mainly mediated by transudative movement of the antibody isotypes IgG1 and IgG2 from

serum to the skin surface (Colditz *et al.*, 1992a; Lloyd *et al.*, 1987). In cattle, there was also active transport of IgA and IgM antibodies through a local secretory process onto the skin (Lloyd *et al.*, 1987). Notably, new technologies have been developed for inducing strong local immune responses in the skin by intradermal injection of antigens, however it is unclear whether these approaches are practical for the protection of sheep from lumpy wool (Wallis *et al.*, 2019; Colditz and Watson, 1993; Gill *et al.*, 1993; Colditz *et al.* 1992a).

In general terms, there is potential for an induced antibody to a *D. congolensis* antigen(s) to bind and neutralize the infectivity of *D. congolensis* at an infection site and thereby generate a protective immunity resulting in control of the incidence of lumpy wool in sheep. However, there are several factors crucial for success that are not yet clear including, identification of specific and effective *D. congolensis* life-stage antigen(s), induction of a relevant antibody isotype in and onto skin, production of sufficient quantity of neutralizing antibody at the skin infection site, and the period of protection of sheep (at least one season but preferably lifetime immunity). In addition, there needs to be an easy, safe, reproducible and cost-effective means of scaling up antigen production and ultimately a commercially acceptable value proposition for vaccine development.

Acquired immunity to a disease agent can also be mediated by specific immune cells. Lymphocytes and macrophages are enriched in skin tissue at sites of *D. congolensis* infection (Ellis *et al.*, 1987) however, there is no direct evidence that these immune cells in skin kill or inhibit the reproduction of *D. congolensis*. There are very few studies reported for this specific area of research in relation to *D. congolensis* infection. Moreover, it is highly unlikely that live and fully functional lymphocytes will be present in dermatophilosis exudate.

Key points on antibody to *D. congolensis* on sheep skin

- Washings from sheep skin affected by lumpy wool identified specific antibodies to *D. congolensis.*

5.7   Experimental vaccines to protect sheep from dermatophilosis

During the late 1980s and early 1990s, there were attempts to produce experimental vaccines that protect sheep from *D. congolensis* infection (Norris *et al.*, 2008). **Table 1** summarizes these investigations. **Table 2** summarizes, more briefly, parallel investigations in cattle. "*Overall conclusions*" in the tables are comments from the authors of this review.

There is an unfiled provisional Australian Patent application (Australian Patent Office Number 1986908866; provisional patent, PH8866; Applicant, The State of Western Australia; 1986) entitled *Dermatophilosis Vaccine*. The absence of full patent filing and particularly the absence of an attempt at commercialisation of the vaccine implies that there were significant technical issues and/or market limitations at the time.

D. congolensis likely secretes proteins, especially proteases to aid removal of the protective outer keratin layer of skin, lipases to remove skin wax and haemolysins to allow bacterial invasion of cells, that collectively facilitate the bacterial invasion of skin. These enzymes may be virulence or pathogenicity factors. They could be specifically targeted using a vaccine to generate antibody-mediated neutralisation of the functions of these factors potentially leading to decreased infectivity and lesion severity (How *et al.*, 1990). However, the identification of these factors has not been a systematic process. None have been proven to be pathogenicity or virulence factors and few individual proteins have been tested in experimental vaccines. Roberts concluded that *D. congolensis* did not produce any factors that resulted in the killing of host phagocytes or leukocytes (Roberts, 1965; Roberts, 1966). This result suggests that *D. congolensis* does not secrete factors that contribute to the pathogenesis of infection as measured by the immune cell killing assay. The inference from this research is that the strong inflammatory response to infection may be attributable to products arising from mild cellular damage

in the skin caused by the penetrating hyphae. However, there are many other ways in which these bacteria could generate pathology via the secretion of bacterial products and hence the absence of secreted pathogenicity factors is unproven and unlikely. Another potential virulence factor is the flagellar protein of zoospores (Hiraizumi and Tagawa, 2014). Antibody-mediated binding to this protein could hinder zoospore motility and therefore infectivity.

Key points on experimental vaccines to protect sheep from *dermatophilosis*

- There were some vaccine effects but these were weak and inconsistent;

- There was no consistently identified antigen that was the most efficacious;

- Most antigens were not trialled in natural challenges in field trials;

- The extent of *D. congolensis* strain variation was unclear and there may have been substantial strain-specific effects both in the formulation of vaccine antigens (potential for immunodominance effects) and experimental and field challenges;

- Most vaccines were formulated to produce neutralising antibody-mediated effects; preferential antibody production and antibody isotype production in skin may have been required;

- The potential for using secreted pathogenicity or virulence factors has not been thoroughly tested;

- The absence of protection of sheep from repeated infections indicated that the specific antibody response to *D. congolensis* detected in the skin is irrelevant. Therefore, a protective vaccine may need to generate specific antibodies on the skin surface that bind to antigens not normally seen by the immune surveillance system during repeated infections.

Specific aspects of these conclusions are also supported by various reviewers of this area of research for both sheep and cattle dermatophilosis (Norris *et al*., 2008; Mine, 1996; Tabar, 1998; Berry and Watt, 2017). Perhaps the most significant limitations in terms of past vaccine development were complications in vaccine design caused by *D. congolensis* strain variation, an inability to reliably reproduce the disease experimentally and a poor understanding of skin immunity.

Table 1. Experimental vaccines tested for control of dermatophilosis in sheep. Ag, antigen(s); Ab, antibodies.

Vaccine antigen (life-stage)	Experimental model	Targeting of virulence factors?	Comments	Ref.
Live *D. congolensis* (zoospores)	3 inoculations with live *D. congolensis*; assessment of dermatophilos after each microbial challenge	unknown	• Strong inflammatory and immune cell responses after 1st inoculation. Resolution of lesions after 14-38 days. • 2nd inoculation 70 d after 1st failed to produce lesions suggesting naturally acquired immunity. • 3rd inoculation at 140 d associated with development of skin lesions, which resolved after a further 13 d. • Subset of sheep with lower inoculation doses did not develop skin lesions after 3rd inoculation, suggesting development of acquired immunity. • Speculation that humoral immunity involving IgA was involved. • **Conclusion**: no strong evidence of acquired immunity from experimental exposure of sheep to live *D. congolensis.*	Ellis *et al.,* 1987
D. congolensis life-stage Ag: Ag A, crude hyphae filaments; Ag B, zoospore protein and mucoid material	Intradermal vaccination of sheep. Assessed sheep for dermatophilos is skin lesions after experimental and natural challenge with *D. congolensis*.	unknown	• Vaccine A sheep had fewer and less severe lesions than vaccine B sheep and controls. • After natural challenge, Vaccine A sheep had the same number of lesions as control sheep. • After natural challenge, Vaccine A sheep were similar to control group. • **Conclusion:** Vaccine A more effective than Vaccine B in experimental challenge; both vaccines had no protective effects in natural challenge.	Ellis *et al.,* 1991
Zoospore, filamentous and soluble Ag (zoospore, hyphae and secreted proteins).	Sheep challenged with *D. congolensis* zoospores	Secreted proteins and filaments could contain virulence or pathogenicity factors.	• 1st Experiment: Number of sheep vaccinated with filamentous Ag and protected was greater than the control group. • 2nd Experiment: filamentous Ag and control sheep groups both developed skin lesions after challenge, but lesions less severe for filamentous group. • Ab present on the skin surface was variable. • **Conclusion:** Partial protection using filamentous antigen. No evidence Ag are virulence factors. Variable Ab responses on skin surface.	Sutherland and Robertson, 1988 Sutherland *et al.,* 1987
Heat-inactivated filamentous phase *D.*	Sheep challenged experimentally with *D.*	No	• Sheep vaccinated with live filaments showed reduction in lesion severity; result specific to only one of the two challenge strains.	Ellis *et al.,* 1991

congolensis or live isolated *D. congolensis* filaments.	*congolensis* zoospores		● **Conclusion:** Partial protection of sheep using dead filamentous Ag or live filaments when experimentally challenged. Vaccine efficacy depended on strain of challenge.	
Live crude filaments or dead zoospore protein and mucoid material	Sheep challenged with *D. congolensis* zoospores and field trial challenge	No	● fewer lesions in group vaccinated with crude live filaments; experimentally challenged ● no difference between sheep vaccinated with filaments compared with control group. ● **Conclusion**: The live filament vaccine did not protect sheep in a field trial.	Sutherland *et al.*, 1991
D. congolensis serine protease	Not tested	possible	● Serine protease identified and cloned. ● Serine protease peptide synthesised; sheep vaccination induced specific Ig. ● Expressed as recombinant protein but not tested as vaccine Ag. ● **Conclusion:** No evidence protease was a virulence factor.	Mine, 1996
Peptides from phage libraries screened with Ig to crude preparation; and recombinant serine protease	Sheep challenged with zoospores from two *D. congolensis* serine protease strains.	possible	● Peptides and recombinant serine protease were antigenic. ● Sheep vaccinated with peptides or recombinant serine protease; increased resolution of lesions when challenged with one strain. ● **Conclusion:** No evidence that secreted enzymes were virulence factors. Faster, but strain-specific resolution of lesions after *D. congolensis* challenge. Weak effects.	Tabar, 1998 Tabar and Carnegie, 2002

Table 2. Experimental vaccines tested for control of dermatophilosis in other species, besides sheep. Ag, antigen(s); Ab, antibodies.

Vaccine antigen (life-stage)	Experimental model	Targeting of virulence factors?	Comments	Ref.
Experimental infection with *D. congolensis.*	Mice challenged to test for acquired resistance	Unknown	• Intact skin resistance to primary infection but abrasion, or methanol/ether washes increased infection rate. • A range of pathogenicity using different mouse genetic lines. • No vaccination trials	Lloyd and Noble, 1982
D. congolensis zoospores (intradermal injection)	Rats challenged with *D. congolensis*	Unknown	• Fewer zoospores on skin.	Davis, 1988
Ag used for vaccination unclear.	Effect of cattle skin washings on motility of zoospores	Yes	• Skin washings from vaccinated cattle had no effect on zoospore motility. • Sera immobilized and clumped zoospores via a coat around the flagella.	Jenkinson *et al.*, 1989
D. congolensis secreted proteins	Experimental and natural challenges of cattle with *D. congolensis*	Unknown	• Sera from infected cattle identified subset of *D. congolensis* secreted proteins. • Positive correlation between severity of infection and number of immunoreactive secreted proteins. • *D. congolensis* strain-specific differences in secreted proteins. • Secreted proteins contain antigens possibly involved in immunity or immunopathogenesis of dermatophilosis. • Immunity to dermatophilosis might involve non-classic responses mediated by gamma-delta T cells but no evidence.	Ambrose, 1996a; Ambrose *et al.*, 1997; 1999
D. congolensis zoospores	Experimental challenge of rabbits with *D. congolensis*	Unknown	• Vaccination caused enhanced resistance to zoospore infection.	Roberts, 1966

6. Advances in enabling technology

6.1 Introduction

Most research on fleece rot and lumpy wool was undertaken about three decades ago. Technology changes over the last 15 years relating to understanding and exploiting biology have been revolutionary and are continuing. New options for vaccine design to precisely elicit specific immune responses are strongly enabled by these recent technological developments. These technologies allow the identification of species and strains of bacteria causing disease through the use of genomics tools (including identification of bacteria that cannot be grown in laboratory culture), the discovery of vaccine antigens (and complete families of antigens through genome sequencing) and the formulation of vaccines to induce specific types of immune responses.

The common scientific theme associated with these technological changes is a transition from reductionist experimental science (typically investigating one molecule or microbial species at a time and then attempting to build an understanding of the complexity of a biological system) to a new approach that measures everything in a biological process, even anonymous molecules or microbes. The latter approach provides a greatly improved understanding of biological complexity. It has been enabled by new technologies characterised by massively parallel high through-put measurements of various classes of molecules e.g., DNA, gene expression (mRNA), proteins, and small molecules (e.g., lipids and sugars). For example, over the last 15 years the technology for DNA sequencing of a large mammalian genome has massively increased in speed whilst its cost per unit of information (nucleotide) decreased more than one million-fold (Giani *et al*., 2020; Heather and Chain, 2016). Presently, the technology has the capacity to fully sequence several bacterial genomes in less than a day; a task that even 15 years ago took more than a year. Moreover, there has been massive development of bioinformatics software that is designed to analyse and interpret genome sequences. A similar technologically-enabled discovery acceleration has occurred with mass spectrometry, which can rapidly identify and quantify large numbers of protein and small biological molecules in parallel analyses. Mass spectrometry is well suited to the identification of small molecules that are secreted by bacteria to enhance their virulence. All these technologies are highly sensitive and quantitative e.g., DNA sequencing can identify and quantify low abundance bacterial species in a complex mixture.

The new technologies for interrogation of biology generate huge quantities of data that are mined by continually improved analytical tools. The process is accelerating not only because of the technological improvements. There are now publicly-available, large and expanding databases populated with reference genomes (tens of thousands of microbial genomes are available), huge numbers of genetic variants within a species population (a measure of population diversity), small molecule reference structures, and statistical measures of units of biological complexity. Thus, biological data is being generated and processed at unprecedented rates.

Over the last two decades, there have also been many developments in vaccine design, composition and delivery that enable improved and longer-lasting vaccine efficacy by tailoring the induced immune response generated by a vaccine to better recognise a threat from an infectious microbial agent (Wallis et al., 2019; Francis, 2018).

With respect to the development of a fleece rot or lumpy wool vaccine, the new technologies will help provide: (i) more rapid and comprehensive identification of microbial populations and their abundances in complex mixtures; (ii) measures of the genetic diversity of individual species of bacteria involved in initiating and sustaining fleece rot or lumpy wool; (iii) a means to monitor antibiotic resistance in bacteria; (iv) more efficient identification and isolation of candidate antigens, particularly molecules secreted from bacteria to enhance their

infectivity and pathogenicity in the natural state of infection (as opposed to secreted proteins identified from laboratory culture of bacteria), and novel undetected antigens discovered using bacterial genomic sequence analyses; (v) improved vaccine formulations to enhance vaccine efficacy and duration of protection; (vi) more efficient means of manufacturing antigens (e.g., by use of recombinant proteins or synthetic chemistry) and; (vii) more efficient quality control of vaccine manufacture.

6.2   Microbial genetics

At present, a completed genome sequence (DNA sequence) of a novel bacterial species can be obtained relatively cheaply and within days (even hours). Importantly, publicly accessible databases contain very large numbers of genome sequences for a broad variety of bacteria and strains within bacterial species. The genome sequence provides a species identification fingerprint and information about all the genes that orchestrate the functions of the bacterial species. In particular, it provides deduced protein coding sequences for all bacterial proteins, including secreted proteins that act as toxins potentially involved in enhancing bacterial infectivity and pathogenesis. The latter information can be rapidly obtained by bioinformatics analyses once a bacterial genome sequence is available. The approach uses a search for bacterial signal sequences for secretion of an encoded protein, which has specific protein sequence characteristics and is located at the beginning (amino-terminal region) of the protein sequence. This bioinformatics strategy has potential to rapidly identify previously unknown secreted proteins, some of which will be virulence factors. Antibody-mediated inactivation of these proteins is likely a high priority strategy for vaccines designed to protect sheep from fleece rot and lumpy wool (see Sections 7 and 8). Moreover, comparisons of genes between different bacterial species may also increase the discovery rate for genes that have potential to encode virulence factors. Once candidate genes are identified, their encoded proteins can be rapidly and artificially produced as recombinant proteins for testing in vaccines. Currently, the genomes from nearly all the bacterial species shown previously to be present in fleece rot lesions and in lumpy wool have been sequenced and hence considerable information is already available. Genome sequences for a range of strains of a bacterial species are generally limited, but can be rapidly generated.

Proteins and small molecules secreted from bacteria to enhance infectivity and pathogenesis can also be rapidly and directly identified using new high sensitivity mass spectrometry (proteomics) techniques using washings from sites showing fleece rot or lumpy wool or laboratory cultures of bacteria causing these infections. The amino acid sequences obtained for these proteins can be compared to database information thereby allowing rapid identification of the bacterial species secreting these proteins. This approach can also generate information about structural variants of these secreted proteins arising from strain variation. In addition, mass spectrometry is well suited to the identification of non-proteinaceous small secreted molecules and their diversity of structural variants. These molecules may also be virulence factors well suited to be tested in vaccines.

Genome sequence information also provides information about genetic diversity in a complex bacterial population, which may translate into the need for diversity of potential target antigens for inclusion in a vaccine. Ideally, a vaccine is designed to target all the genetic diversity in the population of bacteria causing disease. The approach can also identify a candidate vaccine antigen that shows no diversity of structural variation, which potentially may be efficacious in a vaccine that protects an animal from a broad diversity of bacterial strains.

Genome sequencing can additionally comment on types of antibiotic resistance mechanisms present within the bacterial population. This information is essential to enable monitoring of movement of antibiotic resistance genes within a bacterial population and between bacterial species. This knowledge is important from the perspective of the management of livestock and ultimately human health. The use of antibiotics in the

environment of an animal at risk of bacterial infection can potentially change the balance of strains in the bacterial challenge population, which may impact vaccine efficacy and the required diversity of vaccine antigens.

