final report

Project code: B.AHE.0013

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Date published: 20 February 2017

PUBLISHED BY
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NORTH SYDNEY NSW 2059

Importance and epidemiology of mastitis in the Australian sheep flock

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

This project investigated the impact of mastitis on eight meat and wool sheep properties in Australia as there is little published on this disease in Australian sheep. Over 5,200 Merino, Poll Dorset and maternal ewes had milk collected at weaning, along with clinical mastitis samples collected from other flocks. The presence of mastitis at weaning reduced lamb growth and impacted ewe production and welfare. The most common causes of clinical mastitis were *Mannheimia* species with coagulase negative Staphylococci the predominant cause of sub-clinical mastitis. Some antibiotic resistance was found in bacteria causing clinical mastitis, in particular to penicillin, but little resistance found in bacteria causing sub-clinical mastitis. A combination of physical selection, milk testing and recording, vaccination and genetics/genomics may improve the welfare and production of meat and wool sheep by reducing the impact of mastitis.

Executive summary

The objectives of this study were to:

- Evaluate the number of ewes with sub-clinical mastitis at weaning in representative flocks
- Investigate bacterial causes of mastitis (clinical and sub-clinical)
- Review the sensitivity of bacteria causing mastitis to commonly used antibiotics
- Evaluate available “on-farm” diagnostics to aid in treatment decision making
- Record udder and teat phenotype scores and assess association with milk cell count
- Investigate the impact of sub-clinical mastitis on lamb production
- Develop a mammary lymphatic cannulation model for sheep
- Provide guidelines on treatment of ewes with clinical mastitis

This work was needed due to a lack of information on mastitis in the Australian sheep flock with very few published papers in the previous 25 years, combined with data demonstrating that mastitis was a significant problem in some flocks.

This study collected data from five information nucleus flocks (INF) located at Turretfield, Struan, Hamilton, Rutherglen and Armidale. Ewes on INF properties were a mix of Merino and Maternal breeds with all ewes in Victoria and NSW on their first or second lactations. Further data were collected from three Poll Dorset properties located in Central, North East and North West Victoria. In addition to these samples collected at weaning, sample containers were sent to interested producers who collected milk from ewes that had clinical mastitis throughout lactation and this was assessed for the species of bacteria causing clinical mastitis.

Evaluation of the number of ewes with sub-clinical mastitis at weaning in representative flocks
Milk was collected from 1746 ewes on INF properties over two breeding seasons at the time of weaning. Milk was collected over three breeding seasons from Poll Dorset properties with 3,529 ewes sampled. All ewes were milked in the same purpose built sheep milking handler with teats cleaned using 80% ethanol to allow milk collection for both milk cell count and bacterial culture. Milk collections were performed using gloves to further reduce the risk of contaminating samples or potential zoonoses.

There are a number of possible methods for classifying ewes as having mastitis at the time of weaning. The most obvious of these is those ewes that have clinical mastitis as evidenced by physical changes in the udder, teats, lymph nodes and/or milk. Measuring sub-clinical mastitis in this study was done by counting the numbers of cells per millilitre of milk using Fossomatic cell counting and also via culture of milk on agar plates. These two tests of subclinical mastitis give different results based on sensitivity and specificity of the tests. The method of bacterial culture chosen for this study was standard aerobic bacterial culture meaning that some organisms would not grow; suggesting the result from this is less than the true infection rate. Some bacteria are also shed in an intermittent fashion meaning a ewe with a negative bacterial culture may still have sub-clinical mastitis. Using cell count of greater than 500,000 as indicative of mastitis, 15% of Merino and maternal ewes on INF properties had mastitis and the comparable number on Poll Dorset properties was 31%. The equivalent figures using bacterial growth to assess mastitis were 16% on INF properties and 21% on Poll Dorset properties.

Investigation of the bacterial causes of mastitis at weaning
Mastitis at weaning in 8 different flocks including Merino, Maternal and Poll Dorset breeds was assessed. A number of ewes had clinical mastitis at weaning that was not noted prior to assessment of the ewe for the study. In 2012 this figure was 9% of ewes on PD properties

and 4% on INF properties. In 2013 it was 7% of PD ewes and 4% of INF ewes with the figure in 2014 for PD properties 9%. This demonstrates that it is difficult for producers to assess mastitis without a full physical examination of the udder. This is particularly useful at weaning time or "wet and drying". Bacterial causes of mastitis at weaning were similar to previous international sheep mastitis investigations with coagulase negative Staphylococci the most common cause of subclinical mastitis followed by Streptococci and *Mannheimia* species and *Staphylococcus aureus* the more common cause of clinical mastitis at weaning. A range of other less common bacterial species were also found across different properties.

Investigation of bacterial causes of clinical mastitis from different flocks
The majority of research on clinical mastitis of sheep has been conducted in the Mediterranean on dairy breeds of sheep. Previous work in our laboratory demonstrated the majority of clinical mastitis in south east Australia was due to *Mannheimia* species and *Staphylococcus aureus*. Over 1500 sample bottles were sent to producers during this study to collect milk from ewes with clinical mastitis. Milk samples from 49 ewes were returned from 6 properties with 27 of these positive for bacterial growth. The most commonly diagnosed bacteria were *Mannheimia* species, although other reports in Australia and overseas have suggested *Staphylococcus aureus* is more common. Both of these bacteria cause severe mastitis in less than 24 hours and require urgent treatment to save the ewe.

Sensitivity of bacteria to commonly used antibiotics
The bacteria resistance or susceptibility status of 242 bacterial samples was assessed across INF and PD properties in 2012 and showed that less than 10% of bacteria were resistant to any of penicillin, trimethoprim/sulphonamide, cephalosporin, erythromycin, tetracycline or amoxicillin. Penicillin had the most *in vitro* resistance at 10% of bacteria resistant. It was more likely for clinical mastitis isolates to be resistant to commonly used antibiotics such as penicillin so collecting milk from ewes with clinical mastitis and identifying the bacteria and its sensitivity to antibiotics is useful in formulating an optimal flock treatment plan.

Evaluation of "on-farm" diagnostics to aid decision making
A number of "on-farm" diagnostic tests are available in the cattle dairy industry to test for subclinical mastitis and confirm clinical mastitis. These include the California mastitis test and a range of tests of electrical conductivity or pH based tests. We assessed these tests as well as using infrared thermography and the De Laval cell counter for sheep's milk. All of the on-farm diagnostic tests were useful for confirming clinical mastitis although lacked the discriminating power of cell count for the purposes of selection. The California mastitis test is the cheapest of the "on-farm" tests and diagnosed a greater percentage of subclinical mastitis cases than conductivity or pH based tests. The infrared thermography may be useful in situations where stock are regularly monitored or if stock need to walk through a race regularly, however is unlikely to be as useful in lamb and wool production systems. The De Laval cell counter demonstrated good correlation with Fossomatic cell counting at low to moderate cell counts and offers the convenience of on-farm testing of milk and was simple to use.

Impact of mastitis on lamb production
This study has shown for the first time in Australia that the impact of subclinical mastitis on lamb growth was small but significant with a loss of lamb growth rate of 1 gram per day for every increase in cell count of 1,000,000 cells per millilitre. This difference can be difficult to demonstrate as lambs can steal milk from other ewes and also have access to grass in extensive Australian lamb production systems making it more difficult to show lamb growth rate differences compared to sheep in shedded environments.

Develop a mammary lymphatic cannulation model for sheep
There has been little investigation of lymph cells involved in sheep mammary defence in the past thirty years. This project developed a new surgical approach to mammary lymphatic cannulation in the sheep and demonstrated its use in a mastitis infection model. This allowed collection of both milk and lymph over a number of weeks. This technique has the potential to further evaluate immunological parameters occurring in the udder following infection or vaccination as well as investigating antibiotic and anti-inflammatory therapy use.

Guidelines for treatment of ewes with clinical mastitis
On properties managing sheep for wool and/or meat production ewes that develop clinical mastitis are most commonly diagnosed through lameness, anorexia or general lethargy in the paddock. This is usually followed by assessment of the udder and associated lymph nodes, teats and/or milk to confirm mastitis is the cause of the ewe's behaviour. Once bacteria enter the mammary gland a ewe can become very sick in less than 12 hours. Guidelines for treatment therefore are as follows:

1. Collect a sample of milk from the infected gland after first cleaning the teat end with 80% ethanol solution into a sterile container. Place this milk sample in a ziplock bag in a freezer (note that some bacteria are potential zoonoses so are transmissible to people) – if you then get more ewes with mastitis you have a greater number of samples to send for diagnosis. Depending on the cause of mastitis only every second of third sample will yield a positive culture result so more samples results in more accurate diagnosis.
2. Treat the ewe with an effective antibiotic as soon as possible following veterinary advice.
3. Remove the ewe and lamb/s from the flock into a "hospital" paddock when possible as they may act as sources of infection for other ewes/lambs either via direct transfer or transfer via feed or water sources.
4. In most cases ewes will need at least a three day course of antibiotics to cure mastitis so discuss duration of treatment with your veterinarian.

Table of contents

List of figures

List of tables

1 Background

1.1 Definition of mastitis

The technical definition of mastitis is any disease leading to inflammation of the mammary gland. The most common reason for this inflammation is bacterial infection entering via the teat canal, although other infectious and traumatic agents may also lead to mastitis. There are a number of potential methods of further classifying mastitis into different categories. For this report we use the initial categorisation of clinical and sub-clinical mastitis.

1.2 Clinical mastitis

Clinical mastitis is defined as any form of mastitis that can be diagnosed on property without the use of further diagnostic testing. A number of changes can be observed in clinical mastitis.

1.2.1 Changes in udder shape, size, consistency, temperature and colour

One of the easiest ways to note clinical mastitis in a ewe is that in the vast majority of cases it is only one side of the udder that is affected, hence there is a difference in appearance between the two sides (note that if a lamb has a preference for drinking from one side of the udder this may result in a size differential without mastitis). The firmness of the udder may be affected – most commonly this presents as the infected udder being harder than the other side. In certain infections however it may become more flaccid. Similarly the infected udder will generally appear larger than the uninfected side, in part due to lamb/s continuing to suckle the non-infected side. The udder may also have uneven consistency with lumps throughout the mammary tissue or swollen lymph nodes to either side or towards the back of the udder. In severe, peracute mastitis the udder may turn a blue or black colour as blood supply to tissue is lost. The udder temperature may also be hotter or colder than normal, depending on the time post infection and degree of damage to the udder. A scoring system for clinical mastitis in the ewe has been described in New Zealand (Quinlivan 1968a).

1.2.2 Changes in milk

Along with changes in the udder there are frequently changes in milk. A range of changes can be seen including changes in colour and viscosity. Colour may vary from the standard white colour to milk with apparent blood in it, green or blue tinges to milk or milk that is virtually clear along with other possibilities. The thickness of the milk sample may also vary from normal milk viscosity through to milk with viscosity similar to cream or of varying thickness with clots in the milk.

1.2.3 Changes in locomotion and behaviour

One of the more common presentations of peracute mastitis is a ewe with significant lameness that is also anorectic. This is often reported by producers during periods of feed shortage as the ewe is reluctant to move to a feed trail and even when offered grain may not eat. This lameness is presumably due to pain from inflammation of the mammary gland and associated rubbing while walking. In severe cases without early, effective antibiotic administration ewes become recumbent and prognosis for recovery is poor.

1.3 Sub-clinical mastitis

Sub-clinical mastitis requires further diagnostic testing following clinical examination of the animal. A range of tests are available to determine sub-clinical mastitis, some of these can be performed on-farm and some are done in the laboratory as they require significant investment in infrastructure.

1.3.1 Direct microscopic cell count (DMCC)

Direct microscopic counting of cells in milk is the historical gold standard for diagnosis of subclinical mastitis; however automated methods such as the Fossomatic cell counter are routinely used for commercial sample quantities. The DMCC is still used for reference purposes in smaller scale research studies.

1.3.2 Individual sheep cell count (ISCC)

Sheep milk can be assessed by an automated cell count performed through a Fossomatic cell counter in much the same way as a cow's milk test. This test produces a very similar result to that achieved via DMCC (Gonzalo *et al.* 2003). Milk chemistry can be performed at the same time as cell count using equipment such as a CombiFoss to give results for percentage of fat, protein, lactose and solids non-fat in the milk. This allows further analysis of changes in milk quality along with changes in cell count. A number of smaller devices have been developed in the past ten years to allow milk cell counting "on-farm" for sheep and cattle. These have been shown to have potential use for on-farm use in the sheep industry (Gonzalo *et al.* 2006; Gonzalo *et al.* 2008), although are not routinely used on lamb and wool producing properties.

1.3.1 California mastitis test (CMT)

The California mastitis test continues to be used routinely within the cattle and sheep dairy industries as a rapid and cheap assessment of mastitis of animals in dairies. In most studies, results from a CMT as not as accurate at identifying bacterial infection as individual sheep cell count, however this depends on the bacterial isolate (Hueston *et al.* 1986; McDougall *et al.* 2001a). The test also requires some degree of training and relies on accurate visual assessment of results.

1.3.2 Electrical conductivity of milk testing

A range of commercial devices are available for measuring electrical conductivity in milk. These are useful for producing objective measurement of milk samples to improve subjective decision making, however do not have the same accuracy as cell count for investigation of mastitis (Peris *et al.* 1991).

1.4 Previous studies of mastitis in the Australasian sheep population

There has been relatively little published research on sheep mastitis in Australian sheep flocks, given the anecdotal evidence that it causes significant clinical losses and unknown subclinical losses. A number of unpublished studies have investigated sheep mastitis on a limited scale with the only previously peer reviewed published Australian study in the past 40 years from four properties in the Armidale region of NSW (Watson *et al.* 1990a). This study suggested the average subclinical infection rate in ewes was 14%, using infection characterised by significant bacterial growth from milk collection. The only other large scale study in the southern hemisphere was performed in New Zealand, primarily in Romney ewes. This study suggested an overall infection rate of between two to three percent (Quinlivan 1968a), however this was based solely on ability to detect clinical mastitis via palpation and visual observation in the ewe, which is likely to significantly under-report mastitis compared to bacteriological or cytological measures. Both of these methods are likely to under-report total subclinical mastitis in a flock, due to some bacteria being difficult to culture under standard conditions or having low levels within milk in the mammary gland (Winter *et al.* 2003; Hariharan *et al.* 2004). Our laboratory commenced studying sheep mastitis in 2005 and in 2006 was funded via PIRD V04/06 through MLA to investigate clinical mastitis in Poll Dorset flocks in Victoria and southern NSW. This project demonstrated the

importance of both *Mannheimia spp.* and *Staphylococcus aureus* in severe clinical mastitis of sheep. This is in contrast to reports on clinical mastitis of dairy sheep in the European Union where *Staphylococcus aureus* is by far the most common pathogen (Bergonier *et al.* 2003). There have not been any reported studies of milk cell count in the Australian sheep population. The incidence of clinical mastitis varies significantly throughout the Australian sheep flock but it is not uncommon for outbreaks to impact on more than 10% of a flock (Omaleki *et al.* 2016).

1.5 Previous international studies of mastitis in the sheep

The majority of research available on mastitis in the sheep comes from research in the dairy sheep industry or via extrapolation from cattle data. The removal of lambs from sheep in dairy management systems has the potential to significantly change the bacterial dynamics within the flock and the potential damage to the end of the ewe's teat; hence findings from dairy production systems may not apply equally to lamb/wool production systems. Both lentivirus and virulent *Mycoplasma agalactiae* are exotic to Australia so these diseases are not considered within this report.

The major cause of clinical mastitis within dairy sheep production systems is *Staphylococcus aureus*, with relatively few cases caused by *Mannheimia haemolytica* or *M. glucosida (Bergonier et al. 2003)*. The most common cause of subclinical mastitis are coagulase negative staphylococci (CNS). A number of species have been described *including S. epidermidis, S. xylosus, S. chromogenes, S. simulans* and *S. caprae (Bergonier et al. 2003).* Streptococci may also cause subclinical mastitis along with a range of other less common bacterial pathogens (Bergonier *et al.* 2003).