6.3   Microbiomes

Populations of different species of bacteria at different abundances are present in fleece rot lesions and lumpy wool. It should be noted that most past investigations of the bacterial diversity and dynamics in fleece rot and lumpy wool were technically limited by today's standards. The full scope of bacterial numerical and functional diversity in fleece rot samples was likely not accurately or comprehensively ascertained due to: (i) biased laboratory culture conditions for growing bacteria and the inability to grow some bacteria in laboratory culture (for example, for bacterial ecology studies, easily isolatable and cultivable bacteria may represent less than 1% of the total bacterial diversity in a sample (Hugenholtz, 2002)); and (ii) the use of molecular technologies for detection of bacterial species that did not fully capture the diversity of the bacterial population through lack of sensitivity or specificity, and the absence of quantitative analyses. The dynamics of the formation of microbial populations can now be assessed in great detail by microbiome analyses.

A high priority for future research is to use modern and unbiased microbiome analyses (Forbes *et al.*, 2017; Denman *et al.*, 2015; Qin *et al.*, 2010) to detect and quantify *all* bacterial species on normal skin and fleece, and the bacterial dynamics that lead to fleece rot. This approach may also provide additional support for a causal role of *P. aeruginosa* in generating fleece rot and have the added benefit of documenting *P. aeruginosa* strain diversity (bacterial strains are subsets of a species population that have slightly different morphological, metabolic or pathological characteristics associated with small changes in their DNA sequence). The microbiome approach is rapid, comprehensive, quantitative, unbiased, does not require laboratory-culture of bacteria, and it is now the mainstay for investigations of complex microbial communities (Forbes *et al.*, 2017; Denman *et al.*, 2015; Qin *et al.*, 2010; Bharti and Grimm, 2019). The technique relies on massively parallel sampling of DNA sequences from all bacteria in a complex population. The efficiency and utility of this process for the identification of bacteria are further advanced by the availability of whole-genome sequences for a huge variety of bacterial species. The approach is also complemented by mRNA expression analysis and proteomic approaches. In addition, the cost of these technologies per sample has dramatically decreased with time.

Pseudomonas aeruginosa is ubiquitous throughout nature and an opportunistic pathogen with a relatively large genome (~6–7 Mb) allowing it to thrive in many different environments. How it achieves this is starting to be understood through "multi-omic" approaches. *P. aeruginosa* genomes have a mosaic structure composed of a highly conserved core with inserts of flexible (found in multiple isolates) and unique genes (only in single isolates). The Pseudomonas Genome Database (version 19.1 released October 2019, https://www.pseudomonas.com/) (Winsor *et al.*, 2016) currently contains 9,109 *Pseudomonas* spp. of which there are now 4,660 *P. aeruginosa* genomes.

Studies using transposon sequencing have identified 321 essential genes required for growth on five different media substrates (Poulsen *et al.* 2019), while an *in-silico* pan-genome comparison identified 665 core genes representing about 1% of the entire pan-genome (Freschi *et al.*, 2019). Knowledge of the core genome is essential to help define mechanisms to inhibit the proliferation of this bacterial species. Based on average nucleotide identity analysis (ANI), *P. aeruginosa* can phylogenetically be separated into five groups, with most isolates assigned to group 1 and 2. This bias is partly based on the over-representation of clinical isolates, but also reflects the low genomic diversity between isolates. Only genomes for a single isolate from sheep mastitis infection and seven from cattle lung and faecal samples are currently available publicly. Genome sequencing of

isolates relevant to Australian sheep fleece rot is essential if a greater understanding of the transition of environmental strains to disease causative strains is to be achieved.

Although there is minimal correlation between genotypes and habitat, there is evidence of the emergence of dominant phenotypical *P. aeruginosa* isolates from within a community population supporting adaption to niche specialists (Kidd *et al*., 2012; Azimi *et al*., 2020). In particular, within a population of strains in a cystic fibrosis antibiotic-resistant biofilm, evolutionary trajectories were measured using metagenomic methods and identified that 60% of genomic changes to strains had evolved within 10 days (Azimi *et al*., 2020). The overall community function is determined by all the individuals in the population and not a single species. Understanding what environmental factors affect community structure and function remains critical to defining mechanisms to alter/inhibit these communities.

6.4   Vaccine design and delivery

There have been significant technical developments in the design and delivery of vaccines over the last few decades with several excellent reviews of these changes (Wallis *et al.,* 2019; Francis, 2017). These developments have the potential to catalyse the production of new vaccines where there have been past failures in vaccine design. The recent technology improvements are aimed at producing longer-lasting, highly protective specific cellular or humoral immune responses in the tissues affected by infection. The technology improvements were driven by the need for a diversity of vaccine types with each tailored to the specific characteristics of the infective agent. Vaccines are designed for many purposes e.g., killing microbes by cell mediated and/or antibody mediated cytotoxic pathways, decreasing microbial infectivity, decreasing microbial pathogenicity in the host, and neutralising the effects of bacterial toxins secreted by microbes. The specific purpose of a vaccine guides the type of immune response that needs to be generated by the vaccine design and the body tissue location where it needs to be effective. Other technological improvements relate to broadening the range of options for physical administration and better ease of use of a vaccine.

There are no commercial vaccines designed to protect sheep from fleece rot or lumpy wool despite considerable efforts in the late 1980s.

7.   Justification for development of a fleece rot vaccine

7.1   Multiple drivers for development of a fleece rot vaccine

7.1.1   Reduction in incidence of fleece rot and flystrike

The bacterium *P. aeruginosa* is thought to be the primary species that likely initiates and sustains fleece rot, although contributions from other pseudomonad species and other bacterial genera may also be possible (see Sections 4.4 and 4.5.2). At present, the incidence and severity of fleece rot caused by these bacterial species are minimised by managing environmental risk factors e.g., exposure to warm and wet weather, and the timing of shearing. There is no effective antibiotic treatment. The extent of direct loss in industry productivity due to

fleece rot is likely to be small and highly variable year-on-year based on incidence (Section 4.2) and existing management practices.

The development of fleece rot in sheep is a strong predisposing factor for body strike caused by larval infestation of *L. cuprina* or *L. sericata* (Norris *et al.*, 2008; Dai, 1997; Watts and Merritt, 1981a, 1981b; Eisemann, 1988; Raadsma, 1991a; Raadsma, 1991b; Raadsma *et al.*, 1988). Thus, the major driver for development of a fleece rot vaccine is a reduction in the incidence of blowfly strike, which has considerable productivity impact in the industry. Moreover, vaccination as part of an overall integrated pest management strategy has the potential to extend the effective lifetime of existing insecticides used to control flystrike. Vaccination of sheep against fleece rot has potential for commercial success due to new technological advances, and existing know-how and infrastructure for production of other types of vaccines used in the livestock industries.

7.1.2 Maintaining the effectiveness of insecticides for control of flystrike

Insecticides have been widely used in Australia to control blowfly strike in sheep for over seven decades and before that various nonspecific chemical agents were used (Sandeman *et al.*, 2014; Levot, 1995; Shanahan and Roxburgh, 1974). Insecticides have worked well and the sheep wool and meat industries are currently reliant on them for improving industry productivity and maintaining good animal welfare. However, there is a long history of blowfly resistance development to various classes of insecticides. The risk of resistance developing to currently used insecticides emphasises the need for constant vigilance and for use of integrated pest management practices to prolong the utility of insecticides. In this context, a fleece rot vaccine used in conjunction with a blowfly vaccine could complement the existing integrated pest management strategies by providing an additional and indirect means of controlling blowfly strike.

7.1.2.1 *L. cuprina* resistance to insecticides

Insects have short generational times and produce many progeny in each generation. Thus, the large number of individuals in an insect population ensures genetic diversity as random mutations are acquired by some individuals in each generation. Under intense selective pressure caused by use of an insecticide, the survival and reproduction of a minor population can be strongly advantaged by an acquired mutation(s) that causes resistance to the insecticide. That advantage translates to expansion and ultimately dominance of this genetic group in the population. The ability of insects to disperse within the environment enhances the spread of insecticide resistance across the landscape. Continuing control of the insect population then relies on the introduction of another class of insecticide. The response of *L. cuprina* to insecticide use in the sheep industry over a long period of time exemplifies this cyclical process. The past serves as a lesson for the future.

Sandeman *et al.*, (2014), Levot (1995) and Shanahan and Roxburgh (1974) highlighted the long history of development of insecticide resistance in *L. cuprina* in Australia. Initially, from 1955 to 1958 organochlorines such as dieldrin and aldrin were used by the sheep industry but withdrawn due to unacceptable residues in meat. Organophosphates were introduced in 1957. *L. cuprina* resistance was then first detected in 1965. Their effectiveness reduced from 16 weeks (or more) to 2-4 weeks and the resistance quickly became widespread. Resistance remains in the current *L. cuprina* population despite subsequent use of new insecticide classes (Levot, 1995; Sandeman *et al.*, 2014). Some organophosphates are still in use as topical applications on sheep for treatment of flystrike. It is notable that for nearly 15 years from 1965 to 1979, the industry relied on a single insecticide with declining period of efficacy caused by the spread of resistant populations of *L. cuprina*.

Cyromazine, belonging to the triazine class of insecticides was first introduced in 1979 as a larvicidal agent and it has been the mainstay for control of *L. cuprina* for 30-40 years. Cyromazine belongs to a category of insecticide that disrupts normal *L. cuprina* development by interfering with cuticle turnover during ecdysis (moulting and pupation) (Friedel and McDonell, 1985). Its precise mechanism of action is still unknown. Unlike previously used insecticides, cyromazine has an insect-specific mode of action and consequently there is little mammalian toxicity. The major attraction for using cyromazine was its extended period of control of blowfly strike. Although cyromazine has been used for a considerable period in Australia, resistance has not developed in the field until recently (Levot *et al*., 2014; Levot, 2013; Levot, 2012; Sales *et al*., 2020). Levot *et al*. (2014) concluded that the protection period was reduced from 14 weeks to less than 8 weeks and suggested that producers implement management practices to minimise further resistance development. These conclusions came into sharper focus when resistance to dicyclanil was also demonstrated (Levot *et al*., 2014; Sales *et al*., 2020).

Dicyclanil was introduced for flystrike control in 1998. It functions in a similar way to cyromazine as an insect growth regulator but it had ten times greater potency and initially provided season-long protection (18-29 weeks) (Sandeman *et al*., 2014). A laboratory experiment performed in 2005 was a portent of the recently reported field resistance to dicyclanil (Magoc *et al*., 2005; Sales *et al*., 2020). The investigation in 2005 demonstrated cross-resistance to dicyclanil in cyromazine-resistant laboratory-induced mutants of *L. cuprina*. This result proved that the structurally related insecticides dicyclanil and cyromazine worked through a common biological mechanism and that it was possible that resistance mutations could arise. Field resistance of *L. cuprina* to dicyclanil was reported in 2014, which reduced protection of sheep from flystrike from 18-24 weeks to less than 11 weeks (Levot *et al*., 2014). In addition, laboratory reared populations of *L. cuprina* subjected to mutagenesis were able to be selected for resistance to cyromazine and dicyclanil (Magoc *et al*., 2005; Yen *et al*., 1996). More recently, Sales *et al*. (2020) reported blowfly field strains that were resistant to dicyclanil. Although the *in vitro* resistance ratios were relatively low at 13- to 25-fold compared to a susceptible strain, the protection periods for three dicyclanil containing spray-on products were reduced by 69-83% compared to the period claimed by the manufacturer. Cross-resistance to cyromazine and ivermectin was also demonstrated. Ivermectin is a structurally different class of insecticide that works through a different biological mechanism. If confirmed, this suggests that the resistance mutation is not in the gene encoding the protein target for dicyclanil and cyromazine insecticidal action. One possibility is that a mutation has developed in a common insecticide detoxification pathway. Sales *et al*. (2020) concluded that "*dicyclanil resistance is of major concern to the Australian sheep industry*".

Benzoylphenyl urea (diflubenzuron) is another type of insect growth regulator that was used to control blowfly strike and lice in the past. It acts by inhibition of the enzyme chitin synthase, which disrupts ecdysis (moulting) in *L. cuprina* (van Eck, 1979). The insecticide was introduced into Australia for flystrike control in 1991 but rapid high-level resistance developed by 2002, which led to its discontinuance as a flystrike control agent in 2008 (Sandeman *et al*., 2014). Resistance was due to mutations occurring in the chitin synthase gene (Douris *et al*., 2016; Van Leeuwen *et al*., 2012).

At present, a few other insecticides representing different chemical classes can be used as additional options for flystrike control. These include ivermectin (a macrocyclic lactone), alpha-cypermethrin (a synthetic pyrethroid) and spinosad (a bio-insecticide originally derived from a soil fungus). Synthetic pyrethroids have not been widely used to control blowfly strike although one is used as an oviposition suppressant. No field resistance to ivermectin has been demonstrated. Spinosad is an insecticidal agent that provides short-term (4-6 weeks) protection of sheep from flystrike. No *L. cuprina* resistance to spinosad has yet developed. It has a unique mode of action involving inhibition of a specific subunit of the nicotinic acetylcholine receptor and thereby disrupting neural function in the blowfly (Cisneros *et al*., 2002; Khan, 2018; Anstead *et al*., 2015). Spinosad is therefore ideally suited to the process of insecticide rotation as part of an integrated pest management strategy. It has

been used on a broad range of insects, however strong resistance has developed in some species, including the house fly, *Musca domestica* (Khan, 2018; Banazeer *et al*., 2019; Anstead *et al*., 2015). Khan (2018) noted that although strong resistance developed in *M. domestica* in the presence of the insecticide, relaxation of its use resulted in rapid loss of the resistance in the fly population due to a fitness disadvantage in the resistant population i.e., spinosad resistance was unstable in the population. This observation further reinforces the view that spinosad use for control of flystrike is best suited to the rotation of insecticide classes within the framework of integrated pest management.

Integrated pest management for flystrike control emphasises the need for rotation of treatment of sheep with different classes of insecticides, consideration of the insecticides used for lice control and careful application to slow the development and spread of insecticide resistance in *L. cuprina*. Appropriate application minimises the presence of a sublethal dose on sheep, which can increase the risk of resistance development and spread. The ability to maintain control and rotate insecticide use to minimise resistance development is likely to become more constrained with the recent development of resistance to cyromazine and dicyclanil. The importance of integrated pest management is also highlighted by the reduced effectiveness of dicyclanil in sheep with dermatophilosis. At present, various insecticides are preferentially used for long-term prevention and short-term treatment of flystrike. This utility difference of insecticides should be a dominant consideration in integrated pest management strategies. The history of development of *L. cuprina* resistance to insecticides and the lack of new chemical classes of insecticides arriving in the market indicate the need to implement additional procedures to prolong the efficacies of existing insecticides. Vaccines that indirectly reduce the likelihood of flystrike (and reduce risk of dermatophilosis) would likely become an important adjunct in the integrated pest management strategy. The utility of such vaccines for reducing the development of insecticide resistance would likely be enhanced by their combination with a blowfly vaccine.

7.1.3   Improved animal welfare

Blowfly strike itself and some current blowfly control practices used on sheep are looming threats to market access in the wool and sheep meat industries due to animal welfare concerns by markets and the public (Sandeman *et al*., 2014; Lee and Fisher, 2007; Tellam and Bowles, 1997). New and more acceptable approaches for control of blowfly strike are therefore required. The major driver for a fleece rot vaccine is the reduction in the incidence of blowfly strike rather than loss in productivity directly due to fleece rot. Vaccination can be cost-effective, animal welfare friendly, and well accepted by the industry, the wider public and markets. This approach can help to diminish potential market access issues.

7.1.4   Enhanced surveillance for bacterial resistance to antibiotics

There is another, though hidden, driver to develop a vaccine that protects sheep from fleece rot. Some populations of *P. aeruginosa* isolated from the human health environment and a wide range of veterinary sources have both innate and acquired antibiotic resistance, including multi-drug resistance mechanisms (Serrano *et al*., 2017). Therefore, the use of antibiotics in livestock to control unrelated diseases may be promoting *P. aeruginosa* resistance levels or, the antibiotic resistance is a reflection of the wider *P. aeruginosa* population structure (see Section 4.5.4). The former situation could become a future human health concern and thereby impact the sheep industries. Antibiotic resistant *P. aeruginosa* could potentially be directly transferred from sheep to humans or these bacteria could act as reservoir of mobile antibiotic resistance genes that can be

transferred to other *P. aeruginosa* strains or other human pathogens (Partridge *et al.*, 2018). Inevitably, there may be increasing pressure on the wool and sheep meat industries to decrease their reliance on antibiotics and ensure that sheep are not an inadvertent source of antibiotic resistant bacteria with implications for human and livestock health. The development of a vaccine to protect sheep from fleece rot could, as a by-product, enable the development of molecular tools that monitor changes in antibiotic resistance genes and thereby provide a preview of potential future issues.

7.2   Scientific opportunities, challenges and strategy

Development of a new vaccine involves at least four major stages that need to be undertaken re-iteratively: 1) identify appropriate antigens; (2) identify an immune mechanism for protection; 3) establish a clinical measure of vaccine efficacy, and; 4) develop a strategy for vaccine use that is practical for producers while addressing epidemiological characteristics of the disease in the field. Application of the enabling technologies outlined above provide opportunities to address the many challenges in developing a new vaccine against fleece rot.