In sheep meat and wool systems in the northern hemisphere a significant breed affect has been shown as a risk factor of clinical mastitis as well as previous mastitis increasing the risk of mastitis in subsequent lactations (Waage and Vatn 2008). A significant variation between British Breed flocks in clinical mastitis has been noted with up to 11% of ewes clinically infected in late lactation (Grant *et al.* 2016). Nutrition may be a significant component of this variation as underfeeding in late pregnancy and lactation increases the odds of developing clinical mastitis (Grant *et al.* 2016). In the UK, mastitis of ewes appears to be a major reason for culling British Breed ewes with a large number of ewes at slaughter showing signs of mastitis (Conington *et al.* 2008).

International work in dairy sheep breeds (Rupp *et al.* 2009; Genini *et al.* 2011) and meat sheep breeds (Conington *et al.* 2008) demonstrates significant potential in selecting against mastitis in sheep populations while maintaining production. This has not been assessed in multiple sheep flocks within Australia.

1.6 Treatment and prevention options for mastitis

There are very few registered options for treating sheep mastitis – either for clinical or sub-clinical infection. All intramammary products are only registered for use in cattle within Australia, although these products are extensively used within sheep dairies in Mediterranean countries (Bergonier *et al.* 2003). Use of these products is "off-label" in Australia at the discretion of a consulting veterinarian. A review by (Mavrogianni *et al.* 2011) covers various options for treatment. These are limited to treating once the disease is noted, which can be difficult in extensive production systems given the speed of disease onset with cases of peracute clinical mastitis. Ewes are also likely to be treated by whatever antibiotic the producer has available as development of mastitis is rapid and may not allow several hours to lapse before a particular new antibiotic may be obtained. There are also some pathogens with resistance to antibiotics and susceptibility testing of isolates on a property is needed to maximise chances of therapeutic success.

It is possible to use antibiotics at dry-off however if producers are inadequately trained or do not clean teats enough this can result in significant infection. Even without dry-off therapy a significant percentage of ewes are likely to eliminate infection over the dry period (Bergonier *et al.* 2003), so much of this antibiotic therapy is wasted and may significantly increase selective pressure on antibiotic resistance in bacteria.

A preferable option may be to utilise vaccination to reduce disease. There is currently only one vaccine against mastitis commercially available in sheep – Vimco (Hipra, Spain). This vaccine targets both *S. aureus* and CNS. The addition of a *Mannheimia* antigen to this vaccine could provide protection against the major clinical and subclinical pathogens present in mastitis, but this is not currently available. There are no Australian developed vaccines currently available, although there are registered *Mannheimia* vaccines for use in cattle that show some protection against *Mannheimia* pneumonia in sheep (Zheng *et al.* 2015). There has previously been a report of successful vaccination of Australian sheep using an autogenous *Mannheimia* (*Pasteurella*) vaccine (Kabay and Ellis 1989).

1.7 Information needed for the Australian sheep industry

The relative deficiency of knowledge of sub-clinical and clinical mastitis in the Australian sheep flock makes it difficult to accurately predict the total cost of disease. A recent evaluation of the likely cost of mastitis to the Australian sheep industry using assumptions of clinical mastitis rates varying from 1-5% across different breeds and sub-clinical mastitis rates of 7-30% suggested a likely industry cost of over $52 million AUD per annum (Lane *et al.* 2015). This figure however uses assumptions as to the likely growth rate reduction in progeny from dams with subclinical mastitis. This study provides the first insight into expected costs of mastitis across the predominant breed types in the country. It also investigates bacterial resistance to commonly used antibiotics from both clinical and subclinical mastitis pathogens. The utilisation of sheep recorded with Sheep Genetics Australia and also with SNP chips within the Sheep CRC will allow further investigation of potential genetic selection to reduce the impact of mastitis on sheep welfare and production in the future.

2 Project objectives

2.1 Summary of project objectives

The primary objective of this proposal was to give sheep breeders and veterinarians a better understanding of the causes and importance of mastitis in the Australian sheep industry. This information was previously unavailable and is required for breeders and also commercial companies to make decisions on what treatment methodologies may be applicable to reduce the incidence of the disease where this is necessary. The project would also lay the foundation for some tools that may assist producers in reducing the incidence of mastitis using selection. The listed objectives prior to the commencement of the project included:

- Collect over 3100 milk samples from sheep from INF and terminal sire flocks and analyse using Fossomatic cell counting and standard bacterial culture.
- Collect all submitted clinical mastitis samples from across Australia provided by sheep producers and assess using standard plating techniques.
- Provide MLA with guidelines to assist producers in treating clinical mastitis in their flocks to maximise chances of therapeutic success.

- Review the impact that sub-clinical mastitis has based on weaning weight and cell count and if any sires produce progeny that are more likely to develop an increased cell count and/or clinical mastitis.
- Describe the incidence and causes of mastitis in more than 7 representative flocks across Australia to indicate the importance of mastitis to the sheep industry.

2.1.1 Summary of project design

The project design involved:

a. Collecting samples and data from five information nucleus flocks (INF) within the sheep CRC to assess sub-clinical and clinical mastitis and any correlations with udder phenotype.
b. Collecting samples and data in conjunction with three Poll Dorset flocks with a history of some mastitis in the flock, linkage to other flocks and extensive history of using Lambplan recording
c. Assessment of clinical mastitis samples provided by both stud and commercial producers from across Australia over the period of the project
d. Investigate immune responses in the mammary gland using cannulation to collect lymph from the mammary gland

3 Methodology

3.1 Animals

3.1.1 Information Nucleus flock (INF)

At the conclusion of the Sheep Genomics Program (SGP), ewes on INF properties were assigned to the Sheep CRC prior to commencement of this study. Five INF properties collaborated in data collection from ewes for this study. Under the SGP all INF ewes had full pedigree and genomic data collected prior to commencement of this study providing a greater resource for potential future investigation following this mastitis project, if required. The information nucleus flocks used in this study were pastured at Hamilton (VIC), Rutherglen (VIC), Struan (SA), Turretfield (SA) and Armidale (SA) properties. The furthest distance between two properties in this project was more than 1,500 kilometres.

3.1.2 Terminal sire stud flocks

Three Poll Dorset stud flocks were selected as cooperating properties for this study as previous reports suggested the incidence of mastitis in this breed may be higher than in Merino or First Cross ewe flocks (Watson *et al.* 1990b). Flock selection was based on a moderate sized ewe flock of greater than 250 stud ewes with full pedigree and production records available for use, a willingness to be involved in the study and provide some support during milk collection. All flocks were located within Victoria; in the north-east, central and north-west areas of the state. The longest travelling distance between two properties was more than 300 kilometres. All flocks had been in operation for more than twenty years.

3.1.3 Commercial ewes

Milk and udder phenotype measures were collected from a limited number of commercial Poll Dorset and First Cross (Border Leicester X Merino) ewes on PD2 and from Merino and maternal ewes on INF2. It was not possible to link lamb weights with milk data from these ewes as these data were not maintained for these animals.

3.1.4 Ewes for lymphatic cannulation

Four commercial East Friesian milking ewes were purchased to evaluate a novel lymphatic cannulation technique to aid understanding of mammary gland immunology.

3.2 Ethics

All animal work in this report was covered under relevant ethics coverage for each jurisdiction. Animal ethics approval numbers for each state are listed in the following section.

3.2.1 Victoria

University of Melbourne AEC:
1212599.2 – Investigation of bacterial causes of clinical and sub-clinical mastitis in sheep
1312857.2 – Development of a mammary gland lymphatic cannulation model for the study of mastitis-causing pathogens;

DEDJTR AEC:
2012-23 - Mastitis survey - milk sampling to quantify incidence of sub-clinical mastitis

3.2.2 South Australia

PIRSA AEC:
#27/12 - Investigation of clinical and sub-clinical mastitis in sheep

3.2.3 New South Wales

UNE AEC:
AEC13-165 - Investigation of subclinical mastitis in Australian sheep

All work was undertaken according to relevant guidelines for each application. While it would have been beneficial to gain data from West Australia the timeline, budget and geographic challenges didn't allow this to occur.

3.3 Infrastructure – development of a suitable milking platform

Our initial work on sheep mastitis in 2006 required sampling milk from ewes with them standing in a race, however this had associated occupational health and safety issues that made large numbers of milk sample collections difficult or impractical. We then changed to collecting samples from sheep in a conventional shearing position however this required each sheep to be dragged from a catching pen and over a shearing board. This resulted in fewer OHS issues however did not allow a view of the udder in its standard position and had the potential to increase bacterial contamination of samples. Mr Gary Nolan then developed a 360 degree, adjustable height turntable for milking ewes "the Bush-Tiger" milker that removed these issues. This milking platform allowed for good sheep handling and flow with excellent visualisation of the udder and sample collection, however this was not in a safe, transportable format for milking on multiple properties.

This project required the development of a trailer to transport the milking platform between properties to minimise variation in recording ewe udder phenotype and milk collection due to property infrastructure differences. Mr Gary Nolan built a trailer which is able to be towed by a four wheel drive utility and provides a standard, safe platform for the sheep handling unit. This trailer and unit was readily cleaned using high pressure water and disinfectant to enable biosecurity requirements to be maintained between property visits. Figure 1 shows this trailer on the road and Figure 2 shows the trailer in use with one sheep being milked and one in the race leading up to the handling unit.

Figure 1: Image of "Bush-Tiger Milker" in road mode

Sheep walk onto a short race with anti-backing panels and then into the handler. The handler can rotate through 360 degrees and be raised up to chest height to enable good visualisation of the sheep.

Figure 2: Image of "Bush-Tiger Milker" in work mode

Figure 3 demonstrates a sequence of images illustrating the change from "road mode" to when the Bush Tiger Milker is in use. The trailer is first positioned in an appropriate location close to the sheep yards where stock are yarded. The vehicle is then uncoupled from the trailer.

The front, rear and side panels of the trailer can be lifted as shown in the schematic on the left panel of Figure 3. Bolts securing the platform to the trailer base are then removed allowing Happijac motors on each corner of the trailer to lift the platform off the chassis. The trailer base is then removed from under the milking platform. Metal poles are then screwed into the uprights at each corner of the trailer to keep the roof at the same height when the motors lower the base of the unit to the ground. Once the unit has reached ground level the front, rear and side panels are further supported using tent poles. During adverse weather conditions such as wind, rain and dust any of the four sides may be partially or completely closed. This proved particularly useful during inclement weather, although it did slightly reduce numbers of sheep milked per day.

Sheep yard panels were used to connect the unit to the appropriate gate in the sheep yards to maximise ewe flow. The use of the Bush Tiger milker platform allowed for the same flooring and stock handling to be used on every property in the trial with the same staff member milking every animal in the trial. The primary requirements to use the unit on a property were an adequate space to park the trailer, relatively level ground and being in close proximity to yards. This was achieved on all properties involved in the project. The trailer was fitted with LED lights to allow work during poor light conditions. The lights and happijac motors were powered by a standard car battery with a further car battery used for powering vertical lift for the sheep handling unit when required. These batteries were recharged intermittently as required.

Bush tiger milker road mode shown on top left of page to in use on bottom right

Figure 3 Flowchart of changing "Bush-Tiger Milker" from travel mode to working mode

3.4 Milk samples

3.4.1 Samples collected at weaning

All milk samples at weaning were collected from ewes within 12 hours of lambs being removed from their dams. In general, lambs were drafted from ewes in the early morning with milk collections finished by the early evening, depending on how many ewes were in the flock. The teats of each ewe were cleaned with cotton wool swabs soaked in a solution of 80% methylated spirits and 20% sterile water. Teats were cleaned until no further dirt came off the end of the teat. The first two to three squirts of milk were discarded.

Approximately equal volumes of milk from each side of the udder were then collected into a 30mL tube. An 18mg Bronopol tablet (Broad spectrum microtabs) was added to the 30mL sample to limit any potential bacterial growth if testing was delayed. A further 5 mL sterile vial was then used to collect at least one squirt of milk from each side of the udder. Both milk samples were identified using bar code labels and refrigerated within 15 minutes of collection at 4 degrees Celsius. Milk was stored in a portable Engel refrigerator next to the collection area during the day and transferred to a mains powered refrigerator each night for multiple day collection visits.

3.4.2 Clinical sample collection

Sheep producers were contacted by a range of methods to encourage them to submit samples of milk from ewes with clinical mastitis. This included direct contact with a number of producers who had previously submitted samples to our laboratory, via Bestwool/Bestlamb conference, producer groups and also via producers contacting us direct from media articles in “Feedback”, Weekly Times and via contact with the local veterinarian. Each producer was mailed a sampling kit with instructions for how to collect, store and send samples to our laboratory (see section 10.1).

3.5 Milk cell count and analysis

Measurements of milk cell count and components (fat, protein, solids-not-fat (SNF) & lactose) for each 30 mL milk sample were performed at Dairy Technical Services (North Melbourne, VIC, Australia) using a CombiFoss 5000 with standard FOSS reagents and technique, using International Dairy Federation standards. Milk was kept refrigerated at 4 degrees Celsius following collection until delivery to Dairy Technical Services. All samples were delivered within a 7 day period with most delivered within 3-4 days and tested on the day of arrival.

3.6 Bacterial culture and identification

Within one week of milk collection and mostly within one to two days of collection, milk (10µL) from each sheep milk sample was cultured on a sheep blood agar plate (SBA) and incubated at 37°C for 48 hrs. Primary bacterial phenotypic identification was based on cellular and colony morphology and haemolysis on SBA and the results of Gram reaction.

3.6.1 Gram positive cocci (catalase positive)

3.6.1.1 Biochemical tests

All Gram positive cocci isolates testing positive for catalase and coagulase were identified using the ID 32 Staph API system (BioMerieux) at the Clinical Pathology Laboratory, The University Veterinary Hospital, Werribee as per the manufacturer’s instructions. Species identification of staphylococci was obtained using the API database

(apiweb.biomerieux.com). A limited number of coagulase negative isolates were tested using this commercial identification kit.

3.6.1.2 Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) of glyceraldehyde-3-phosphate dehydrogenase (gap) gene

The catalase positive Gram positive cocci that were found to be coagulase negative (CNS) were tested by Polymerase Chain Reaction (PCR) for the presence of the glyceraldehyde-3-phosphate dehydrogenase (*gap*) gene (Yugueros et al., 2000).

Genomic DNA was extracted from a LB broth culture (~10^9 cells) generated from a single well isolated colony and incubated for 18 hrs. The cell pellet was washed and resuspended in PBS (200 µL). Lysozyme (1 mg/mL) and lysostaphin (0.1 mg/mL) were added to the suspension and incubated at 37°C for 1 hr. Following the cell lysis step, genomic DNA was purified using the High Pure PCR template preparation kit (Roche) according to the manufacturers' instructions.

For amplification of *gap* gene, 0.2 µM GF-1 (5'-ATGGTTTTGGTAGAATTGGTCGTTTA-3') and GR-2 (5'-GACATTTCGTTATCATACCAAGCTG-3′) oligonucleotide primers were used. The PCR was carried out as described previously with minor modifications (Yugueros *et al.* 2000). The conditions of the reaction were an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 20 sec, 55°C for 30 sec and 72°C for 40 sec and final extension step at 72°C for 4 min using a T100 thermal cycler (Bio-Rad).

For the RFLP, PCR amplicons of *gap* gene (15 µL) were digested with the endonuclease restriction enzyme, 10 units of *Alu*I (New England BioLabs) at 37°C for 12 hrs. The DNA fragments were separated by electrophoresis on a 2% agarose gel and photographed using the ChemiDoc XRS+ imaging system (Bio-Rad). The restriction profiles were compared with those previously published (Yugueros *et al.* 2000).

3.6.1.3 Molecular identification of coagulase negative Staphylococci

The amplification of universal bacterial gene targets (16s rRNA and rpoB) were performed to identify CNS isolates collected in 2014. The template was prepared from a well isolated single colony, resuspended in 10 mM Tris-HCl buffer, pH 8 (100 µL) and incubated at 95°C for 5 min. The suspension (2 µL) was added to the PCR. All reactions were performed using 1.25 U GoTaq® DNA polymerase with 1x GoTaq® flexi buffer (Promega), 2 mM MgCl2, 0.2 mM dNTPs and gene specific oligonucleotide primers.