7.2.1 Identification of appropriate antigens

There is a need to use modern microbiome analyses to investigate the dynamics and diversity of bacteria involved in initiating and sustaining fleece rot and to confirm that *P. aeruginosa* is the primary bacterial agent. This type of analysis measures all bacteria including all strains of *P. aeruginosa* simultaneously and generates unprecedented fine granularity of data in a semiquantitative manner. The goal is to identify the diversity of bacteria and their exoproducts that need to be included in a vaccine. In this stage, bacterial serotype or strain diversity analyses are important because they allow identification of the comparable variations in the structures of antigens that are included in the vaccine. A priority question for antigen identification is the contribution of secreted virulence and pathogenicity factors (i.e., toxins, exoproducts (e.g., enzymes), and communication molecules (bacteria to bacteria and, bacteria to blowfly communication molecules), to progression of fleece rot and the creation of a niche for egg deposition by female blowflies.

Antigen identification can also be enhanced by mRNA gene expression analyses and targeted proteomics. The former, only samples genes that are actively transcribing mRNA under a particular environmental condition while the latter can directly confirm the presence of candidate proteins derived from genome bioinformatic analyses. Both approaches are complementary and can assess strain specific variation in candidate antigens.

A challenge accompanies the potential need to include several antigens in a vaccine that represent different serotypes or strains. Increased complexity of a vaccine has impacts on the manufacturing process, vaccine formulation and cost. A concern here is that competition between antigens for recognition by the immune system reduces vaccine efficacy compared to a vaccine formulated with antigens from a single bacterial serotype and challenged with the same bacterial serotype. One strategy may be to identify essential exoproducts that contribute to infectivity or pathogenicity but which show the least structural variation across different serotypes or strains.

7.2.2 Identify an immune mechanism for protection

A number of experiments point to IgG antibody in serous exudate on the skin surface being able to provide protection against disease progression, however this remains to be conclusively demonstrated. Salient supportive findings include:

- there is no bacterial invasion of the epidermis, so there is little opportunity for leukocytes to control bacteria by phagocytosis or release of bactericidal products;

- when it occurs, exudate is serous not purulent (or serosanguinous) so there is little chance of leukocyte function on the skin surface (probably wouldn't be even if exudate was purulent) (There is a generally pale coloured crust formation, not brown scab formation, at the lesions);

- there is some IgM and IgA in natural skin secretions (probably sweat (suint)) but these Igs are better at agglutinating bacteria than neutralising toxins;

- IgG1 and IgG2 are the Igs of choice for neutralising toxins and exoproducts from bacteria;

- while there is evidence of potential to induce the local production of antibodies in the skin, achieving this across the whole of the skin surface is very problematic, and there is no mechanism for active transport of IgGs into skin secretions. Blood supply to the skin is excellent (especially during dermatitis), so delivery of IgGs to dermal vasculature is not limiting the availability of IgGs in the serous exudate;

- high titres of IgGs can be achieved by a conventional systemic vaccination route with a potent adjuvant for antibody production;

- natural boosting of the immune system by bacteria on skin may help lengthen the duration of protection following vaccination, but this is largely an unknown.

Establishing a mechanism of immune protection paves the way for designing a vaccine to activate this defence pathway. If IgG antibody in serous exudate proves to be protective, there is an excellent choice of commercial vaccine adjuvants registered for use in livestock that can generate high titre antibodies of this immunoglobulin isotype. There is also substantial knowledge regarding antigen formulation, suitable administration sites on the animal, injection regime (number of injections and timing), and longevity of effective IgG antibody responses for this immune defence mechanism, and therefore longevity of protection.

An important unknown is the potential for antibody present in skin secretions prior to protracted wetting of skin to prevent the development of fleece rot. Is it necessary for a serous exudate to develop before sufficient antibody is present to control bacterial activity? This leads to the third challenge of vaccine development.

7.2.3 Establish a clinical measure of vaccine efficacy

Clarity over the goal of vaccination is a prerequisite for developing a clinical measure of vaccine efficacy. Is reduction in wool pigmentation, crusting and fibre damage (aka fleece rot) a sufficient goal to justify development of a new vaccine, or are reduction in body strike and potentially also breech strike necessary criteria of vaccine efficacy?

Several issues are important here. Firstly, is it necessary to prevent bacterial proliferation or is it adequate to inactivate the exoproducts that confer virulence to the bacteria? As fleece rot typically resolves spontaneously

when weather conditions permit the fleece to dry, it may be sufficient to inactivate exoproducts without killing or inactivating bacterial growth.

Secondly, if the goal is reduction in body strike, the relationship between severity of fleece rot and susceptibility to body strike may create a threshold for vaccine efficacy. This relationship has been studied in most detail by Raadsma *et al.* (1988). In a study of 176 ten month old wethers with four months wool undergoing artificial wetting, these authors observed that susceptibility to body strike was linearly related to severity of fleece rot. Body strike only occurred in sheep with exudative fleece rot (fleece rot scores 3 to 6; **Figure 3**) or with intense staining of wool in bands greater than 10 mm wide in the absence of exudate (fleece rot score 2). None of 48 of the 176 wethers with no fleece rot or with mild wool discolouration in bands less than 10 mm wide developed body strike (fleece rot scores 0 and 1, respectively; **Figure 3**). If this relationship holds in larger numbers of sheep with various wool types and wool lengths, then it suggests that the prevention of exudation is an important clinical measure of vaccine efficacy for control of body strike, while prevention of strong wool discoloration greater than 10 mm would provide a more stringent clinical measure. It is noteworthy that previous research on experimental blowfly vaccines indicates that antibody provides the mechanism of immune defence against larvae (Casu *et al.*, 1997; Tellam *et al.*, 2001). The strong association between the presence of exudate and susceptibility to body strike suggests that in sheep vaccinated against blowfly larvae, antibody would be present in exudate on the skin surface at the time larvae begin to feed on the host.
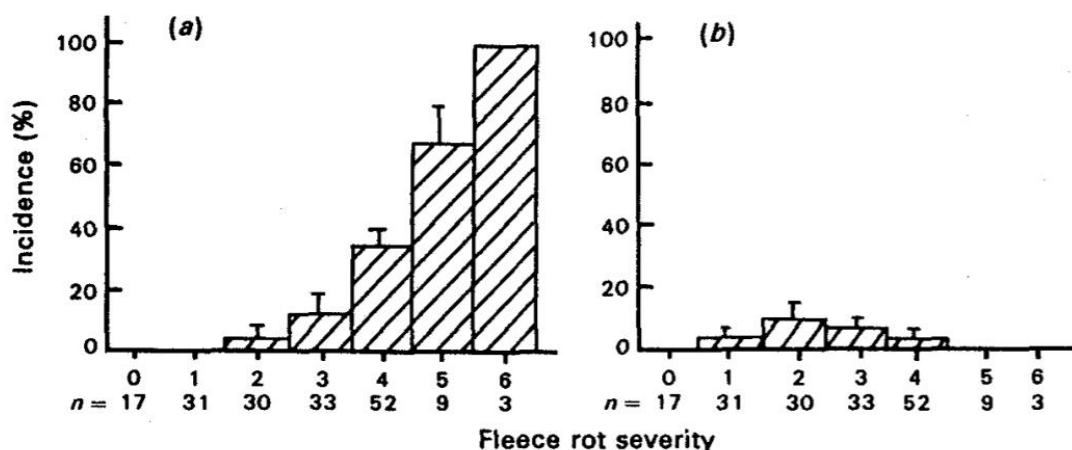
Incidence of (*a*) body strike and (*b*) other forms of fly strike as a function of fleece rot severity grading in experiment 3 (*n* = number of sheep scored in each grade).

Figure 3. Figure taken from Raadsma *et al*. (1988).

A third question is the role of odours in initiation of body strike and whether prevention of odour development during fleece rot is an important goal of vaccination. A recent AWI review by James *et al.* (2019) examined the role of odours in predisposition to blowfly strike, and the reader is referred to that document for technical details. These authors concluded that blocking odours, for example by vaccination, may play a role in control of susceptibility to fly strike. They noted:

"In sheep, odours associated with bacterial growth, particularly when in association with urine staining, scouring and diseases such as fleece rot and dermatophilosis, are critical in determining susceptibility to strike. However, there is little evidence to suggest that differences in attraction of flies to sheep, or innate odour differences between sheep, are key factors in breech strike susceptibility, other than when associated with differences in

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known predisposing conditions. In addition, any innate differences in sheep odour are likely to be overwhelmed by the effects of bacterial odours during strike waves" James *et al.* (2019).

Assessment of vaccine efficacy requires a disease challenge model. The contemporary environment for ethical use of animals in experiments could limit the use of artificial disease challenges, for instance in a wetting shed. In-principle approval of disease challenge experiments by an Animal Ethics Committee could be a valuable preliminary step in developing a new vaccine research program. Studies employing natural field challenge would be feasible but would provide a slower means of assessing efficacy. *In vitro* correlates of efficacy, for instance models of antibody effects on bacterial function or on inhibition of attraction of gravid female blowflies to bacteria-generated odorants could play an important role in fast-tracking vaccine development. It would need to be kept in mind, however, that *in vitro* correlates, on account of their specificity, may not cover the full spectrum of activities through which a vaccine confers protection. For instance, a vaccine might not only affect production of odours that attract female flies but also influence the generation of cues for oviposition and the exudation of serum used by larvae as a food source.

7.2.4   Develop a strategy for vaccine use in the field

Practical issues to address here include the timing of vaccination to fit with routine husbandry practices if possible, while accommodating the epidemiological characteristics of the disease in the field. Some of these details can only be solved when earlier stages of vaccine development are completed. For instance, longevity of protection will influence frequency and time of year (or occurrence of weather conditions) when vaccination is necessary.

Strategies required for successful use and adoption of a new vaccine could include:

- A stand-alone fleece rot vaccine with a prime boost administration regimen in lambs followed by annual boost at the beginning of seasonal period of risk in environments with a seasonal risk profile (e.g., Mediterranean), plus strategic boost administration before Bureau of Meteorology forecast periods of greatly enhanced risk due to major wet weather events;

- A fleece rot vaccine combined with a multivalent clostridial vaccine administered in the typical clostridial vaccine regimen;

- A fleece rot vaccine combined with a potential flystrike vaccine administered prior to onset of peak flystrike season (early Spring) coinciding with sheep care, including crutching and drenching;

- A combined fleece rot and lumpy wool vaccine administered as per the first dot point.

7.3   Commercialisation challenges and opportunities

Currently, there are commercialisation challenges and significant opportunities associated with the development of a vaccine to protect sheep from fleece rot. Ultimately, the impact of the vaccine will be measured by a reduction in body strike in sheep, maintenance of insecticide efficacy, reduction in insecticide use and the removal of external market threats. The extent of impact of the vaccine in these areas would interact with new and independent efforts to directly control body strike.

Ideally, an efficacious vaccine will be integrated with existing management practices to ensure good market penetration and minimisation of labour time. The optimum process would incorporate effective fleece rot antigens into an existing vaccine protecting sheep from multiple unrelated diseases (e.g., the 5 in 1 vaccine). This outcome would increase the value of the existing vaccine. The efficacies of all antigenic components in this multi-disease vaccine would need to be unaffected by the inclusion of the fleece rot antigens. Importantly, there is the implicit assumption that the protective immune responses generated in sheep for each antigen in the multi-disease vaccine would involve a similar type of immune response. Before considering this process, the efficacy of a fleece rot vaccine would need to be independently demonstrated under a range of circumstances. The inclusion of a fleece rot vaccine into existing unrelated vaccines should be considered but may not be practical for a variety of reasons.

The likely immune response required for an effective fleece rot vaccine will be one focussed on antibody generation in the blood or possibly skin that then releases antibody into fleece rot exudate at the skin surface. This type of immune response may be different from that primarily generated by existing commercial multi-valent disease vaccines for control of other diseases. The efficacies of some of the latter vaccines depend on antibody titre although not antibody in skin. Thus, there is a possibility of compatibility with existing vaccines. An alternative approach is the production of a stand-alone fleece rot vaccine i.e., a strategic application not integrated with existing vaccines. The fleece rot vaccine will require at least two injections aimed at developing a minimum of season-long protection particularly in the at-risk period of the year (warm and wet conditions). In addition, the vaccine will likely need inclusion of multiple pseudomonad antigens to ensure broad protection against multiple *Pseudomonas* species and *P. aeruginosa* strains that contribute to fleece rot. This possibility will impact the design and cost of vaccine manufacture. A vaccine company will also require the infrastructure and know-how to isolate and formulate the effective antigens in a vaccine.

7.3.1   Market size

The potential market size for a fleece rot vaccine is commercially important but difficult to calculate. There is considerable data available on the prevalence of fleece rot in Australian sheep, however, these data primarily reveal that prevalence is highly variable, particularly in relation to weather and location (Norris *et al*., 2008). Perhaps the best data obtained over a 17 year period at Trangie (NSW) and over six years at Longford (NSW) are prevalence values of 23.5% and 26.7%, respectively, but with very large year to year ranges (Norris *et al*., 2008). Market up-take could be geographically variable, being influenced by climatic conditions and management practices in at-risk periods. Uptake could also be influenced by fleece type and sheep genetics. The primary driver for vaccine development is not the direct value loss associated with fleece rot in sheep, rather the associated increased risk of flystrike and the need to maintain long-term efficacy of insecticides in the face of a long history of development of insecticide resistance. The cost of flystrike control in Australia has been estimated at $173 million per year, but some control practices are also major external marketing threats to the Australian wool and sheep meat industries (Peachey, 2018; Sandeman *et al*., 2014). There is some quantitative information about the relationship between fleece rot incidence and severity with flystrike incidence (see Section 7.2.3; Raadsma *et al*., 1988), which would be valuable for estimation of market size. The major markets for the fleece rot vaccine would probably align with those countries producing large quantities of wool i.e., Australia, New Zealand, South Africa and the United Kingdom. There is also a small possibility that knowledge generated producing an efficacious fleece rot vaccine for use in sheep could aid the development of a vaccine protecting humans against *P. aeruginosa* infections and therefore generate access to the lucrative human health care market.

7.4 History of past patents and commercialization efforts

A large research effort was undertaken in the 1980s and early 1990s to develop vaccines against *P. aeruginosa* for fleece rot control. Details describing these experimental vaccines are listed in Section 4.11. The text below highlights possible reasons for past commercial failures for a fleece rot vaccine.

Australian and New Zealand patents entitled *Improved Vaccine* were granted in 1988 but are now expired (Burrell and MacDiarmid, 1988) (application date, 17/1/1986; publication date, 29/2/1988; patent reference numbers, AU PG8941 19850118; NZ 214858A; inventors, D.H. Burrell and J.A. MacDiarmid; applicants, CSIRO and Biotech Australia Pty Ltd). A related patent submitted by the same inventors and applicants was filed in 1986 (Burrell and MacDiarmid, 1986) (granted 1986; patent reference numbers, AU 8652530-A, ZA 8600374-A; inventors, D.H. Burrell and J.A. MacDiarmid; applicants, CSIRO and Biotech Australia Pty Ltd). The commercial partner in both instances was Biotechnology Australia Pty Ltd (Sydney; 1988 - ~1992). The major patent claim was for a vaccine that reduces predisposition to flystrike by reducing microbial activity in skin i.e., "*The vaccine is useful for reducing the predisposition of sheep to fly strike (claimed). Action is by protecting the sheep against microbial activity in the fleece (fleece rot) and skin, preventing dermatitis lesions which attract the blowfly to oviposit*". The vaccine could include soluble antigens or outer membrane antigens from *P. aeruginosa* alone or in combination with similar antigens from *P. putida, P. stutzeri* or *P. maltophlia* (Burrell and MacDiarmid, 1988). The vaccine was not commercialised by Biotech Australia Pty Ltd. We made contact with a former scientist associated with the "Burrell" vaccine and a former affiliate of Biotechnology Australia Pty Ltd. The scientist noted that the vaccine was more efficacious when it contained antigens from multiple *P. aeruginosa* serotypes, and the vaccine worked in a field trial. The scientist also suggested that Biotechnology Australia Pty Ltd, at the time, was not focused on the development of the vaccine due to structural difficulties that eventually led to its closure in about 1992. The affiliate of Biotechnology Australia Pty Ltd indicated that field trials were performed with the vaccine but it was not sufficiently efficacious. From a commercial perspective, the affiliate indicated that the vaccine, at that time, would be unable to supplant the use of insecticides used for control of blowfly strike (the major (indirect) product competition in the market). We speculate that additional factors contributing to commercial failure at the time likely included *P. aeruginosa* strain variation (impacts vaccine efficacy and manufacture) and market size.

Several experimental vaccines for fleece rot protection have been investigated in the past but few were tested in field trials and none was commercialised, although one was patented but not tested for efficacy against fleece rot (Chin, 1995; WO 95/27508). Clinical trials using antigens derived from *P. aeruginosa* to protect humans from several infectious diseases caused by this bacterium have been disappointing and no commercial vaccine for human use has been developed (Hoggarth *et al.*, 2019; Merakou *et al.*, 2018; Priebe and Goldberg, 2014). Notably, several specific potential antigens have been identified.