The small subunit ribosomal RNA, 16S rRNA gene, was amplified using universal oligonucleotide primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR was carried out as described previously with minor modifications (Weisburg *et al.* 1991). The reaction mix included 0.2 µM of 27F and 1492R oligonucleotide primers. The PCR cycling conditions were an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec and final extension at 72°C for 7 min.

The RNA polymerase beta-subunit encoding gene, rpoB, was amplified using the oligonucleotide primers 0.4 µM 2643F (5′-CAATTCATGGACCAAGC-3′) and 3241R (5′-GCIACITGITCCATACCTGT-3′). The PCR was carried out as described previously with minor modifications (Drancourt & Raoult, 2002; Supre et al., 2010). The PCR cycling conditions were an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 45°C for 30 sec and 72°C for 30 sec and final extension at 72°C for 5 min.

The 1.5 kb PCR products generated by the 16S rRNA reaction and the 598 bp amplicons of the rpoB PCR were purified using the Ultraclean® PCR clean-up kit (MOBIO) and sequenced using the BigDye® Terminator v3.1 sequencing system (Applied Biosystems), with oligonucleotide primers used to generate each product. Reactions were analysed using an Applied Biosystems 3100 Genetic Analyser. The sequences were compared to the available sequence using the Greengenes website for 16S rRNA (DeSantis et al., 2006) and the rpoB sequences were compared with those listed in Genbank using BLAST. Sequences with similarity of 98% or higher were matched to the species (Drancourt and Raoult 2002; Supre et al. 2010).

3.6.2 Gram positive cocci (catalase negative)

The Gram positive cocci that tested negative for the catalase enzyme were tested by a panel of biochemical tests in addition to amplification and DNA sequencing of *rpo*B and 16S rRNA genes.

3.6.2.1 Biochemical tests

The panel of biochemical tests included growth on MacConkey agar; hydrolysis of aesculin and hippurate; fermentation of the carbohydrates inulin, arabinose, mannitol, trehalose, sorbitol, ribose, raffinose and salicin.

A limited number of samples were tested using the rapid ID 32 STREP V4.0 (BioMerieux) at the Clinical Pathology Laboratory, The University Veterinary Hospital, Werribee as per the manufacturer's instructions.

3.6.2.2 Identification using molecular techniques

SplrpoAF and SplrpoAR oligonucleotide primers designed to amplify the RNA polymerase alpha subunit *rpo*A gene of *Streptococcus pluranimalium* (LMG 13592) were used. For identification of Enterococcus, oligonucleotide primers designed to the 16S rRNA, E1 and E2 were used (Deasy *et al.* 2000). The multiplex PCR OneTaq Quick-Load 2× master mix with standard buffer (New England BioLabs) was used for the amplification. Each PCR mixture contained 12.5 µL of 1X One Taq Quick-Load 2 × master mix, 0.8 µM of SplrpoAF, SplrpoAR primers and 0.4 µM of E1, E2 primers, single colony in milliQ water to a final volume of 25 µL were used. The PCR cycling conditions were an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec and final extension at 72°C for 5 min.

The DNA sequence of the PCR products was determined using the methods previously described. The isolates were identified by comparing these DNA sequences with those held within public sequence databases.

3.6.3 Gram negative bacteria

Gram negative bacteria were grown on SBA and MacConkey agar. Isolates were identified by observation of colony morphology and biochemical tests: oxidase, ornithine decarboxylase, β-glucosidase, acid from fermentation of arabinose, trehalose, maltose, rhamnose, sorbitol, salicin and xylose and hydrolysis of esculin. Analysis of DNA sequence generated from amplification of 16s rRNA and *rpo*B genes as previously described for CNS were used for identification of a limited number of isolates to confirm identification.

3.7 On farm data collection

Information that was collected from INF and PD properties included the following:

Dam ID - Management tag of each ewe

Lamb ID - Management tag of each lamb

Dam eID – Electronic ID of each ewe (where available)

Lamb eID – Electronic ID of each lamb (where available)

BWT - Birth weight

BT - Birth type (Single, Twin, Triplet, Quadruplet)

RT - Rearing type (1, 2, 3 or 4)

DOB - Date of birth

DOW - Date of weaning

WWT - Weaning weight

SireID - Sire of lamb

SoD - Sire of Dam

Birth weights were collected at least once a day on all properties and lambs identified to their dams. For PD properties this data was transferred to the Sheep Genetics Australia (SGA) database and for INF properties this went to the CRC database. Subsequently the management tag of each ewe on INF properties was related back to the sheep eID and the management tag from each Poll Dorset property related to their 16 digit SGA code.

3.8 Phenotype data

Data were collected describing the phenotype of ewes enrolled in this study. The method of (Casu *et al.* 2006a) was used to evaluate udder morphology as shown in .

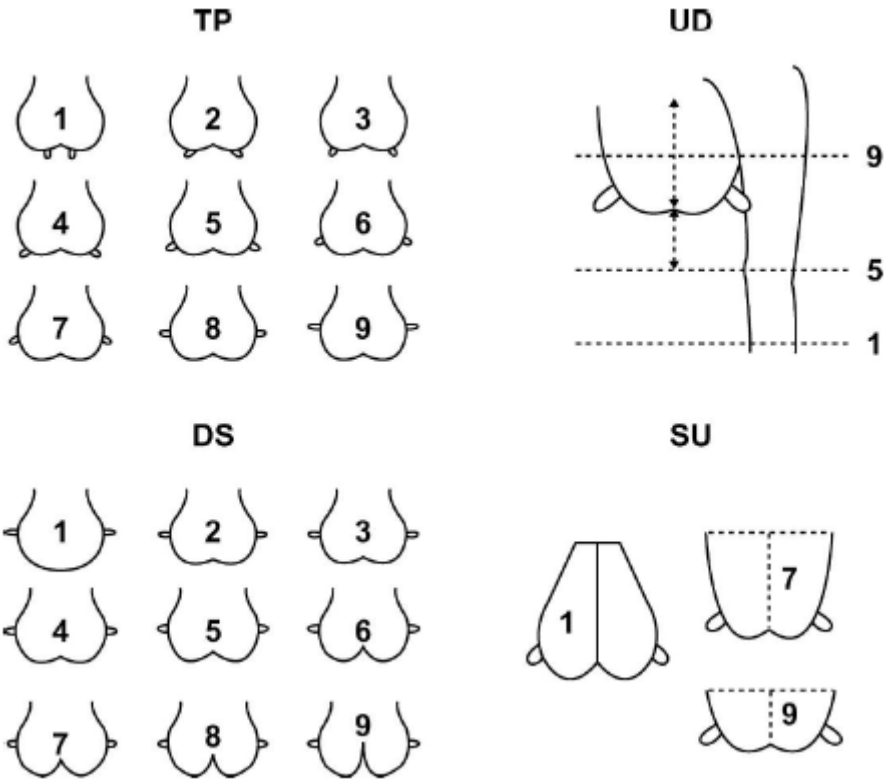


Figure 4 Udder morphology measurement from (Casu *et al.* 2006b). A nine-point linear scale was used for teat placement (TP), udder depth (UD), degree of separation (DS) and degree of suspension of the udder (SU)

In addition to these four phenotype measurements the length of the teat from its attachment to the udder to the tip of the teat was measured in millimetres using a 15 cm ruler. In 2013 and 2014 additional teat measurements of teat width at the point of attachment to the udder and the midpoint teat width were also measured. All teat measurements were taken without applying pressure to the teat to avoid increasing the measurements given the elastic nature of teat tissue. If the udder and teats were normal the left teat was measured, however if any abnormality was noted in the teat or udder on the left side then the right teat was measured.

3.9 On-farm tests for mastitis

Within the cattle dairy industry there are a number of products used for “on-farm” diagnosis of mastitis. While the use of on-farm diagnostics was not a primary research outcome from this project it was clear that the addition of this work to the program would be of potential benefit to sheep producers.