7.5 Progress with vaccines against human diseases caused by *P. aeruginosa*

P. aeruginosa normally does not infect healthy people. It is a pathogen that exploits opportunities arising in individuals with injuries, chronic health conditions or deficient immune systems. Typically, *P. aeruginosa* infects broken human skin, particularly resulting from burns, and most epithelial tissue surfaces such as the lower respiratory tract (especially people with cystic fibrosis), urinary tract, gastrointestinal tract, ear and cornea. The bacterium is one of the most prevalent causes of infectious diseases in humans and responsible for considerable morbidity and mortality (Merakou et al., 2018). The metabolic and genetic versatility of *P. aeruginosa* is the reason why it can successfully proliferate in diverse tissues each with different local environmental

characteristics. The feature common to all infections is the breaking down of the barrier function of epithelial tissue layers and the promotion of a moist environment in which *P. aeruginosa* proliferates using cellular debris as a nutrient source. Treatment of infected people is difficult as infections are often resistant to multiple classes of antibiotics and the bacterium readily avoids immune defence responses in the host by using a formidable array of strategies (Döring and Pier, 2008; Sharma *et al.,* 2011; Merakou *et al.*, 2018). Hence, antibiotic treatments are usually aggressive and often do not fully eradicate the bacterium thereby leading to long term chronic infection.

Since the 1970s, there have been numerous and continuing effects to develop vaccines to protect at-risk individuals from *P. aeruginosa* infections. To date, no vaccine has become commercially available although promising results have been obtained (Döring and Pier, 2008; Sharma *et al.,* 2011; Merakou *et al.,* 2018). One reason for the lack of success is that *P. aeruginosa* chronic infections often occur in people whose immune system is compromised by other health factors and hence they cannot mount an adequate immune response to an administered vaccine. Moreover, for some triggers of infection, like burns and injuries, there is insufficient time for a strategically administered vaccine to induce an effective immune response. In the latter instance, passive immunization involving the transfer of effective antibodies from another source to the injured person is a quicker and likely more effective strategy.

The ability to test a new vaccine in a relevant human cohort is difficult due to limited patient numbers who often receive variable, and therefore confounding, additional treatments, particularly antibiotic treatments. These factors in clinical trials often lead to statistically inconclusive results or non-confirmation in subsequent trials. There are also ethical and clinical issues associated with the use of human control groups that remain unvaccinated. Further, the results for vaccines tested in animal models of human diseases caused by *P. aeruginosa* often do not translate well when tested in humans.

The search for effective vaccines has focussed on identifying protective antigens from *P. aeruginosa*, generating specific immune responses by optimising vaccine formulations and administration routes, designing vaccines to manage *P. aeruginosa* strain variation, and producing different vaccines for each type of medical condition. Effective vaccines against *P. aeruginosa* should be characterised by several features. First, the vaccine should elicit an antibody response that mediates opsonophagocytic killing by neutrophils and macrophages and neutralise virulence factors. Second, the vaccine antigen should be present in the *P. aeruginosa* strain causing disease or the antigen should be conserved in structure and thereby generate cross-reactive protection against multiple *P. aeruginosa* strains. Alternatively, the vaccine antigen may contain the spectrum of structural forms of an antigen that are present in multiple strains causing disease. Third, the target antigens in *P. aeruginosa* need to be expressed in sufficient quantities in the bacterium and available for recognition by antibodies. Fourth, the vaccine should also generate a T cell response (T_H17), although there is some debate as to whether this is of primary importance (Merakou *et al.*, 2018).

Numerous antigens from *P. aeruginosa* have been tested in experimental and clinical vaccines designed to protect humans from *P. aeruginosa* infections. The antigens with some extent of protection include lipopolysaccharide, polysaccharide repeating unit of LPS (O-antigen), outer membrane proteins, mucoid exopolysaccharides (alginate), polysaccharide-protein conjugates, flagella proteins, pili proteins, whole dead *P. aeruginosa*, live-attenuated *P. aeruginosa*, attenuated *Salmonella* and attenuated adenovirus engineered to express various *P. aeruginosa* antigens, surface-expressed T3SS proteins involved in type 3 secretion of bacterial virulence proteins directly into epithelial cells, exotoxin A and secreted proteases (Döring and Pier, 2008; Sharma *et al.*, 2011; Merakou *et al.,* 2018). Some of the tested vaccines included combinations of antigens. In the list of antigens, there is an emphasis on virulence factors that help *P. aeruginosa* adhere to cell surfaces, damage tissues for the dissemination of nutrients or increase the bacterial survival rate. **Table 3** summarises the tested antigens (taken from reviews by Döring and Pier, 2008; Sharma *et al.*, 2011; Merakou *et al.,* 2018). For simplicity, the table lists classes of antigens and does not contain antigens tested by passive immunisations. The latter would not be practical for livestock animals.

Table 3. **Summary of *P. aeruginosa* antigens tested in human clinical trials and animal models of human infections.** The table contains merged and abbreviated information from reviews by Döring and Pier, 2008; Sharma *et al.,* 2011; Merakou *et al.,* 2018. Ab, antibodies; Ag, antigen.

Antigen	Function in *P. aeruginosa*	Advantage	Limitation
Live-attenuated *P. aeruginosa*	Live *P. aeruginosa* missing aroA gene	Presentation of multiple Ag to immune system; 0psonophagocytic Ab (i.e. pathogen marked by Ab for ingestion by phagocytes)	Residual virulence
Killed *P. aeruginosa*	-	Presentation of multiple Ag to immune system	Toxic
Lipopolysaccharide (LPS)	Potent toxin causing tissue inflammation	Generation of high levels of opsonic Ab	Toxic; pyrogenic; high heterogeneity; low immunogenicity
O-polysaccharides (O-Ag)	Polysaccharide repeating unit of LPS	Generation of high levels of opsonic Ab	Toxic; pyrogenic; high heterogeneity; low immunogenicity
Alginate (mucoid polysaccharide that is primary determinant of serogroup)	Secreted exopolysaccharide; confers mucoid phenotype; immobilises diffusion of antibiotics, Ab and phagocytes; responsible for biofilms (retain hydration)	Generation of opsonic Ab; low structural heterogeneity; Ab-binding enables phagocytosis by neutrophils; Ab prevents *P. aeruginosa* attachment to epithelial surface	Ab show variable opsonic ability
Flagella proteins	Motility; attaches to mucin for mucin colonisation; chemotaxis; invasiveness	Adjuvant effect; moderate heterogeneity	*P. aeruginosa* can shed flagella
Pilin proteins	Adhesion to epithelial cells; biofilm formation; twitching motility	High immunogenicity; multiple proteins	High heterogeneity; cell receptor binding site hidden
T3SS translocator proteins	Translocates bacterial proteins into host cells to invade tissue; virulence factor	Induces T_H17 immune cells; blockage by Ab binding enhances phagocytosis	Not widely tested yet
Outer membrane proteins	Structural and functional components of bacterial cell wall; biofilm formation; virulence factor by binding innate defence components (C3b adhesion factor; IFN gamma)	Highly conserved and immunogenic; quorum-sensing through IGF-gamma binding; surface exposed	nil
Exotoxin A (mono-ADP-ribosyltransferase) and proteases (elastase and alkaline protease)	Virulence factors; initiate tissue invasion, cell death, availability of	Ab binding neutralises cytotoxic effects and pathology	Less effective in bacterial clearance; Ag requires inactivation to be used in vaccine

	nutrients for bacterial proliferation		
Attenuated *Salmonella* and attenuated adenoviral vectors engineered to express various *P. aeruginosa* Ag	-	High immunogenicity and adjuvant properties	Pre-existing anti-adenovirus immunity; *Salmonella* vector restricted to mucosal immunity

8. Justification for development of a lumpy wool vaccine

8.1 Introduction

A commercial vaccine that protects sheep from lumpy wool is not available. Many of the comments regarding the justification for the development of a fleece rot vaccine outlined in the previous section also pertain to the development of a vaccine for control of lumpy wool, although there are some important differences.

Lumpy wool causes wool production losses and increased risk of blowfly strike, especially on the sheep body, back and sides (Berry and Watt, 2017; Bateup and Edwards, 1990; Edwards, 1985; Edwards *et al.*, 1985; Gherardi *et al.*, 1981; Gherardi *et al*, 1983; NSW Industry and Investment, 2010; Wilkinson, 1979). However, the extent of its contribution to blowfly strike risk is unclear and may be less than fleece rot. Lumpy wool is currently managed by monitoring the natural self-healing of sheep and by using antibiotics in severe cases. Moreover, good management practices can limit the spread of lumpy wool within a flock. The implication is that the market size for a vaccine protecting sheep from lumpy wool is relatively small and can be quantified from knowledge of antibiotics use and cost of labour for this control purpose. A much larger market is possible if vaccine efficacy extended to other animals, particularly livestock (cattle) and pets.

The bacterial species causing lumpy wool in sheep, *D. congolensis,* causes dermatophilosis in a wide range of animals but it is not an important pathogen in humans (Amor *et al.*, 2011). Strain variation changes in sheep could be occurring as a result of antibiotic use for lumpy wool control and thereby influence vaccine design. In addition, livestock industries may be required in the future to monitor antibiotics resistance development in bacterial populations relevant to the control of livestock diseases to offset future infection risks in livestock and the risk to human health through indirect transfer of antibiotic resistance genes to other bacterial species.

8.2 Scientific opportunities and challenges

The scientific challenges associated with the development of a vaccine to protect sheep from lumpy wool are similar to those listed for a fleece rot vaccine. One common aspect includes the need for a sheep immune response to bacterial antigens that results in the production of antibodies that can be released onto the skin. One difference from fleece rot, however, is that the invasive hyphae of *D. congolensis* penetrate the upper skin layer and are therefore more exposed to the sheep's immune surveillance system than *P. aeruginosa*. As a consequence, phagocytic activity and cell mediated toxicity could contribute to vaccine induced immune defence against *D. congolensis*. A vaccine against *D. congolensis* may also require consideration of the diversity

of *D. congolensis* strains in the vaccine formulation. The identification of specific protective antigens from *D. congolensis* will require consideration of life stage and potential antigen function. The priority strategy for antigen identification for inclusion in a vaccine should focus on secreted virulence and pathogenicity factors and the inactivation of their normal activities by specific IgG antibodies induced in skin or present in serous exudate on the skin surface.

8.3   Commercialisation challenges and opportunities

8.3.1   Market size

The commercialisation issues for a vaccine protecting sheep from lumpy wool will need to consider market size and penetration, as most affected sheep self-heal. The cost of administering antibiotics to severely affected sheep is the direct market competition for a vaccine. The average yearly incidence of lumpy wool in the national flock is unclear due to considerable year to year and geographical variation. Berry and Watt (2017) reported incident rates in lambs from a number of mobs on two properties in the central tablelands of NSW. The incident rates in lambs from all mobs ranged between 10-80% with most mobs averaging 10-30%. Most affected sheep self-healed and remained untreated with antibiotics with the exception of one mob where 80% of lambs (2,600 lambs) were severely affected and treated. However, lamb treatment rates in most mobs ranged between 0-13% and likely averaged ~ 5-10%. If these numbers translate across the industry then the total treatment cost is likely to be, in aggregate, relatively small, as most affected lambs self-heal. Moreover, older sheep are more resistant to lumpy wool. Thus, there is unlikely to be a large market for a stand-alone lumpy wool vaccine unless its utility extends to dermatophilosis in cattle and pets. Most other commercialisation factors are similar to those listed above for a fleece rot vaccine. It is also noteworthy that the efficiency of the treatment of choice for sheep severely affected by lumpy wool in a small study undertaken by Berry and Watt (2017) was 64 %. However, at shearing 40% of the untreated sheep were incompletely shorn due to active lesions, compared to 10% for those that were treated with the antibiotic i.e. antibiotic treatment for control of lumpy wool increased total fleece value. A lumpy wool vaccine that indirectly decreased the incidence of flystrike would enhance its intrinsic value. Currently, the quantitative relationship between lumpy wool incidence and severity with flystrike incidence is unknown.

8.3.2   Combination vaccines

One commercial possibility is that a vaccine could be developed that combined antigens based on secreted products from *D. congolensis* and *P. aeruginosa* to produce a vaccine protecting sheep from both fleece rot and lumpy wool. The impetus for this approach is the likely common requirement of both vaccines to produce IgG antibody on skin that represses the biological functions of secreted virulence products, the likelihood of compatibility of antigens in the vaccine and commercial efficiency. Both diseases also have similar timing in relation to weather events and season and hence the seasonal requirements for timing of maximum vaccine efficacy are aligned. However, individual protective vaccines would first need to be developed before the antigens were trialled together. A further possibility is that vaccine antigens protecting sheep from *D. congolensis* and *P. aeruginosa* could also be combined into the vaccine being developed to directly protect sheep from fly strike or existing commercial vaccines for control of other diseases in sheep. For any of these combination vaccine possibilities, there will be a need for a staged strategy of vaccine development that is ultimately based on vaccine efficacy.

9. Strengths, Weaknesses, Opportunities and Challenges

An analysis of strengths, weaknesses, opportunities and threats for vaccines protecting sheep from fleece rot and lumpy wool is shown in **Table 4**. The table summarises core conclusions.

Table 4: SWOT analysis for fleece rot and lumpy wool vaccines

Strengths	Weaknesses
● Improve control of body strike ● Minimise major market risks ● Improved animal health and welfare ● Enhanced public and market image ● Increase productivity ● Decrease reliance on insecticides for control of flystrike and prolong the utility of current insecticides where resistance has developed. ● Decrease reliance on antibiotics (for control of Lumpy Wool) ● Existing companies have relevant vaccine manufacturing infrastructure, know-how and marketing abilities	● Small markets for stand-alone vaccines ● Market penetration for efficacious vaccines unclear ● Identification of protective antigens is the core challenge ● Generating sufficient neutralizing IgG on skin ● Bacterial strain variation ● Antigenic competition ● Generating sufficient vaccine efficacy to minimize flystrike over at least one season ● Ability to mass produce antigen(s) on the scale needed ● Alignment of vaccination timing with management procedures ● Proximate costs to producer of vaccine and vaccination
Opportunities	**Threats**
● Microbiome analyses ● Identification of candidate antigens from genome sequences ● New technologies for vaccine adjuvants and delivery ● Utility of virulence factors in the same species of bacteria infecting humans ● Increased market sizes by identifying opportunities in other species ● Future need to monitor antibiotic resistance genes in bacteria causing livestock diseases ● No direct market competition ● Combination vaccines and incorporation into existing vaccines ● Improve control of breech strike	● Change in bacterial strain variation with time and geography ● Existing disease controls perceived by producers as sufficient and acceptable resulting in small market penetration ● New antibacterial agents (low likelihood)

10. Animal welfare considerations

Freedom from disease, the associated pathologies and clinical outcomes is a component of any concept of animal welfare. In the case of fleece rot and lumpy wool, these diseases emerge after sheep are exposed to prolonged periods of wetting and result in dermatitis and disruption of fleece and skin barrier integrity. More importantly, these diseases predispose the infected sheep to blowfly body strike that results in wound exacerbation, agitation, odour and matted wool. Ultimately fly strike can be lethal. A core objective of AWI and Australian sheep producers generally is the elimination of fly strike in order to optimise the welfare status of sheep. The focus in recent decades has been on the control of breech strike. Increasing adoption of genetic solutions to breech strike and new methods for breech modification are likely to reduce the occurrence of breech strike. In this scenario, the *relative* importance of body strike as a cause of fly-related disease and death in sheep is likely to increase. Thus, fleece rot and body strike may gain more prominence as diseases of commercial significance and ethical concern.

In this report we detail opportunities for new research, specifically the development of vaccines for controlling fleece rot and lumpy wool. A consideration for undertaking this work is that unless suitable laboratory-based tests of efficacy can be developed (such as larval growth inhibition assays performed in the flystrike vaccine project), there will be a need for trials testing the efficacy of any candidate vaccines and these are likely to require specific disease challenge involving the wetting of sheep. This procedure in itself can be negatively perceived by the public as there is a small risk that animals may become hypothermic, and at elevated risk to flystrike or other parasitic infections. These risks can all be mitigated through well-developed protocols, controlled environments and frequent monitoring of animals. Dr Sue Mortimer has kindly provided a detailed experimental protocol for sheep wetting trials that has been approved previously by animal ethics committees. This protocol is attached in **Appendix 2**. Importantly, this demonstrates that the trials required to test a fleece rot or lumpy wool vaccine can be carried out under conditions approved by an appropriate animal ethics committee such as the CSIRO Armidale AEC.

The consideration described above is not dissimilar to that faced in developing therapeutics or vaccines for any disease of humans or animals. Ultimately the efficacy of the product needs to be tested in a challenge trial, that cannot be conducted without animals potentially attaining some level of disease. The decision to proceed must ultimately be based on whether this action can be justified through the potential for developing a product that may prevent or alleviate suffering in all other animals.

11. Recommendations

1. *Undertake an investigation of microbial ecology and pathogenesis of dermatitis in order to identify appropriate vaccine antigens*

   a. Comprehensive microbiome analysis of microbial ecology in fleece rot, lumpy wool and the breech of sheep susceptible to breech strike. A microbiome analysis will greatly assist the identification of bacterial isolates required for *in vitro* or challenge efficacy trials and antigenic targets for vaccine development.

   b. Compile a list of possible vaccine antigens by undertaking comprehensive bioinformatics analyses of publicly available *Pseudomonas aeruginosa* and *Dermatophilus congolensis* genome sequences. Identify all secreted proteins, all known infectivity and pathogenicity protein factors, and known antibiotics resistance genes. Cross-reference information obtained with strains of *P. aeruginosa* and *D. congolensis* identified by experimental microbiome analysis of fleece rot and lumpy wool. From the list of antigens, identify strain-specific and species-specific structural variants.

   c. Identify non-protein secreted products from *Pseudomonas aeruginosa* and *Dermatophilus congolensis*. The secreted microbial products will include a subset of infectivity and pathogenesis factors, which are prime candidates for inclusion in respective vaccines. Mass spectrometry can experimentally identify the secreted factors. Structural differences in non-protein antigens for different bacterial strains will need to be determined.

   d. Finger-print *Pseudomonas aeruginosa* strain specific genetic differences in fleece rot samples. The approach will determine *Pseudomonas aeruginosa* population structures (strains) in fleece rot samples, antibiotic resistance profiles and similarities of population structures with *Pseudomonas* populations that infect humans. Strain variation will be important from the perspective of fleece rot vaccine design.