Two flocks, PD1 and PD2, were used for testing a range of “on-farm” diagnostic aids that are commercially available for producers in the dairy industry. Each ewe had milk collected from both left and right udder halves after the teats were cleaned with 80% methylated spirits (ethanol). The first squirt of milk was discarded and then 30-35mL of milk was collected for

use in a range of “on-farm” diagnostics and subsequent Fossomatic cell count. After this initial collection a further 2-5mL of milk was collected for bacteriological analysis into a sterile 5 mL tube. Milk for on-farm testing using the following devices, except the De Laval cell counter, was tested within four hours of collection from the ewe. Milk for use in the De Laval cell counter was refrigerated for 24 hours to simulate the same conditions as that used for Fossomatic cell counting.

Samples collected for bacteriology were refrigerated for up to 3 days at 4 degrees Celsius prior to plating on Sheep Blood Agar using a 10 ul loop of milk for PD1 and within 24 hours for PD2. The number of bacterial colonies were counted and Gram stain performed on all samples.

Milk was collected from a total of 976 udder halves at the time of weaning on the two properties with 338 from PD2 in 2013 (25/10/13 and 7/11/2013) and 638 from PD1 in 2013 (26-28/8/2013) and 2014. A number of on-farm diagnostic tests for sub-clinical mastitis were assessed on PD1 and PD2 as described in the following sections.

3.9.1 California mastitis test

The California mastitis test (CMT) requires use of a detergent reagent to mix with milk in equal volumes to assess the formation of precipitate or gel. In this test the CMT detergent reacts with the DNA of cell nuclei to provide a measure of somatic cells in milk (Ruegg and Reinemann 2002). A standard CMT paddle (Bovivet CMT test) with four chambers was used to assess milk sample within one hour post collection. Three millilitres of milk and the same volume of detergent were added to a paddle well and swirled. Results were reported as + (trace), 1, 2 or 3 (gel formation).

3.9.2 Tests relying on milk conductivity and pH

A number of tests relying on pH and conductivity or resistance of milk are available in either handheld devices or for use as “in-line” measurement on dairy properties. These tests rely on reductions in lactose and potassium during infection with concurrent increase in sodium and chloride due to increased blood capillary permeability and damage to ion-pumps (Kitchen *et al.* 1980). In dairy cattle mastitis these tests in general are considered less accurate than the CMT (Ruegg and Reinemann 2002). Results from milk conductivity tests had not been published from extensive Australian sheep production systems prior to this study.

3.9.2.1 pH paper

On PD1 Bovivet pH paper (Shoof International) was used to test sheep milk within one hour post collection. A few drops of milk were added to pH paper and colour changes noted according to product instructions.

3.9.2.2 Draminski electronic mastitis tester

A minimum of 15 mL of milk was added to the Draminski mastitis tester and the test switch was turned on. Once the result was available on screen the datum was recorded and the milk was returned to the original tube and the Draminski unit rinsed with water prior to addition of the next sample.

3.9.2.3 Mas-D-Tec

A few mL of milk was poured into the Mas-D-Tec collection cup. The screen reading (from 0-9) on the side of the Mas-D-Tec was recorded. Milk poured into the Mas-D-Tec was discarded.

3.9.2.4 Milkchecker

A minimum of 20mL of milk was added to the Milkchecker collecting cup. The test button was then pressed and screen result was recorded. Milk was then transferred back to its tube and the Milkchecker was rinsed in water. The reset button was pressed between each milk sample.

3.9.3 Infrared thermography

Infrared thermography relies on an infection eliciting an elevated udder temperature that is measured by increased skin surface temperature over the mammary tissue. A Flir E5 camera was used to record an infrared image of the ewe's udder from a caudal view to capture the entire view of the udder on PD1. A measure of ambient temperature in the shed was recorded at the same time as the image was collected. A corresponding visual image was collected at the same time as the infrared image using a JVC 3CCD camera mounted on a tripod behind the Bush-Tiger Milker. The maximum temperature for each half of the udder was recorded using FLIR tools software and then the largest circle that could be transcribed onto the bare surface of the udder was drawn and the software calculated the average temperature over this region. The same size circle and region was used to measure average temperature on each side of the udder.

3.9.4 On farm cell counting

A sub-group of ewes on PD1 were assessed using the De Laval Cell Counter DCC. A sample of milk that had been prepared for Fossomatic cell counting was used to measure cell numbers in the De Laval Cell Counter DCC. The cassette for insertion into the DCC was used to take in milk from the tube and was inserted into the De Laval Cell Counter DCC. A sheep protocol setting was used for cell counting and the cell count from the screen was noted for each udder half for comparison with the Fossomatic method.

3.10 Diagnostic parameters for mastitis

Most authors cite either positive bacterial culture or a threshold cell count as measures for defining sub-clinical mastitis, while clinical mastitis is defined by disease that can be physically assessed on-farm without further testing (Bergonier *et al.* 2003). For the purposes of this study we used cell count as the primary measure of sub-clinical mastitis with the cell count threshold set at 500,000 cells per mL. For this study this figure refers to the cell count of milk collected from both halves of the udder, hence implies a significantly higher figure for the infected gland as most ewes only have one half of the udder infected. We assessed cell counts in both sides of the udder in a subset of ewes in section 3.9. Bacterial culture was also performed to review causes of sub-clinical mastitis but was not used as the defining criteria in this study for subclinical mastitis as previous studies have demonstrated a significant number of ewes with subclinical mastitis are not diagnosed utilising standard aerobic bacterial culture conditions even with ideal milk collection technique and storage when using a single milk collection at the time of weaning.

3.11 Birth weight and weaning weight

All lambs were weighed within 24 hours of birth on both PD and INF properties. Lambs were then weighed at weaning when milk was collected from ewes. Lamb bodyweight was recorded to one decimal place with lambs weighed using electronic scales. Bodyweight data was collated on the Sheep Genetics Database for PD properties and in the Sheep CRC database for INF properties.

3.12 Developing a mammary lymphatic cannulation method

Material from this section (Section 3.12) has been published in BMC Veterinary Research: Development of an ovine efferent mammary lymphatic cannulation model with minimal tissue damage. Authors: Hung-Hsun Yen, Elizabeth Washington, Wayne Kimpton, Evan Hallein, Joanne Allen, Silk Yu Lin, Stuart Barber: Yen et al. BMC Veterinary Research (2016).

An understanding of mammary immunology, both innate and acquired, is important in improving the ability to reduce mammary disease (Meeusen *et al.* 2013). There are only 4 previous reports of mammary efferent lymphatic cannulation described in peer reviewed journals since its first report in 1960 (Linzell 1960; Lascelles and Morris 1961a; Watson and Davies 1985; McKeever and Reid 1987). Part of the reason for the lack of use of mammary lymphatic cannulation is the relative difficulty of the technique.

We developed a novel surgical approach to the mammary lymph node efferent lymphatics. This approach reduced the skin and muscle damage associated with earlier methods (Lascelles and Morris 1961b), although it is still a time consuming technique with a surgical team able to cannulate both mammary lymphatics (left and right) in one day. This surgical model was developed by first reviewing the lymphatic anatomy using three cadavers from other research trials.

Subsequently, four lactating East Friesian cross ewes, aged approximately 2 years and on their first or second lactation were used for this study. The ewes had been lactating between 94 to 127 days prior to arrival at the animal house and were fed on chaff and then introduced to sheep pellets and chaff mix for the duration of the trial. They were milked by hand twice daily and acclimatised for a minimum of seven days before lymphatic cannulation surgery. Prior to each milking the teat ends were disinfected with 80% ethanol soaked swabs. Milk volume was determined and 30 mL of milk was set aside for cell count and component analysis following the first few squirts of milk. An 18 mg bronopol tablet (Broad spectrum microtabs, Advanced Instruments) was added to the 30mL tube to allow samples to be sent for weekly analysis with milk refrigerated within 1 hour of collection. Measurements of milk cell count and components (fat, protein, solids-not-fat (SNF) & lactose) for each udder half were performed at Dairy Technical Services (North Melbourne, VIC, Australia) using a CombiFoss 5000 with standard FOSS reagents and technique, using International Dairy Federation standards.