2. *Identify mechanism(s) of immune-mediated protection induced by vaccination.*

   a. Use candidate antigens from results of microbial analyses, and establish a primary goal of vaccination: to control fleece rot, lumpy wool and body strike, or body strike and some breech strike.

   b. Develop *in vivo* models to assess mode of vaccine action for use in stage 3.

3. *Develop in vitro correlates of immune-mediated protection*

   a. Use *in vitro* immune function correlates of protection (e.g. antibody mediated neutralisation of bacterial exoproduct activity) to inform adjuvant choice and vaccine delivery route and regimen.

   b. Use an *in vitro* model to examine potential duration of action in the field.

   c. If fleece rot vaccine shares a common mode of immune-mediated protection (e.g. antibody in skin secretions and exudates) with the Blowfly Vaccine Project (AWI Project ON-00619), integrate the two projects.

4. *Undertake vaccination efficacy trials and develop field strategies for use of the vaccine*
    a. Establish clinical efficacy in the field against primary and secondary vaccine indications: i.e., to control fleece rot, lumpy wool and body strike, or body strike and some breech strike as per 2a).
    b. Determine duration of vaccine action.
    c. Determine whether the fleece rot vaccine can be combined/formulated with other current sheep vaccines.
    d. Develop protocols for vaccine use that accommodate the influence of management practices, sheep age, duration of action, and climatic variables on practicality and efficacy of vaccination.

12.   Acknowledgements

The aim

13. References

Al-Mamun, H.A., Kwan, P., Clark, S.A., Ferdosi, M.H., Tellam, R., Gondro, C., 2015. Genome-wide association study of body weight in Australian Merino sheep reveals an orthologous region on OAR6 to human and bovine genomic regions affecting height and weight. Genet. Sel. Evol. 47, 66.

Ambrose, N., 1996a. Antigenic, pathogenic and virulence factors of *Dermatophilus congolensis*. URL http://www.vet.ed.ac.uk/ctvm/AHP/ahp/finalAHPelectronic/025.pdf

Ambrose, N.C., 1996b. The pathogenesis of dermatophilosis. Trop. Anim. Health Prod. 28, 29S-37S.

Ambrose, N., Lloyd, D., Maillard, J.C., 1999. Immune responses to *Dermatophilus congolensis* infections. Parasitol. Today 15, 295–300.

Ambrose, N.C., El-Jack, M.A., McOrist, S., Boid, R., 1997. Electrophoretic and antigenic characterisation of *Dermatophilus congolensis* extracellular products. Vet. Microbiol. 59, 37–51.

Amor, A., Enríquez, A., Corcuera, M.T., Toro, C., Herrero, D., Baquero, M., 2011. Is infection by *Dermatophilus congolensis* underdiagnosed? J. Clin. Microbiol. 49, 449–451.

Anstead, C.A., Korhonen, P.K., Young, N.D., Hall, R.S., Jex, A.R., Murali, S.C., Hughes, D.S.T., Lee, S.F., Perry, T., Stroehlein, A.J., Ansell, B.R.E., Breugelmans, B., Hofmann, A., Qu, J., Dugan, S., Lee, S.L., Chao, H., Dinh, H., Han, Y., Doddapaneni, H.V., Worley, K.C., Muzny, D.M., Ioannidis, P., Waterhouse, R.M., Zdobnov, E.M., James, P.J., Bagnall, N.H., Kotze, A.C., Gibbs, R.A., Richards, S., Batterham, P., Gasser, R.B., 2015. *Lucilia cuprina* genome unlocks parasitic fly biology to underpin future interventions. Nat. Commun. 6, 7344.

Atkins, K., McGuirk, B., 1979. Selection of Merino sheep for resistance to fleece-rot and body strike. Wool Technology and Sheep Breeding 27, 15-19.

Australian Wool Innovation Limited, 2019. Managing breech flystrike. URL https://www.wool.com/globalassets/wool/sheep/research-publications/welfare/improved-breech-flystrike-management/gd2428-2019-managing-flystrike-manual_11.pdf

Awad, W., Abdou, N.E., El-Sayed, A., 2007. Diagnosis and treatment of bovine, ovine and equine dermatophilosis. Journal of Applied Sciences Research 4, 367–374.

Azimi, S., Roberts, A.E.L., Peng, S., Weitz, J.S., McNally, A., Brown, S.P., Diggle, S.P., 2020. Allelic polymorphism shapes community function in evolving *Pseudomonas aeruginosa* populations. bioRXiv, April, 1–14.

Banazeer, A., Afzal, M.B.S., Ijaz, M., Shad, S.A., 2019. Spinosad resistance selected in the laboratory strain of *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae): studies on risk assessment and cross-resistance patterns. Phytoparasitica 47, 531–542.

Baran, A., Świderska, M., Bacharewicz-Szczerbicka, J., Myśliwiec, H., Flisiak, I., 2017. Serum fatty acid-binding protein 4 is increased in patients with psoriasis. Lipids 52, 51–60.

Bateup, B., Edwards, J., 1990. Processing of wool contaminated with dermatophilosis scab. Australian Veterinary Journal 67, 154–155.

Belschner, H.G., 1937. Observations on fleece-rot and body strike in sheep, particularly in regard to their incidence, type of sheep susceptible and economic importance. New South Wales Department of Agriculture Science Bulletin 54, 61-95.

Berggren-Thomas, P.L., Kaattari, S., Hohenboken, W.D., Shrestha, J.N.B., Heaney, D.P., 1987. Inheritance of active and acquired immunity traits in sheep. Journal of Animal Science 64, 1302-1312.

Berghof, T., Bovenhuis, H., Mulder, H., 2019. Body weight deviations as indicator for resilience in layer chickens. Frontiers in Genetics 10, 1216.

Berghof, T., Poppe, M., Mulder, H., 2018. Opportunities to improve resilience in animal breeding programs. Frontiers in Genetics 9, 692.

Berry, W., Watt, B., 2017. High prevalence mild dermatophilosis (lumpy wool) in unweaned merino lambs following a wet spring. URL http://www.flockandherd.net.au/sheep/ireader/dermatophilosis.html

Bharti, R., Grimm, D.G., 2019. Current challenges and best-practice protocols for microbiome analysis. Brief. Bioinform. (in press).

Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., Decreusefond, C., 1971. Genetic regulation of the function of antibody-producing cells. In: Progress in Immunology, Elsevier, pp. 529-545.

Bull, L.B., 1929. Dermatomycosis of the sheep (lumpy or matted wool) due to *Actinomyces dermatonomus*. Australian Journal of Experimental Biology and Medical Science 6, 301–314.

Bull, L.B., 1931. Some observations on dermatitis of the folds in the breech of sheep and its possible relationship to blowfly strike. Australian Veterinary Journal 7, 143-148.

Burrell, D.H., 1985. Immunisation of sheep against experimental *Pseudomonas aeruginosa* dermatitis and fleece-rot associated body strike. Australian Veterinary Journal 62, 55–57.

Burrell, D.H., 1990. The bacteriology and pathogenesis of fleece rot and associated myiasis; control of these diseases with *Pseudomonas aeruginosa* vaccines. Advances in Veterinary Dermatology 1, 347-358.

Burrell, D.H., MacDiarmid, J., 1984. Characterisation of isolates of *Pseudomonas aeruginosa* from sheep. Australian Veterinary Journal 61, 277-279.

Burrell, D.H., and J.A. MacDiarmid., 1986. Vaccine containing *Pseudomonas maltophilia* antigens to reduce pre-disposition of sheep to fly strike. Australian Patent Office patent number AU8652530-A and ZA8600374-A, filed 17 January 1986, and issued 1986.

Burrell, D.H., MacDiarmid, J.A., 1988. Vaccines against flystrike. New Zealand Patent Office, patent number NZ214858A, issued 1988.

Burrell, D.H., MacDiarmid, J., Smith, A., 1992. The impact of fleece rot on the wool industry. Wool Technology and Sheep Breeding 40, 117-122.

Burrell, D.H., Merritt, G.C., Watts, J.E., Walker, K.H., 1982a. Experimental production of dermatitis in sheep with *Pseudomonas aeruginosa*. Australian Veterinary Journal 59, 140-144.

Burrell, D.H., Merritt, G.C., Watts, J.E., Walker, K.H., 1982b. The role of *Pseudomonas aeruginosa* in pathogenesis of fleece-rot and the effect of immunisation. Australian Veterinary Journal 58, 34-35.

Burrows, L.E.R., 2018. Molecular genetics of flystrike susceptibility in New Zealand sheep, PhD Thesis. Lincoln University.

Casu, R., Eisemann, C., Pearson, R., Riding, G., East, I., Donaldson, A., Cadogan, L., Tellam, R., 1997. Antibody-mediated inhibition of the growth of larvae from an insect causing cutaneous myiasis in a mammalian host. Proc. Natl Acad. Sci. 94, 8939–8944.

Chapman, R.C., Hollis, D.E., Hemsley, J.A., 1984. How quickly does wetting affect the skin of Merino sheep? Animal Production in Australia 15, 290-292.

Chen, D., Colditz, I.G., Glenn, G.M., Tsonis, C.G., 2000. Induction of systemic immune responses in sheep by topical application of cholera toxin to skin. Veterinary Immunology and Immunopathology 77, 191-199.

Chen, D., Colditz, I.G., Glenn, G.M., Tsonis, C.G., 2002. Effect of transcutaneous immunization with co-administered antigen and cholera toxin on systemic and mucosal antibody responses in sheep. Veterinary Immunology and Immunopathology 86, 177-182.

Chin, J.C., Watts, J.E., 1988. Biological properties of phospholipase C purified from a fleecerot isolate of *Pseudomonas aeruginosa*. Microbiology 134, 2567-2575.

Chin, J.E., Watts, J.E., 1991. Dermal and serological response against *Pseudomonas aeruginosa* in sheep bred for resistance and susceptibility to fleece-rot. Aust. Vet. J. 68, 28–31.

Chin, J.C., Watts, J.E., 1992. Relationship between the immune response of sheep and the population dynamics of bacteria isolated from fleecerot lesions. Veterinary Microbiology 32, 63-74.

Chin, J., 1995. Composition for use in raising an immune response. Patent WO 95/27508.

Chin, J.C., Dai, Y., Watts, J.E., 1995. Antibody response against *Pseudomonas aeruginosa* membrane proteins in experimentally infected sheep. Vet. Microbiol. 43, 21–32.

Chin, J., Gil, F., Eamens, G., Djordjevic, S., Simecka, J., Duncan, J., Mullbacher, A., 1996. Manipulating systemic and mucosal immune responses with skin-deliverable adjuvants. Journal of Biotechnology 44, 13-19.

Cisneros, J., Goulson, D., Derwent, L.C., Penagos, D.I., Hernández, O., Williams, T., 2002. Toxic effects of spinosad on predatory insects. Biological Control 23, 156–163.

Colditz, I.G., 2008. The costs of immune responses. In: Rauw, W.M. (Ed.), Resource Allocation Theory Applied to Farm Animal Production, CABI Publishing, Wallingford, UK, pp. 192-209.

Colditz, I.G., Altmann, K.G., Watson, D.L., 1992a. Intradermal and percutaneous transudation of IgG1 and transferrin in sheep. Immunol. Cell Biol. 70 (Pt 5), 323-327.

Colditz, I.G., Woolaston, R.R., Lax, J., Mortimer, S.I., 1992b. Plasma leakage in skin of sheep selected for resistance or susceptibility to fleece rot and fly strike. Parasite Immunology 14, 587-594.

Colditz, I.G., Eisemann, C.H., Tellam, R.L., McClure, S.J., Mortimer, S.I., Husband, A.J., 1996a. Growth of *Lucilia cuprina* larvae following treatment of sheep divergently selected for fleece rot and fly strike with monoclonal antibodies to T lymphocyte subsets and interferon gamma. International Journal for Parasitology 26, 775-782.

Colditz, I.G., Watson, D.L., Gray, G.D., Eady, S.J., 1996b. Some relationships between age, immune responsiveness and resistance to parasites in ruminants. International Journal for Parasitology 26, 869-877.

Colditz, I.G., Hine, B.C., 2016. Resilience in farm animals: biology, management, breeding and implications for animal welfare. Anim. Prod. Sci. 56, 1961-1983.

Colditz, I.G., Lax, J., Mortimer, S.I., Clarke, R.A., Beh, K.J., 1994. Cellular inflammatory responses in skin of sheep selected for resistance or susceptibility to fleece rot and fly strike. Parasite Immunology 16, 289-296.

Colditz, I.G., Paull, D.R., 2010. Needle-free vaccination in sheep. Australian Veterinary Journal 88, 368.

Colditz, I.G., Piper, L.R., Atkins, K.D., 2001. Breeding for resistance to fly strike. In: Champion, S. (Ed.), FLICS: Fly and Lice IPM Control Strategies, Tasmanian Institute of Agricultural Research, University of Tasmania, Hobart, pp. 383-394.

Colditz, I.G., Watson, D.L., 1993. Immunisation to induce antibody production in skin of sheep. In. Proceedings of Vaccines '93, Lorne House, Victoria (Abstract J94).

Colditz, I.G., Watson, D.L., Eisemann, C.H., Tellam, R.L., 2002. Production of antibodies to recombinant antigens from *Lucilia cuprina* following cutaneous immunisation of sheep. Veterinary Parasitology 104, 345-350.

Cottle, D.J., 1996. Selection programs for fleece rot resistance in Merino sheep. Australian Journal of Agricultural Research 47, 1213-1233.

Cope, R.B., Colditz, I.G., 2000. Effect on systemic antibody concentrations of topical application of cholera toxin to skin of sheep. Australian Veterinary Journal 78, 121-123.

Dai, Y., 1997. Potential role of *Pseudomonas aeruginosa* in sheep fleece rot and the associated serological response. PhD thesis, University of Wollongong.

Dantzer, R., Kelley, K.W., 1989. Stress and immunity: an integrated view of relationships between the brain and the immune system. Life Sciences 44, 1995-2008.

Davis, D., 1988. Experimental vaccination of rats with *Dermatophilus congolensis* zoospores. Res. Vet. Sci. 44, 400–401.

Denman, S.E., Martinez Fernandez, G., Shinkai, T., Mitsumori, M., McSweeney, C.S., 2015. Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. Front. Microbiol. 6.

Dixon, T.J., Mortimer, S.I., Norris, B.J., 2007. 16S rRNA gene microbial analysis of the skin of fleece rot resistant and susceptible sheep. Aust. J. Agric. Res. 58, 739–747.

Döring, G., Pier, G.B., 2008. Vaccines and immunotherapy against *Pseudomonas aeruginosa*. Vaccine 26, 1011–1024.

Douris, V., Steinbach, D., Panteleri, R., Livadaras, I., Pickett, J.A., Van Leeuwen, T., Nauen, R., Vontas, J., 2016. Resistance mutation conserved between insects and mites unravels the benzoylurea insecticide mode of action on chitin biosynthesis. Proc. Natl Acad. Sci. USA 113, 14692–14697.

Dowling, M.E., Schlink, A.C., Greeff, J.C., 2006. Inheritance of moisture content in greasy and clean wool and its relationship to other wool traits in Merinos. Australian Journal of Experimental Agriculture, 46, 933–936.

Dwyer, C.M., Bornett, H.L.I., 2004. Chronic stress in sheep: assessment tools and their use in different management conditions. Animal Welfare 13, 293-304.

East, I.J., Eisemann, C.H., Vuocolo, T., Pearson, R.D., Donaldson, R.A., Cadogan, L.C., 1992. Vaccines against blowfly strike: the effect of adjuvant type on vaccine effectiveness. International Journal for Parasitology 22, 309-314.

Edwards, J.R., 1985. Sale and processing of wool affected with dermatophilosis. Aust. Vet. J. 62, 173–174.

Edwards, J.R., Gardner, J.J., Norris, R.T., Love, R.A., Spicer, P., Bryant, R., Gwynn, R.V.R., Hawkins, C.D., Swan, R.A., 1985. A survey of ovine dermatophilosis in Western Australia. Australian Veterinary Journal 62, 361-365.

Eisemann, C.H., 1988. Upwind flight by gravid Australian sheep blowflies, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae), in response to stimuli from sheep. Bulletin of Entomological Research 78, 273–279.

Elgersma, G.G., De Jong, G., Van der Linde, R., Mulder, H.A., 2018. Fluctuations in milk yield are heritable and can be used as a resilience indicator to breed healthy cows. J. Dairy Sci. 101, 1240-1250.

Ellis, T.M., Masters, A.M., Sutherland, S.S., Carson, J.M., Gregory, A.R., 1993. Variation in cultural, morphological, biochemical properties and infectivity of Australian isolates of *Dermatophilus congolensis*. Vet. Microbiol. 38, 81–102.

Ellis, T.M., Robertson, G.M., Sutherland, S.S., Gregory, A.R., 1987. Cellular responses in the skin of merino sheep to repeated inoculation with *Dermatophilus congolensis*. Vet. Microbiol. 15, 151–162.

Ellis, T.M., Sutherland, S.S., Davies, G., 1991. Strain variation in *Dermatophilus congolensis* demonstrated by cross-protection studies. Vet. Microbiol. 28, 377–383.

Ellis, T.M., Sutherland, S.S., Gregory, A.R., 1989. Inflammatory cell and immune function in Merino sheep with chronic dermatophilosis. Vet. Microbiol. 21, 79–93.

Ellis, T.M., Sutherland, S.S., Turnor, R., Krueger, R., 1992. Relationship between chronic ovine dermatophilosis and levels of T6 lymphocyte antigen staining in peripheral blood mononuclear cells. Vet. Microbiol. 30, 281–287.

Emmens, R.L., Murray, M.D., 1983. Bacterial odours as oviposition stimulants for *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae), the Australian sheep blowfly. Bulletin of Entomological Research 73, 411–415.

Engwerda, C.R., Dale, C.J., Sandeman, R.M., 1998. IgE, TNFa, IL1b, IL4 and IFNg gene polymorphisms in sheep selected for resistance to fleece rot and flystrike. International Journal for Parasitology 26, 787-791.

Escayg, A.P., Hickford, J.G., Bullock, D.W., 1997. Association between alleles of the ovine major histocompatibility complex and resistance to footrot. Res. Vet. Sci. 63, 283–287.

Faris, B., Hollis, L., 2013. Dermatophilosis in sheep and goats. Kansas State University Agricultural Experiment Station and Cooperative Extension Service. URL https://bookstore.ksre.ksu.edu/pubs/MF3118.pdf

Finney, J., Yeh, C.-H., Kelsoe, G., Kuraoka, M., 2018. Germinal center responses to complex antigens. Immunol. Rev. 284, 42–50.

Forbes, J.D., Knox, N.C., Ronholm, J., Pagotto, F., Reimer, A., 2017. Metagenomics: the next culture-independent game changer. Front. Microbiol. 8, 1069.

Francis, M.J., 2018. Recent Advances in Vaccine Technologies. Vet. Clin. North Am. Small Anim. Pract. 48, 231–241.

Freney, M.R., 1940. Chemical investigations on the fleece of sheep. Council for Scientific and Industrial Research Bulletin 130.

Freschi, L., Bertelli, C., Jeukens, J., Moore, M.P., Kukavica-Ibrulj, I., Emond-Rheault, J.-G., Hamel, J., Fothergill, J.L., Tucker, N.P., McClean, S., Klockgether, J., de Soyza, A., Brinkman, F.S.L., Levesque, R.C., Winstanley, C., 2018. Genomic characterisation of an international *Pseudomonas aeruginosa* reference panel indicates that the two major groups draw upon distinct mobile gene pools. FEMS Microbiol. Lett. 365, 14.

Freschi, L., Vincent, A.T., Jeukens, J., Emond-Rheault, J.G., Kukavica-Ibrulj, I., Dupont, M.J., Charette, S.J., Boyle, B., and Levesque, R.C., 2019. The *Pseudomonas aeruginosa* pan-genome provides new insights on its population structure, horizontal gene transfer, and pathogenicity. Genome Biology and Evolution 11 (1): 109–20.

Friedel, T., McDonell, P.A., 1985. Cyromazine inhibits reproduction and larval development of the Australian sheep blow fly (diptera: Calliphoridae). J. Econ. Entomol. 78, 868–873

Furuhashi, M., Matsumoto, M., Murase, T., Nakamura, T., Higashiura, Y., Koyama, M., Tanaka, M., Moniwa, N., Ohnishi, H., Saitoh, S., Shimamoto, K., Miura, T., 2019. Independent links between plasma xanthine oxidoreductase activity and levels of adipokines. J. Diabetes Investig. 10, 1059–1067.

Gardiner, M.R., 1971. Mycotic dermatitis (lumpy wool) of sheep. Journal of the Department of Agriculture, Western Australia 12, 5.

Gherardi, S.G., Monzu, N., Sutherland, S.S., Johnson, K.G., Robertson, G.M., 1981. The association between body strike and dermatophilosis of sheep under controlled conditions. Aust. Vet. J. 57, 268–271.

Gherardi, S.G., Sutherland, S.S., Monzu, N., Johnson, K.G., 1983. Field observations on body strike in sheep affected with dermatophilosis and fleece-rot. Aust. Vet. J. 60, 27–28.

Giani, A.M., Gallo, G.R., Gianfranceschi, L., Formenti, G., 2020. Long walk to genomics: History and current approaches to genome sequencing and assembly. Computational and Structural Biotechnology Journal 18, 9–19.

Gill, H.S., Colditz, I.G., Watson, D.L., 1993. Immune responsiveness of lambs selected for resistance to haemonchosis. Res. Vet. Sci. 54, 361–365.

Gilruth, J.A., Tillyard, R.J., Seddon, H.R., Gurney, W.B., Mackerras, I.M., 1933. The sheep blowfly problem, Report no 1 of the Joint Blowfly Committee. NSW Department of Agriculture Science Bulletin 40, 1-136.

Glass, E.J., 2004. Genetic variation and responses to vaccines. Animal Health Research Reviews 5, 197-208.

Glenn, G.M., Rao, M., Matyas, G.R., Alving, C.R., 1998. Skin immunization made possible by cholera toxin. Nature 391, 851.

Glenn, G.M., Scharton-Kersten, T., Vassell, R., Matyas, G.R., Alving, C.R., 1999. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins as antigens and adjuvants. Infection and Immunity 67, 1100-1106.

Gogolewski, R.P., Mackintosh, J.A., Wilson, S.C., Chin, J.C., 1992. Immunodominant antigens of zoospores from ovine isolates of *Dermatophilus congolensis*. Vet. Microbiol. 32, 305–318.

Gogolewski, R.P., Nicholls, P.J., Mortimer, S.I., Mackintosh, J.A., Nesa, M., Ly, W., Chin, J.C., 1996. Serological responses against *Pseudomonas aeruginosa* in Merino sheep bred for resistance or susceptibility to fleece rot and body strike. Australian Journal of Agricultural Research 47, 917-926.

Goodrich, B.S., Lipson, M., 1978. Easter splitting of wool wax in fleece-rot and mycotic dermatitis. Australian Veterinary Journal 54, 43.

Hammond, S.A., Tsonis, C., Sellins, K., Rushlow, K., Scharton-Kersten, T., Colditz, I., Glenn, G.M., 2000. Transcutaneous immunization of domestic animals: opportunities and challenges. Advanced Drug Delivery Reviews 43, 45-55.

Hay, J.B., Mills, S.C., 1982. Chemical changes in the wool of adult Merino sheep during prolonged wetting and prior to development of fleece rot. Australian Journal of Agricultural Research 33, 335-346.

Hayman, R.H., 1953. Studies on fleece-rot in sheep. Australian Journal of Agricultural Research 4, 430-468.

Hayman, R.H., 1955. Effects of wet weather on the fleece. Wool Technology 2, 31-35.

Heather, J.M., Chain, B., 2016. The sequence of sequencers: The history of sequencing DNA. Genomics 107, 1–8.

Hedegaard, C.J., Heegaard, P.M., 2016. Passive immunisation, an old idea revisited: Basic principles and application to modern animal production systems. Veterinary Immunology and Immunopathology 174, 50-63.

Hernandez, A., Quinton, M., Miglior, F., Mallard, B.A., 2006. Genetic parameters of dairy cattle immune response traits. In: International Committee for World Congress on Genetics Applied to Livestock Production, Belo Horizonte, MG, Brazil, pp. 15-18.

Hine, B., Ingham, A., Smith, J., Colditz, I., 2017. Associations between immune competence, health and performance of sheep in the Resource Flock. In: https://www.mla.com.au/download/finalreports?itemId=3635 (accessed 29 September 2019).

Hine, B.C., Bell, A.M., Niemeyer, D.D.O., Duff, C.J., Butcher, N.M., Dominik, S., Ingham, A.B., Colditz, I.G., 2019. Immune competence traits assessed during the stress of weaning are heritable and favourably genetically correlated with temperament traits in Angus cattle. Journal of Animal Science 97, 4053-4065.

Hiraizumi, M., Tagawa, Y., 2014. Isolation and characterization of flagellar filament from zoospores of *Dermatophilus congolensis*. Vet. Microbiol. 173, 141–146.

Hoggarth, A., Weaver, A., Pu, Q., Huang, T., Schettler, J., Chen, F., Yuan, X., Wu, M., 2019. Mechanistic research holds promise for bacterial vaccines and phage therapies for *Pseudomonas aeruginosa*. Drug Des. Devel. Ther. 13, 909–924.

Holdaway, F.G., Mulhearn, G.R., 1934. Field observations on weather stain and blowfly strike of sheep with special reference to body strike. CSIR Pamphlet 48.

Hollis, D.E., Chapman, R.E., Hemsley, J.A., 1982. Effects of experimentally induced fleece rot on the structure of the skin of merino sheep. Aust. J. Biol. Sci. 35, 545-556.

How, S.J., Lloyd, D.H., Sanders, A.B., 1990. Advances in Veterinary Dermatology, Volume 1. London, UK.

Hugenholtz, P., 2002. Exploring prokaryotic diversity in the genomic era. Genome Biol. 3, reviews0003.

Hunt, J.D., Jackson, D.C., Wood, P.R., Stewart, D.J., Brown, L.E., 1995. Immunological parameters associated with antigenic competition in a multivalent footrot vaccine. Vaccine 13, 1649–1657

James, P., Brien,T., Anderson, A., 2019. Review of predisposing factors for breech strike. Australian Wool Innovation. URL https://www.wool.com/globalassets/wool/sheep/research-publications/welfare/flystrike-research-update/review-predisposing-factors-breech-flystrike-final-report.pdf

James, P.J., Ponzoni, R.W., 1992. Fiber diameter variability in South Australian Merinos - phenotypic and genetic-relationships with wool quality parameters and fleece rot resistance. Wool Technology and Sheep Breeding 40, 25-30.

James, P.J., Ponzoni, R.W., Walkley, J.R.W., Smith, D.H., Stafford, J.E., 1984a. Preliminary estimates of phenotypic and genotypic parameters for fleece rot susceptibility in the South Australian Merino. Wool Technology and Sheep Breeding 31, 152-157.

James, P.J., Warren, G.H., Neville, A., 1984b. The effect of some fleece characters on the skin wax layer and fleece rot development in Merino sheep following wetting. Australian Journal of Agricultural Research 35, 413-422.

James, P.J., Ponzoni, R.W., Walkley, J.R.W., Whiteley, K.J., Stafford, J.E., 1987. Fleece rot in South Australian Merinos: heritability and correlations with fleece characters. Merino Improvement Programs in Australia. Proceedings of a National Symposium, Leura, New South Wales, 1987, 341-345.

James, P.J., Warren, G.H., 1979. Effect of disruption of the sebaceous layer of the sheep's skin on the incidence of fleece-rot. Australian Veterinary Journal 55, 335-338.

Jansen, B.C., Hayes, M., 1987. The relationship between the skin and some bacterial species occurring on it in the Merino. Onderstepoort Journal of Veterinary Research 54, 107.

Jenkinson, D.M., Menzies, J.D., Pow, I.A., Inglis, L., Lloyd, D.H., Mackie, A., 1989. Actions of bovine skin washings and sera on the motile zoospores of *Dermatophilus congolensis*. Res. Vet. Sci. 47, 241–246.

Jiang, Y., Xie, M., Chen, W., Talbot, R., Maddox, J.F., Faraut, T., Wu, C., Muzny, D.M., Li, Y., Zhang, W., Stanton, J.-A., Brauning, R., Barris, W.C., Hourlier, T., Aken, B.L., Searle, S.M.J., Adelson, D.L., Bian, C., Cam, G.R., Chen, Y., Cheng, S., DeSilva, U., Dixen, K., Dong, Y., Fan, G., Franklin, I.R., Fu, S., Guan, R., Highland, M.A., Holder, M.E., Huang, G., Ingham, A.B., Jhangiani, S.N., Kalra, D., Kovar, C.L., Lee, S.L., Liu, W., Liu, X., Lu, C., Lv, T., Mathew, T., McWilliam, S., Menzies, M., Pan, S., Robelin, D., Servin, B., Townley, D., Wang, Wenliang, Wei, B., White, S.N., Yang, X., Ye, C., Yue, Y., Zeng, P., Zhou, Q., Hansen, J.B., Kristensen, K., Gibbs, R.A., Flicek, P., Warkup, C.C., Jones, H.E., Oddy, V.H., Nicholas, F.W., McEwan, J.C., Kijas, J., Wang, J., Worley, K.C., Archibald, A.L., Cockett, N., Xu, X., Wang, Wen, Dalrymple, B.P., 2014. The sheep genome illuminates biology of the rumen and lipid metabolism. Science 344, 1168–1173.

Kaminski, G.W., Suter, I.I., 1976. Human infection with *Dermatophilus congolensis*. Med. J. Aust. 1, 443–447.

Keown, A.M., Reid, T.C., 1997. An investigation of techniques used to predict fleece rot incidence in sheep. Proceedings of the New Zealand Society of Animal Production 57, 83.

Khan, H.A.A., 2018. Spinosad resistance affects biological parameters of *Musca domestica* Linnaeus. Scientific Reports 8, 14031.

Kidd, T.J., Ritchie, S.R., Ramsay, K.A., Grimwood, K., Bell, S.C., Rainey P.B., 2012. *Pseudomonas aeruginosa* exhibits frequent recombination, but only a limited association between genotype and ecological setting. PLOS ONE 7 (9): e44199.

Kingsford, N.M., Raadsma, H.W., 1995. Detection of *Pseudomonas aeruginosa* from ovine fleece washings by PCR amplification of 16S ribosomal RNA. Veterinary Microbiology 47, 61–70.

Kingsford, N.M., Raadsma, H.W., 1997. The occurrence of *Pseudomonas aeruginosa* in fleece washings from sheep affected and unaffected with fleece rot. Veterinary Microbiology 54, 275-285.

Kupper, T.S., Ballard, D.W., Chua, A.O., McGuire, J.S., Flood, P.M., Horowitz, M.C., Langdon, R., Lightfoot, L., Gubler, U., 1986. Human keratinocytes contain mRNA indistinguishable from monocyte interleukin 1 alpha and beta mRNA. Keratinocyte epidermal cell-derived thymocyte-activating factor is identical to interleukin 1. The Journal of Experimental Medicine 164, 2095-2100.

Larrasa, J., Garcia, A., Ambrose, N.C., Alonso, J.M., Parra, A., de Mendoza, M.H., Salazar, J., Rey, J., de Mendoza, J.H., 2002. A simple random amplified polymorphic DNA genotyping method for field isolates of *Dermatophilus congolensis*. J. Vet. Med. B Infect. Dis. Vet. Public Health 49, 135–141.

Larrasa, J., García-Sánchez, A., Ambrose, N.C., Parra, A., Alonso, J.M., Rey, J.M., Hermoso-de-Mendoza, M., Hermoso-de-Mendoza, J., 2004. Evaluation of randomly amplified polymorphic DNA and pulsed field gel electrophoresis techniques for molecular typing of *Dermatophilus congolensis*. FEMS Microbiol. Lett. 240, 87–97.

Larsen, C.G., Anderson, A.O., Oppenheim, J.J., Matsushima, K., 1989. Production of interleukin-8 by human dermal fibroblasts and keratinocytes in response to interleukin-1 or tumour necrosis factor. Immunology 68, 31.

Lee, C., Fisher, A.D., 2007. Welfare consequences of mulesing of sheep. Aust. Vet. J. 85, 89–93.

Leoni, A., Fadda, M., Nieddu, A.M., Pittau, M., Sanna, E., Pirino, S., Contini, A., 1993. Dermatophilosis in sheep: first report in Italy, experimental reproduction and evaluation of immune response. Boll. Soc. Ital. Biol. Sper. 69, 775–782.

Levot, G.W., 2013. Response to laboratory selection with cyromazine and susceptibility to alternative insecticides in sheep blowfly larvae from the New South Wales Monaro. Australian Veterinary Journal 91, 61–64.

Levot, G.W., 2012. Cyromazine resistance detected in Australian sheep blowfly. Australian Veterinary Journal 90, 433–437.

Levot, G.W., 1995. Resistance and the control of sheep ectoparasites. International Journal for Parasitology, Australian Society for Parasitology Proceedings of the Scientific Meeting 25, 1355–1362.

Levot, G.W., Langfield, B.J., Aiken, D.J., 2014. Survival advantage of cyromazine-resistant sheep blowfly larvae on dicyclanil- and cyromazine-treated Merinos. Australian Veterinary Journal 92, 421–426.

Lewer, R.P., Gherardi, S.G., Sutherland, S.S., 1987. Realised heritability estimates for resistance to dermatophilosis in Merino sheep., in: Merino Improvement Programs in Australia: Proceedings of a National Symposium, Leura. pp. 347–350.