Ewes were fasted overnight and provided water *ad libitum* until the time of surgery. Anaesthesia was induced by intravenous injection of 1.0-1.5 mg thiopentone sodium (Boehringer Ingelheim, Australia) in 20 to 30 mL distilled water per sheep and then maintained with isoflurane (1.5-2.5%) and oxygen following intubation. The general surgical procedures and method for securing the bottles for lymph collection has been described in previous publications (Yen *et al.* 2006; Yen *et al.* 2009). We used clear vinyl cannulae (internal diameter 0.58 mm; external diameter 0.96 mm, Dural Plastics, Australia) coated with bioactive heparin for all lymphatic cannulation surgeries. The bottles for lymph collections were secured on an animal with two size 6, Surgifix tubular elastic net bandages.

Figure 5 Position of collection bottle in netting demonstrating lymph flow into bottle

Each ewe was administered one injection of Temgesic (2.2 mg/kg) intramuscularly per day for the first two days post-surgery for pain relief post surgery. Following cannulation, ewes were maintained on *ad libitum* feed and water in individual pens in sight of other ewes.

Lymph was collected twice daily in 100 or 250 mL sterile, polypropylene collection bottles (Plastilab, Kartell Labware, Noviglio Italy) containing 1000 or 2000 IU of heparin. The bottle was fixed to the netting surrounding the sheep's abdomen to avoid inadvertent removal of the bottle or tube. At each lymph collection the bottle on both left and right sides of the sheep were removed and replaced with clean, sterile bottles containing heparin. The fresh bottles were secured to the netting with the same strings. The free end of the cannula was disinfected with 0.5% w/v Hibitane in 70% v/v alcohol before inserting it into the bottle through the small opening of the cap and sealed with adhesive tape. The total volume of lymph collected for each duct was measured and the average rate of lymph flow following previous collection was determined.

Cells from a 50 µl sample of lymph were counted using a model Z1 Beckman Coulter Particle Counter. Cells in lymph were then washed 3 times in PBS containing 2% BSA, 0.4% EDTA and 0.1% azide (FACS wash) and stained for flow cytometric analysis of lymphocyte subsets. Monoclonal antibodies (mAb) against the T cell subsets CD4 (44-38), CD8 (38-65) and γδTCR (86D) were obtained from Dr Scheerlinck (Centre for Animal Biotechnology, The University of Melbourne) and have been described previously (Maddox *et al.* 1985, 1987; Mackay *et al.* 1989). They were used as cell culture supernatants and detected with PE-conjugated sheep anti-mouse immunoglobulin (Ig) (Chemicon, Australia).

Cells were analysed fresh on a FACSCalibur Cytometer equipped with argon and red diode lasers (BD Immunocytometry Systems, USA). The instrument was calibrated with Calibrite Beads (BD Biosciences) and samples were collected and analysed using CellQuest Pro software (BD). Forward and side scatter were used to exclude dead cells.

3.12.1 Establishment of a modified mammary lymphatic cannulation

Examination of cadaver anatomy prior to live animal surgery showed that efferent mammary lymphatics coursed with the external pudendal vessels in parallel underneath the aponeurosis of the external abdominal oblique muscle entering the abdomen through the inguinal canal. The mammary lymphatics could have multiple branches and could be either cranial or caudal to the external pudendal vessels. Our surgical approach required a ventro-

cranial to dorso-caudal orientated skin incision approximately 6 cm in length on the abdominal wall, cranio-medial to the inguinal pouch.

After skin incision, blunt dissection was performed to penetrate the subcutaneous fat and the superficial fasciae to approach the aponeuroses of the internal and external abdominal oblique muscles. The lymphatics under the aponeurosis of the external abdominal oblique muscles were then identified. After identifying all branches of the mammary lymphatics, the external pudendal vessels and the lymphatics were detached from the aponeurosis of the external abdominal muscles using blunt dissection. By cutting through the caudoventral insertion of the aponeurosis of the external abdominal muscle to the fasciae connected to the rectus abdominal muscle, more space was created to access the segments of mammary lymphatics adjacent to the mammary lymph nodes. Multiple branches of similarly sized mammary lymphatic vessels were found during some surgeries while in other surgeries one mammary lymphatic vessel was tightly attached to the external pudendal vein. This lymphatic vessel was the largest lymphatic branch in this surgery with a smaller lymphatic vessel next to the vein. It was necessary to identify all lymphatic branches and ligate them, with the largest lymphatic selected for cannula insertion.

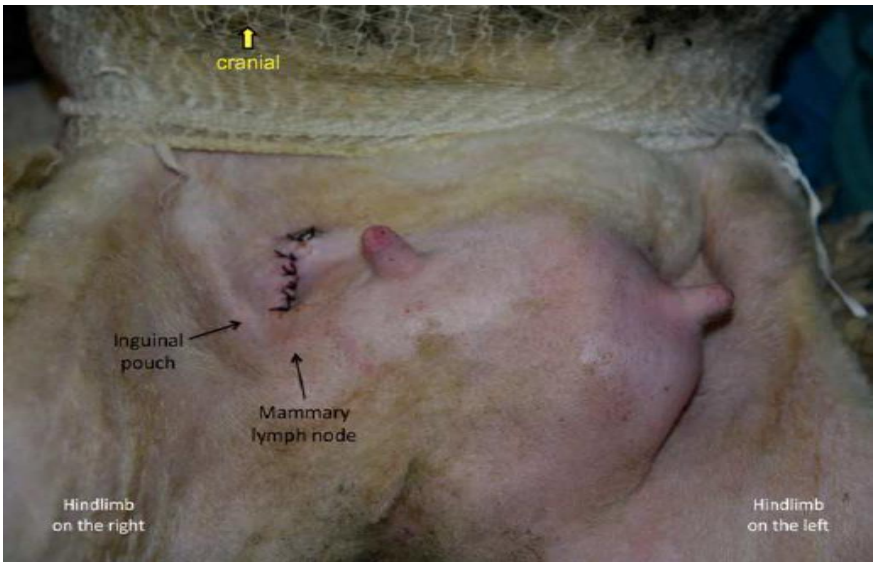


Figure 6 Position of surgical incision for lymphatic cannulation

The bevelled end of a cannula was placed beneath the aponeurosis of the external abdominal oblique muscle via a small stab incision at the dorsal part of the aponeurosis before inserting it into the lymphatic. The basic technique for inserting a cannula into a lymphatic has been described in previous publications (Schwartz-Cornil *et al.* 2006; Yen *et al.* 2006; Yen *et al.* 2009). Briefly, the procedures of cannula insertion into a lymphatic as were:

1. To place two pre sutures around the lymphatic upstream from its ligation,
2. To make a cut in the lymphatic using a pair of corneal scissors and then to insert the cannula into the lymphatic,
3. To secure the cannula in the lymphatic with the preplaced sutures.

After cannula insertion into the mammary lymphatic, the free end of the cannula was threaded through the skin near the wing of the ilium. The cannula was secured using a

purse-string suture at its skin opening ventral to the wing of the ilium and craniodorsal to the pre-femoral lymph node(s) after exteriorising its free end. An additional suture was made to secure the cannula on the skin. Following surgery, the success of mammary lymphatic cannulation was confirmed in all ewes by the presence of blue dye in the cannula following an injection of 1 mL (0.5g patent blue violet in 10 mL PBS) into the mammary tissues dorso-cranial to the teat and lymph dripping from the cannula.

The four sheep that were previously cannulated were infected with an isolate of *M. haemolytica* (1-2B), originally collected from a clinical case of ovine mastitis (Omaleki *et al.* 2010). To prepare the inoculum, colonies were directly harvested from a freshly inoculated sheep blood agar plate and resuspended in saline (2.5 mL). The suspension was diluted to achieve a concentration of 2,125 bacterial cells/0.5 mL. The titres of the initial suspension and the inoculum were confirmed by viable cell count. One teat was chosen for infection of each ewe and 0.5mL of inoculum was placed 2mm inside the teat using an 18G catheter. The teat chosen was based on maximising the ability to collect lymph from that side of the udder. The same volume of PBS was inserted 2mm into the other teat. This gave a total number of 8 glands, with half infected with *Mannheimia* and half used as control.