Li, Y., Swan, A., Purvis, I., 1999. Genetic variation in resistance to fleece rot in CSIRO's fine wool flock. Proceedings of the Association for the Advancement of Animal Breeding and Genetics 13, 524-527.

Lipson, M., Hilton, R.A., Watts, J.E., Merritt, G.C., 1982. Factors influencing fleece rot in sheep. Australian Journal of Experimental Agriculture and Animal Husbandry 22, 168-172.

Lloyd, D.H., Jenkinson, D.M., 1981. Serum and skin surface antibody responses to intradermal vaccination of cattle with *Dermatophilus congolensis*. British Veterinary Journal 137, 601-607.

Lloyd, D.H., Jenkinson, D.M., Miller, P.N., Sykes, T.J., 1987. Sequential production of specific antibodies in serum and at the skin surface of cattle following intradermal vaccination with *Dermatophilus congolensis*. Veterinary Immunology and Immunopathology 16, 251-257.

Lloyd, D.H., McEwan Jenkinson, D., Mabon, R.M., 1979. Location of immunoglobulins in the skin of cattle and sheep. Res. Vet. Sci. 26, 47-52.

Lloyd, D.H., Noble, W.C., 1982. *Dermatophilus congolensis* as a model pathogen in mice for the investigation of factors influencing skin infection. British Veterinary Journal 138, 51–60.

London, C.J., Griffith, I.P., 1984a. Characterization of pseudomonads isolated from diseased fleece. Applied and Environmental Microbiology 47, 993-997.

London, C.J., Griffith, I.P., 1984b. Characterization of pseudomonads isolated from diseased fleece. Appl. Environ. Microbiol. 47, 993–997.

Lozano, C., Azcona-Gutiérrez, J.M., Van Bambeke, F., Sáenz, Y., 2018. Great phenotypic and genetic variation among successive chronic *Pseudomonas aeruginosa* from a cystic fibrosis patient. PLoS One 13, e0204167.

Lung, L.H.D.S., Carvalheiro, R., Neves, H.H.D.R., Mulder, H.A., 2019. Genetics and genomics of uniformity and resilience in livestock and aquaculture species: A review. Journal of Animal Breeding and Genetics. In press.

Lyczak, J.B., Cannon, C.L., Pier, G.B., 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect. 2, 1051–1060.

Lyness, E.W., Pinnock, D.E., Cooper, D.J., 1994. Microbial ecology of sheep fleece. Agriculture, Ecosystems & Environment 49, 103-112.

MacDiarmid, J., Burrell, D.H., 1992. Degradation of the wool fibre by bacteria isolated from fleece rot. Wool Technology and Sheep Breeding 40, 123-124.

MacDiarmid, J.A., Burrell, D.H., 1986. Characterization of *Pseudomonas maltophilia* isolates from fleece rot. Appl. Environ. Microbiol. 51, 346–348.

Magoc, L., Yen, J.L., Hill-Williams, A., McKenzie, J.A., Batterham, P., Daborn, P.J., 2005. Cross-resistance to dicyclanil in cyromazine-resistant mutants of *Drosophila melanogaster* and *Lucilia cuprina*. Pesticide Biochemistry and Physiology 81, 129–135.

Maillard, J.-C., Berthier, D., Chantal, I., Thevenon, S., Sidibé, I., Stachurski, F., Belemsaga, D., Razafindraïbé, H., Elsen, J.-M., 2003. Selection assisted by a BoLA-DR/DQ haplotype against susceptibility to bovine dermatophilosis. Genet. Sel. Evol. 35 Suppl 1, S193-200.

Mallard, B.A., Wilkie, B.N., Kennedy, B.W., Quinton, M., 1992. Use of estimated breeding values in a selection index to breed Yorkshire pigs for high and low immune and innate resistance factors. Animal Biotechnology 3, 257-280.

Marsella, R., 2016. Dermatophilosis. Veterian Key. URL https://veteriankey.com/dermatophilosis/ (accessed 3.7.20).

Masters, A. M., Ellis, T.M., Grein, S.B., 1997a. *Dermatophilus congolensis*: strain differences in expression of phospholipase activities. Vet. Microbiol. 57, 199–213.

Masters, A. M., Ellis, T.M., Groth, D., 1997b. Difference between lambs with chronic and mild dermatophilosis in frequency of alleles of CD3γ. Veterinary Microbiology 57, 337–345.

Masters, D.G., Ferguson, M.B., 2019. A review of the physiological changes associated with genetic improvement in clean fleece production. Small Ruminant Research 170, 62-73.

McColl, K.A., Gogolewski, R.P., Chin, J.C., 1997. Peripheral blood lymphocyte subsets in fleece rot-resistant and -susceptible sheep. Australian Veterinary Journal 75, 421-423.

McGuirk, B.J., Atkins, K.D., 1984. Fleece rot in Merino sheep. I The heritability of fleece rot in unselected flocks of medium-wool Peppin Merinos. Aust. J. Agric. Res. 35, 423-434.

McGuirk, B.J., Atkins, D.A., Kowal, E., Thornberry, K., 1978. Breeding for resistance to fleece rot and body strike - the Trangie programme. Wool Technology and Sheep Breeding 26(iv), 17-24.

Merakou, C., Schaefers, M.M., Priebe, G.P., 2018. Progress toward the elusive *Pseudomonas aeruginosa* vaccine. Surg. Infect. (Larchmt) 19, 757–768.

Merritt, G.C., Watts, J.E., 1978a. An in-vitro technique for studying fleece-rot and fly strike in sheep. Australian Veterinary Journal 54, 513–516.

Merritt, G.C., Watts, J.E., 1978b. The changes in protein concentration and bacteria of fleece and skin during the development of fleece-rot and body strike in sheep. Aust. Vet. J. 54, 517-520.

Mine, O.M., 1996. Molecular cloning and characterisation of *Dermatophilus congolensis* serine protease genes., PhD thesis, Murdoch University.

Moriello, K., 2020. Dermatophilosis in Animals - Integumentary System. Merck Veterinary Manual. URL https://www.merckvetmanual.com/integumentary-system/dermatophilosis/dermatophilosis-in-animals. (accessed 3.9.20).

Morrow, C.K., Colditz, I.G., Cope, R.B., 2001. Simulated solar UVB exposure inhibits transcutaneous immunization to cholera toxin via an irradiated skin site in cattle. Veterinary Immunology and Immunopathology 83, 107-114.

Mortimer, S.I., 2001. Flystrike resistance in the breeding programs of ram breeding flocks. In: Champion, S. (Ed.), University of Tasmania, Hobart, pp. 406-413.

Mortimer, S.I., Atkins, K.D., Raadsma, H.W., 1998. Responses to selection for resistance and susceptibility to fleece rot and body strike in Merino sheep. In: Proceedings of the 6th World Congress on Genetics Applied to Livestock Production. Vol. 27. pp. 283–286. (University of New England: Armidale, NSW).

Naval-Sanchez, M., Nguyen, Q., McWilliam, S., Porto-Neto, L.R., Tellam, R., Vuocolo, T., Reverter, A., Perez-Enciso, M., Brauning, R., Clarke, S., McCulloch, A., Zamani, W., Naderi, S., Rezaei, H.R., Pompanon, F., Taberlet, P., Worley, K.C., Gibbs, R.A., Muzny, D.M., Jhangiani, S.N., Cockett, N., Daetwyler, H., Kijas, J., 2018. Sheep genome functional annotation reveals proximal regulatory elements contributed to the evolution of modern breeds. Nat. Commun. 9, 859.

Nay, T., Watts, J.E., 1977. Observations on the wool follicle abnormalities in Merino sheep exposed to prolonged wetting conducive to the development of fleece-rot. Australian Journal of Agricultural Research 28, 1095-1105.

Nesa, M.-L., 1994. The immune and inflammatory responses of Merino sheep bred for resistance and susceptibility to fleece rot and flystrike: the Trangie R and S flocks. In: La Trobe University, pp. 1-181.

Netea, M.G., Domínguez-Andrés, J., Barreiro, L.B., Chavakis, T., Divangahi, M., Fuchs, E., Joosten, L.A., van der Meer, J.W., Mhlanga, M.M., Mulder, W.J., 2020. Defining trained immunity and its role in health and disease. Nature Reviews Immunology, 1-14.

Nguyen, T.C., 1984. The immune response in sheep: analysis of age, sex and genetic effects on the quantitative antibody response to chicken red blood cells. Veterinary Immunology and Immunopathology 5, 237-245.

Norris, B.J., Colditz, I.G., Dixon, T.J., 2008. Fleece rot and dermatophilosis in sheep. Vet. Microbiol. 128, 217–230.

NSW Industry and Investment, 2010. Lumpy wool - a skin disease of sheep. Primefacts 3. URL https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0013/314320/9819-Lumpy-wool---Primefact-986.pdf

Partridge, S.R., Kwong, S.M., Firth, N., Jensen, S.O., 2018. Mobile genetic elements associated with antimicrobial resistance. Clinical Microbiology Reviews 31, e00088-17.

Pascoe, L., 1982. Measurement of fleece wettability in sheep and its relationship to susceptibility to fleece rot and blowfly strike. Australian Journal of Agricultural Research 33, 141-148.

Paynter, J.R., 1961. Investigations of fleece rot discoloration in greasy wool. Journal of the Textile Institute 52, 64-72.

Peachey, B., 2018. Breech flystrike. Australian Wool Innovation. URL /sheep/welfare/breech-flystrike/ (accessed 4.20.20).

Poppe, M., Veerkamp, R.F., Van Pelt, M.L., Mulder, H.A., 2020. Exploration of variance, autocorrelation, and skewness of deviations from lactation curves as resilience indicators for breeding. J. Dairy Sci. 103, 1667-1684.

Poulsen, B.E., Yang, R., Clatworthy, A.E., White, T., Osmulski, S.J., Li, L., Penaranda, C., Lander, E.S., Shoresh, N., Hung, D.T., 2019. Defining the core essential genome of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U.S.A. 116, 10072–10080.

Priebe, G.P., Goldberg, J.B., 2014. Vaccines for *Pseudomonas aeruginosa*: a long and winding road. Expert. Rev. Vaccines 13, 507–519.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, Shaochuan, Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.-M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, Shengting, Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, Songgang, Qin, N., Yang, H., Wang, Jian, Brunak, S., Doré, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., MetaHIT Consortium, Bork, P., Ehrlich, S.D., Wang, Jun, 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65.

Raadsma, H.W., 1989. Fleece rot and body strike in Merino sheep. III. Significance of fleece moisture following experimental induction of fleece rot. Australian Journal of Agricultural Research 40, 897-912.

Raadsma, H.W., 1991a. Fleece rot and body strike in Merino sheep. V. Heritability of liability to body strike in weaner sheep under flywave conditions. Aust. J. Agric. Res. 42, 279–293.

Raadsma, H.W., 1991b. The susceptibility to body strike under high rainfall conditions of flocks selected for and against fleece rot. Aust. J. Exp. Agric. 31, 757–759.

Raadsma, H.W., 1992. Genetic improvement in resistance to body strike in Merino sheep: where are we at with indirect selection. Proc. Assoc. Adv. Anim. Breed. Genet. 10, 143–146.

Raadsma, H.W., 1993. Fleece rot and body strike in Merino sheep. VI. Experimental evaluation of some physical fleece and body characteristics as indirect selection criteria for fleece rot. Australian Journal of Agricultural Research 44, 915-931.

Raadsma, H.W., Attard, G.A., Nicholas, F.W., Egerton, J.R., 1996. Disease resistance in Merino sheep. V. Genetic heterogeneity in response to vaccination with *Dichelobacter nodosus* and clostridial antigens. Journal of Animal Breeding and Genetics 113, 181-199.

Raadsma, H., Gilmour, A., Paxton, W., 1988. Fleece rot and body strike in Merino sheep. I. Evaluation of liability to fleece rot and body strike under experimental conditions. Aust. J. Agric. Res. 39, 917.

Raadsma, H.W., Gilmour, A.R., Paxton, W.J., 1989. Fleece rot and body strike in Merino sheep. II. Phenotypic and genetic variation in liability to fleece rot following experimental induction. Australian Journal of Agricultural Research 40, 207-220.

Raadsma, H.W., McEwan, J.C., Stear, M.J., Crawford, A.M., 1999. Genetic characterisation of protective vaccine responses in sheep using multi-valent *Dichelobacter nodosus* vaccines. Veterinary Immunology and Immunopathology 72, 219-229.

Raadsma, H.W., Rogan, I.M., 1987. Genetic variation in resistance to blowfly strike. In: McGuirk, B.J. (Ed.), Merino Improvement Programs in Australia. Proceedings of a National Symposium, Leura, NSW, Melbourne, Australian Wool Corporation, pp. 321-340.

Raadsma, H.W., Watts, J.E., Warren, H., 1987. Effect of wither malformation on the pre-disposition of sheep to fleece rot and body strike. Australian Journal of Experimental Agriculture 27, 503-511.

Raadsma, H.W., Wilkinson, B.R., 1990. Fleece rot and body strike in Merino sheep. IV Experimental evaluation of traits related to greasy wool colour for indirect selection against fleece rot. Australian Journal of Agricultural Research 41, 139-153.

Rauw, W.M., Kanis, E., Noordhuizen-Stassen, E.N., Grommers, F.J., 1998. Undesirable side effects of selection for high production efficiency in farm animals, a review. Livestock Production Science 56, 15-33.

Reed, Steven G., Mark T. Orr, and Christopher B. Fox. Key roles of adjuvants in modern vaccines. *Nature Medicine* 19, no. 12 (December 2013): 1597–1608.

Roberts, D.S., 1965. Penetration and irritation of the skin by *Dermatophilus congolensis*. Br. J. Exp. Pathol. 46, 635–642.

Roberts, D.S., 1966. The phagocytic basis of acquired resistance to infection with *Dermatophilus congolensis*. Br. J. Exp. Pathol. 47, 372–382.

Roberts, D.S., 1967. Chemotherapy of epidermal infection with *Dermatophilus congolensis*. Journal of Comparative Pathology 77, 129.

Radostits, O.M., Gay, C.C., Hinchcliff, K.W., Constable, P.D., 2006. Veterinary Medicine: a textbook of the diseases of cattle, horses, sheep, pigs and goats., 10th ed. Elsevier Health Sciences, United Kingdom.

Sales, N., Suann, M., Koeford, K., N.D., 2020. Dicyclanil resistance in the Australian sheep blowfly, *Lucilla cuprina*, substantially reduces flystrike protection by dicyclanil and cyromazine based products. International Journal for Parasitology: Drugs and Drug Resistance (in press). https://doi.org/10.1016/j.ijpddr.2020.04.005

Sandeman, R.M., Levot, G.W., Heath, A.C.G., James, P.J., Greeff, J.C., Scott, M.J., Batterham, P., Bowles, V.M., 2014. Control of the sheep blowfly in Australia and New Zealand – are we there yet? International Journal for Parasitology, The Australian Society for Parasitology 50th Anniversary Special Issue 44, 879–891.

Schiller, B., Buchowicz, I., Korbecki, M., Lysakowska, E., Rudnicka, W., Robaczewska, E., 1981a. Anti-*Pseudomonas* immunoglobulin I. Immunization of sheep. Arch. Immunol. Ther. Exp. (Warsz.) 29, 627–633.

Schiller, B., Lysakowska, E., Owczarska, K., 1981b. Immunogenic properties and specificity of *Pseudomonas aeruginosa* polyvalent vaccine. Med. Dosw Mikrobiol. 33, 91–96.

Scrivener, C.J., Vizard, A.L., 1995. Efficacy of a single dose of erythromycin or penicillin/streptomycin for the treatment of ovine dermatophilosis. Aust. Vet. J. 72, 475–476.

Seddon, H.R., 1927. Dermatitis of sheep. NSW Depart. Agric. Vet. Res. Rep. 5, 10–17.

Seddon, H.R., Belschner, H.G., Mulhearn, C.R., 1931a. Observations on the susceptibility of sheep to blowfly attack. NSW Department of Agriculture Science Bulletin 37, 3-23.

Seddon, H.R., Belschner, H.G., Mulhearn, C.R., 1931b. The influence of breeding as a factor in the sheep blowfly problem. NSW Department of Agriculture Science Bulletin 37, 24-30.

Seddon, H.R., McGrath, T.T., 1929. Green colouration in wool. Agricultural Gazette of New South Wales 40, 206.

Serrano, I., De Vos, D., Santos, J.P., Bilocq, F., Leitão, A., Tavares, L., Pirnay, J.-P., Oliveira, M., 2017. Antimicrobial resistance and genomic rep-PCR fingerprints of *Pseudomonas aeruginosa* strains from animals on the background of the global population structure. BMC Vet. Res. 13, 58.

Shanahan, G.J., Roxburgh, N.A., 1974. The sequential development of insecticide resistance -problems in *Lucilia cuprina* Wied. in Australia: PANS Pest Articles & News Summaries: Vol 20, No 2. URL https://www.tandfonline.com/doi/abs/10.1080/09670877409413633 (accessed 5.8.20).

Sharma, A., Krause, A., Worgall, S., 2011. Recent developments for *Pseudomonas* vaccines. Human Vaccines 7, 999–1011.

Sheep CRC. Dermatitis (Lumpy Wool). FlyBoss, 2020. URL http://www.flyboss.com.au/sheep-goats/files/pages/susceptibility/body-strike/Dermatitis_Pdf_download_130410.pdf

Silby, M.W., Winstanley, C., Godfrey, S.A.C., Levy, S.B., Jackson, R.W., 2011. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiol. Rev. 35, 652–680.

Smith, J.L., 2010. Breeding for breech strike resistance. Final Report to AWI, EC940, November 2010.

Smith, J.L., 2016. Breeding for breech flystrike resistance, Phase3 CSIRO. Final Report to AWI, WP468, August 2016.

Smith, W.J., Li, Y., Ingham, A., Collis, E., McWilliam, S.M., Dixon, T.J., Norris, B.J., Mortimer, S.I., Moore, R.J., Reverter, A., 2010. A genomics-informed, SNP association study reveals FBLN1 and FABP4 as contributing to resistance to fleece rot in Australian Merino sheep. BMC Veterinary Research 6, 27.

Stuart, T.P.A., 1894. On green-producing chromogenic micro-organisms in wool. Journal and Proceedings of the Royal Society of New South Wales 28, 320-322.

Sutherland, S.S., Ellis, T.M., Edwards, J.R., 1991. Evaluation of vaccines against ovine dermatophilosis. Vet. Microbiol. 27, 91–99.

Sutherland, S.S., Ellis, T.M., Robertson, G.M., Gregory, A.R., 1987. Serum and skin surface antibody responses in merino sheep given three successive inoculations with *Dermatophilus congolensis*. Vet. Microbiol. 15, 209–218.

Sutherland, S.S., Robertson, G.M., 1988. Vaccination against ovine dermatophilosis. Vet. Microbiol. 18, 285–295.

Tabar, G.R.H., 1998. Selection of peptides from random peptide libraries for a recombinant vaccine against dermatophilosis, PhD thesis, Murdoch University.

Tabar, G.R.H., Carnegie, P., 2002. Immunization of sheep with phage mimotopes against dermatophilosis. Iranian Biomedical Journal 6, 129–134.

Talbot, G.H., Bradley, J., Edwards, J.E., Gilbert, D., Scheld, M., Bartlett, J.G., Antimicrobial Availability Task Force of the Infectious Diseases Society of America, 2006. Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin. Infect. Dis. 42, 657–668.

Tellam, R.L., Bowles, V.M., 1997. Control of blowfly strike in sheep: current strategies and future prospects. International Journal for Parasitology 27, 261–273.

Tellam, R.L., Eisemann, C.H., Vuocolo, T., Casu, R., Jarmey, J., Bowles, V., Pearson, R., 2001. Role of oligosaccharides in the immune response of sheep vaccinated with *Lucilia cuprina* larval glycoprotein, peritrophin-95. International Journal for Parasitology 31, 798–809.

Trojnar, M., Patro-Małysza, J., Kimber-Trojnar, Ż., Leszczyńska-Gorzelak, B., Mosiewicz, J., 2019. Associations between fatty acid-binding protein 4, a proinflammatory adipokine, and Insulin resistance, gestational and type 2 diabetes mellitus. Cells 8, 227.

Van Eck, W.H., 1979. Mode of action of two benzoylphenyl ureas as inhibitors of chitin synthesis in insects. Insect Biochemistry 9, 295–300.

Van Leeuwen, T., Demaeght, P., Osborne, E.J., Dermauw, W., Gohlke, S., Nauen, R., Grbic, M., Tirry, L., Merzendorfer, H., Clark, R.M., 2012. Population bulk segregant mapping uncovers resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods. Proc. Natl. Acad. Sci. U.S.A. 109, 4407–4412.

Wallis, J., Shenton, D.P., Carlisle, R.C., 2019. Novel approaches for the design, delivery and administration of vaccine technologies. Clin. Exp. Immunol. 196, 189–204.

Watson, D.L., 1992. Biological half-life of ovine antibody in neonatal lambs and adult sheep following passive immunization. Vet Immunol. Immunopathol. 30, 221-232.

Watson, D.L., Colditz, I.G., Andrew, M., Gill, H.S., Altmann, K.G., 1994. Age-dependent immune response in Merino sheep. Res. Vet. Sci. 57, 152-158.

Watson, D.L., Lea, J., Burke, G.L., 1992. A method for collecting interstitial fluid from skin of sheep. Aust. Vet. J. 69, 14-15.

Watts, J.E., Merritt, G.C., 1981a. Body strike in sheep. Veterinary Science Proceedings 58, 177–191.

Watts, J.E., Merritt, G.C., 1981b. Leakage of plasma proteins onto the skin surface of sheep during the development of fleece-rot and body strike. Australian Veterinary Journal 57, 98-99.

Wilkie, B., Mallard, B., 1999. Selection for high immune response: an alternative approach to animal health maintenance? Veterinary Immunology and Immunopathology 72, 231-235.

Wilkinson, F.C., 1979. Dermatophilosis of sheep association with dipping and effects on production. Australian Veterinary Journal 55, 74–76.

Willadsen, P., Eisemann, C.H., Tellam, R.L., 1993. "Concealed" antigens: expanding the range of immunological targets. Parasitology Today 9, 132-135.

Winsor, G.L., Griffiths, E.J., Lo, R., Dhillon, B.K., Shay, J.A., Brinkman, F.S.L., 2016. Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. Nucleic Acids Res. 44, D646-653.

Winstanley, C., Rumbaugh, K.P., 2018. Editorial: complexity and adaptability: an introduction to the special thematic issue on the genus *Pseudomonas*. FEMS Microbiol. Lett. 365, 18.

Yen, Y.L., Batterham, P., Gelder, B., McKenzie, J.A., 1996. Predicting resistance and managing susceptibility to cyromazine in the Australian sheep blowfly *Lucilia cuprina*. Aust. J. Exp. Agric. 36, 413–420.

Young, Alan J., 2019. Adjuvants: what a difference 15 years makes! The Veterinary Clinics of North America. Food Animal Practice 35, no. 3 (November 2019): 391–403.

Zaria, L.T., 1993. *Dermatophilus congolensis* infection (Dermatophilosis) in animals and man! An update. Comp. Immunol. Microbiol. Infect. Dis. 16, 179–222.

14. Appendices

Appendix 1. People contributing to the report

CSIRO

Dr Stuart Denman: Stuart is a microbiologist and molecular biologist with over 25 years experience in conducting livestock research with emphasis in developing a much deeper understanding of the complex rumen of cattle. Stuart has expertise in microbial metagenome analyses and has been responsible for the introduction and implementation of innovative molecular biology and "omics' driven technologies to deliver a greater understanding of complex microbial gut systems pertaining to livestock production and human health.

Dr Aaron Ingham: Aaron has a longstanding commitment to research projects that enhance our understanding of disease resistance and feed efficiency in livestock. He has been involved in and led immunological research projects in a range of livestock species and was a key member of a team that developed a porcine pleuropneumonia vaccine, identified factors that regulate fleece development in sheep and identified antimicrobial peptides for controlling bacterial infections.

Tony Vuocolo: Tony is a molecular biologist with extensive technical expertise in research and development of vaccines against a variety of ectoparasites. Tony currently leads an AWI and CSIRO funded research project developing a vaccine to protect sheep from flystrike. He has a 30 year history in livestock production and health research with many publications in these areas. Tony has developed projects with VetPharma companies and has led science impact training and reporting in the CSIRO livestock program for the past several years.

Dr Gene Wijffels: Gene is a clinical immunologist and biochemist with a research career in the area of protein chemistry and molecular immunology associated with livestock parasites and livestock production systems. Gene has led research projects in vaccine development against endo and ectoparasites and runs a research project developing a deeper understanding of the biological parameters associated with heat stress in cattle.

QAAFI

Dr Peter James: Peter has a long and distinguished career in the biology and control of parasites, disease vectors and nuisance flies associated with livestock. Peter has published extensively in highly relevant areas of research and his expertise in Australian livestock production systems is regularly called upon to provide consultancy services to the animal industries and veterinary pharmaceutical companies.

External Consultants

Dr Ian Colditz: Ian has experience in innate and adaptive immune defence mechanisms in skin against fleece rot and fly strike, vaccine development, immune competence, animal welfare, analgesia for painful husbandry procedures, and resilience in farm animals to environmental and husbandry-induced stressors. Ian has published extensively in these research areas and in his recent retirement maintains a strong link to research groups he has worked with at CSIRO and University of New England.

Dr Ross Tellam AM: Ross is a recently retired scientist with 29 years of broad experience in several CSIRO livestock research areas, and previous adjunct appointments at Griffith University and the University of South Australia. He was a lead scientist in the development of a commercial vaccine against ticks for use in the cattle industry (1987-1993), a CSIRO Sub-Programme Leader for Insect Vaccines (1989-1997) and former leader of Blowfly Vaccine group (1989- 1997). Ross has extensive experience in insect and tick biology, parasitology,

immunology, vaccinology, genetics, genomics, molecular biology and protein chemistry. Ross has published extensively in peer-reviewed international journals and has five patents; many publications relate to early efforts to develop vaccines that protect livestock from ticks and insects that feed on livestock.

Appendix 2. Experimental protocol for sheep wetting trials previously approved by animal ethics committees.

The following protocol has been used by NSW DPI for artificially wetting sheep to study their susceptibility to fleece rot at Trangie. A version of this protocol was used by CSIRO to assess fleece rot susceptibility of sheep in Armidale.

Operation of Wetting Shed and fleece rot induction

DESCRIPTION OF SHED AND EQUIPMENT

The Wetting Shed consists of:
i)   The Wetting Shed proper for fleece rot induction in sheep by wetting.
ii)   An annex that contains the power switches and automatic timer unit that control wetting in the shed. The annex also provides an area for preparation of materials and equipment to be used in the shed e.g. fly raising and handling.

The layout of the shed, with location of equipment, is shown in the Appendix (insert to document).

WETTING SHED

The wetting shed contains six pens to house animals during the wetting period. Pens can be pulled apart and reassembled if necessary, e.g. during repairs.

Each pen contains:
i)   One water trough with floater system. The trough is automatically filled when water levels are below a certain point.
ii)   One large feeder trough which is filled manually.
iii)   Two rain gauges mounted around the pen to measure daily rainfall.
iv)   An individual shower head sprinkler connected to its own tap. The volume of water distributed by the sprinkler can be controlled.
v)   If required, the boundaries of each pen can be separated by suspending canvas or other waterproof material to prevent water from splashing between pens.

The Wetting Shed is fitted with an evaporative cooler ("Braemar"), with a temperature control system ($10^{o}C$ to $30^{o}C$) operated by flicking the appropriate switches. A gas heating unit also is fitted which can be used if flies are released in the shed during the cooler months of the year. A "Simon Insecto-Cutor" is fitted to allow the control of flies and other insects in the shed during wetting.

Annex

The annex contains some equipment useful for the raising and handling of flies and fly larvae. The equipment includes:
i)   Two heat fans – "Xpelair".
ii)   Thermostat – control from –20° C to 70° C.
iii)   Relay – optional automatic or manual control of the thermostat, which controls the heat fans. The thermostat also controls the relay. The relay switch will bypass the thermostat if it is on "manual".
iv)   Two ceiling fans which maintain air flow around the annex.
v)   Two fluorescent lights.

vi)   Automatic timer unit.

The automatic timer unit is used to control the amount and frequency of rainfall in the wetting pens. The timer system is an "ELECTROMATIC S-SYSTEM" (model SB 105 220) which has the capacity to maintain rainfall in the pens anywhere from 8 to 180 seconds at intervals of every 0 – 120 minutes. The timer can be set up to seven days in advance. Each of the above pieces of equipment can be activated by flicking the power switches clearly marked on the control panel (see diagram). The automatic timer system is also activated by two power switches near the timer itself. The intervals between sprays are set by depressing the appropriate times required on the timer dial. Also, the length of time the sprinklers are operating is set by adjusting the appropriate dial on the automatic timer system (see diagram).

METHOD OF WETTING

i)   The day before wetting commences:
- Check that feed troughs and water troughs are clean;
- Set the timer system to the appropriate requirements and check that each sprinkler is delivering the same amount of water. This can be done by running the timer manually about six times then reading the rain gauges in each pen. If water delivery is not even, adjust the taps to the pens and the timer accordingly.
- Ensure that the rain gauges are empty
- Turn on the INSECTOR-CUTOR.

ii)   Fill troughs with pelleted ration (see Animal Health section).

iii)   Allocate animals to pens. The numbers per pens depends on the age and size of the animals, e.g. with hoggets, allocate 18-20 animals per pen.

iv)   "Prime" animals ie. Run the sprinklers two to three times consecutively to wet the animals to skin level.

v)   Switch on the automatic sprinklers.

vi)   Measure rain gauges at the same time each day and empty them after measurement.

vii)   Clean water troughs each day.

viii)   Push down feed each day and remove any wet feed from troughs.

ix)   Turn off sprinklers at power points at the end of the required wetting period.

x)   Leave animals in the shed for 24 hours after turning off the sprinklers to allow animals to 'dry out' before releasing them.

xi)   Shut down all equipment and clean out the shed (see Cleaning of the Shed section).

ANIMAL HEALTH

i)   Animal Ethics Committee now requires that animals are pre-fed for a period of at least seven days before entering the shed for wetting.

ii)   A pelleted ration of 80% lucerne 20% oats is fed *ad lib*.

iii)   Watering troughs are filled automatically and need to be cleaned every day.

iv)   A Wetting Shed operation sheet is to be completed during the operation of the Wetting Shed (see Appendix 2). As well as recording of 'rainfall' out put during wetting of the animals, observations on the state and welfare of the animals is to be recorded. A tag list of all animals in the shed will also be located with this sheet.

v)   Remove any sick animals immediately for appropriate treatment.

vi)   It is essential to keep the animals dry once they are out of the shed for a period of ten days until the post-wetting scores are recorded. It is necessary to monitor the animals closely during this period as they are more susceptible to flystrike.

CLEANING OF THE SHED

i)   Empty and clean the feeders

ii)   A high-pressure hose is used to clean out the shed. The floor is designed so that water flows towards the drain in the middle of the shed. Always keep the drain filled with water to ensure a constant flow of excreta out of the shed. Excreta then flow into a pit, located outside the wetting shed. When cleaning out the shed keep the pit filling with clean water from the outside hose. Ensure the pump is turned on before cleaning begins.

iii)   Hose down the gratings in the floors of the pens thoroughly and carefully, ensuring that no excreta are left behind.

iv)   Scrub out watering troughs.

v)   Empty rain gauges.

vi)   Once the shed is hosed out, run water down the drain for about 30 minutes to flush out the pit. It may be necessary to shovel out the majority of the solid matter from the pit to allow the pit to empty properly.

MAINTENANCE

i)   Distribute "Ratsac" abundantly around the shed when not in use.

ii)   Pump should be checked annually to ensure that it is maintaining a constant pressure.

iii)   Shed should be checked annually to assess maintenance needs, with a report provided to the relevant researcher.

HANDY HINTS

i)   In hot or humid weather open the windows into the shed to allow airflow but in windy weather make sure that they are closed.

ii)   When the shed is being operated, always keep the door between the annex and the wetting area closed.

iii)   Do not allow any metal or hard objects into the drain

iv)   Do not feed out hay or straw in the shed

v)   Always clean out the shed as soon as possible after the animals are let out of the shed (ensures easier cleaning).

vi)   If it is not possible to clean out the shed soon after use, on the day before cleaning turn on the sprinklers to soften the excrement.

This document was prepared initially by M. Nesa. This edition was revised by S. Mortimer.

In-principle approval of a wetting-shed protocol for inducing fleece rot, prior to commencing a new project, would be valuable. Studies employing natural field challenge would be feasible but would provide a much slower means for assessing vaccine efficacy.

Appendix 3. Abbreviations

16S rRNA   a specific size of ribosomal RNA used to monitor bacterial populations

Ab   antibody(ies)

ADP   adenosine diphosphate

Ag   antigen(s)

ANI   average nucleotide identity

AroA   gene encoding 3-phosphoshikimate 1-carboxyvinyltransferase; the enzyme is responsible for aromatic amino acid synthesis; knockout of *AroA* is used to produce live attenuated *P. aeruginosa*

BARE   bacterial ADP-riboslyating exotoxins

boLA-DR/DQ   bovine genetic marker in the MHC region of the genome

C3b   major protein fragment of complement C3; potent opsonin targeting microbes bound with antibody for phagocytosis and death

CD5+   lymphocytes expressing the cell surface marker Cellular Differentiation Antigen 5

DNA   deoxyribonucleic acid, the genetic code of bacteria, animals and plants

FcRn   neonatal Fc receptor

FABP4   gene encoding the protein fatty acid binding protein 4

Ig   immunoglobulin (antibody of isotypes IgG1, IgG2, IgA, IgM, IgE)

IGF-gamma   Insulin like growth factor gamma

IFN   interferon

ISCOMs   immunostimulating complexes

MHC   major histocompatibility complex

MHC-DQA2   major histocompatibility locus in the sheep genome

Montinide   types ISA61VG and ISA50V2 are water-in-oil vaccine adjuvants

mRNA   messenger RNA produced from a gene

PCR   polymerase chain reaction (technique for amplifying specific segments of DNA)

Quil A   saponin-based vaccine adjuvant

RNA   ribonucleic acid

rRNA   ribosomal RNA

SDS-PAGE   sodium dodecyl sulphate polyacrylamide gel electrophoresis (technique to separate proteins based on their size)

TCI   transcutaneous immunization