One sheep on arrival at the centre was already infected with a coagulase negative Staphylococci hence the *Mannheimia* was inserted in the opposite gland. Identification of bacteria was assessed at twelve hours with the bacterial count in milk varying from 540000 to 20,800,000 colony forming units per millilitre of milk. The gland infected by coagulase negative staphylococci had a count of 54500 colony forming units per millilitre of milk. By 24 hours post infection milk volume was below the required amount for fossomatic assessment and most samples were clotted on collection so further cell counts were not possible. Lymph was collected at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14.5, 22 & 26 hours post bacterial challenge. The volume of lymph collected at each time point was recorded. The numbers of cells in lymph were assayed using a model Z1 Coulter Particle Counter (Beckman Coulter). At the completion of these timepoints sheep were humanely euthanased with pentobarbitone.

3.13 Testing for antibiotic resistance

Bacterial isolates that were grown in 2012 were assessed using the CDS method for bacterial resistance or susceptibility to antibiotics. The antibiotic discs chosen were amoxicillin, erythromycin, penicillin G, cephalothin, tetracycline and trimethoprim/sulphomethoxazole as these were reported to be the most commonly used antibiotics in practice. The method used was as per CDS guidelines (Bell et al. 2006).

3.14 Statistical analysis

The INF properties were chosen for this study based on geographic spread in South Australia, Victoria and NSW as well as significant data available on ewes and their progeny. The biggest difference in INF sheep compared to standard Merino or maternal ewes is that for INF1 to INF4 all ewes were either on first or second lactation and ewes in all flocks were monitored more intensively than on commercial flocks. The three PD properties chosen for this study were based in three broadly different rainfall areas of Victoria (NE, NW and central regions) with different histories of the incidence of clinical mastitis. These flocks are marginally larger than the average Poll Dorset flock, but similar to many commercial scale Poll Dorset enterprises (Roe 2016).

On PD flocks every lactating ewe on the property was sampled at weaning. On PD3 this was only possible for spring born lambs in 2012 and 2013 but all other ewes were sampled on PD1 and PD2. All INF ewes were sampled on INF1, INF2, INF3 and INF4 in 2012 and 2013. Three flocks of ewes on INF5 with approximately 200 in each mob were selected for

milk sampling with all ewes in each mob sampled. All ewes on INF1, INF2, INF3 and INF4 were either first or second parity while ewes on INF5, PD1, PD2 and PD3 ranged from first parity up to ewes of greater than 10 years of age.

On-farm data were collected in hard copy and then transferred to excel sheets for each farm visit. Milk data from DTS laboratories were then uploaded and cross matched via bar code. Three spreadsheets were produced; dam information (genetics, age), lamb information and milk information. These spreadsheets were subsequently imported into a Microsoft Access database for further analysis.

Each of the continuously distributed explanatory variables were plotted as a function of log transformed individual milk cell count and the association between the two measures quantified using Pearson's correlation coefficient. Acknowledging the relationship between each of the explanatory variables and log transformed individual milk cell count was not linear, a non-parametric (loess) regression curve was superimposed on each plot.

Ewe age at the time of lambing was categorized into terciles (1 – 2 years, 3 – 4 years and > 4 years) and the significance of the association between ewe age and average daily gain quantified using analysis of variance.

Explanatory variables that were associated with average daily gain at an alpha level of less than 0.10 (flock, individual sheep milk cell count, number of lambs born, birth weight and age of ewe at lambing) were included in a linear regression model of the following form:

$$y_i = \beta_0 + (\beta_1 x_{1i} + \cdots + \beta_m x_{mi}) \qquad \text{Equation 1}$$

In Equation 1 yi represents average daily gain for the i^{th} lamb, β_0 is an intercept term and $\beta_1, \ldots \beta_m$ are the regression coefficients for each of the m explanatory variables (x) included in the model. Flock was included in the model as a fixed effect term comprised of eight levels (INF1, INF2, INF3, INF4, INF5, PD1, PD2 and PD3).

The significance of each explanatory variable in the model was tested using an analysis of variance. Explanatory variables that were not statistically significant were removed from the model one at a time, beginning with the least significant, until the estimated regression coefficients for all variables retained were significant at an alpha level of less than 0.05.

Frequency histograms of the residuals from the linear regression model and plots of the residuals versus predicted values were constructed to check that the assumptions of normality and homogeneity of variance had been met.

4 Results

4.1 Collection of milk samples from information nucleus flock ewes

4.1.1 Numbers and description of ewes

Ewes on INF properties were either Merino or Maternal lines of ewes. Ewes were pastured at Struan and Turretfield in South Australia and at Hamilton and Rutherglen in Victoria. In these four flocks all ewes collected were either first or second lambing ewes with samples collected in 2012 and 2013. All INF ewes on INF1, INF2, INF3 and INF4 were collected in 2012 and 2013. A final group of ewes were pastured at Armidale in NSW and collected in January 2014 with a broader representation of mixed age ewes. These five properties were

allocated the descriptors INF1, INF2, INF3, INF4 and INF5. Note that these descriptors were in order of date of collection and do not correlate with CRC numbering of properties. The total number of ewes sampled is shown in Table 1.

4.1.1 Collection of samples

It was possible to collect milk from up to 200 ewes in 12 hours using the "Bush-Tiger Milker" with two staff and a good race leading to the sampling area, once ewes had been yarded and lambs were weaned. Setting up the Bush-Tiger Milker and cleaning equipment and closing the Milker for road transport added a further two hours with preparation of milk tubes an additional one to two hours. The design of the Bush-Tiger milker allowed safe collection of samples in a range of environmental conditions including rain, wind, dust and high temperatures. Minor adjustments were made during the project to improve safety and useability. Adequate milk samples were collected from almost all Merino and Maternal ewes for cell count and bacterial testing, excepting from ewes with mastitis where either no milk or a small amount of milk for culture was collected. Where lambs were weaned for at least two hours pre milking this timing facilitated easier milk collection.

4.1.2 Physical assessment of clinical mastitis

Each ewe was assessed for clinical mastitis. This assessment included mammary abnormalities, lymph node abnormalities and teat abnormalities but did not include any obvious shearing damage such as cut teats. It also did not include ewes that had a dry half of the udder without further abnormality as this can be due to lack of suckling by a single lamb. Ewes that died during lactation or that were dry at the time of weaning were also not included in this analysis. On INF properties the percentage of clinical mastitis ranged from a low of 1% to a maximum of 6% (Table 1) at a particular visit.

Table 1 Collection of milk from Information Nucleus Flock (INF) properties

Property name	Date	Ewes assessed	Samples collected	Clinical mastitis	ISCC >500,000
INF1	27/9/2012	166	139	8 (5%)	23 (17%)
INF2	6/11/2012	196	193	11 (6%)	22 (11%)
INF3	6/12/2012	156	153	6 (4%)	46 (30%)
INF4	19/12/2012	181	154	4 (2%)	17 (11%)
INF2	22/10/2013	144	141	4 (3%)	18 (13%)
INF1	19/11/2013	137	132	2 (1%)	43 (33%)
INF4	20/11/2013	49	49	1 (2%)	1 (2%)
INF3	22/11/2013	106	104	2 (2%)	16 (15%)
INF5	14-16/01/2014	611	583	34 (6%)	57 (10%)
	TOTAL	**1746**	**1648**	**72 (4%)**	**243 (15%)**
	Average ewes per day	**159**	**150**		

Note: The majority of clinical mastitis sheep are also included in the ISCC>500,000 column. On some properties both dry and lactating ewes were present in a single mob, hence assessed ewe figures being significantly higher on some flocks compared to sample collection.

4.1.3 Assessment of subclinical mastitis on INF properties

The percentage of subclinical mastitis, assessed as those ewes with SCC > 500,000 cells/ml of milk varied substantially across flocks and years. The lowest percentage was in INF4 with 2% of ewes with counts above 500,000 cells per ml and the highest in INF1 with 33% of

ewes having a cell count greater than 500,000 cells per millilitre of milk. The average across flocks was 15% of Merino and Maternal ewes with cell counts greater than 500,000 as shown in

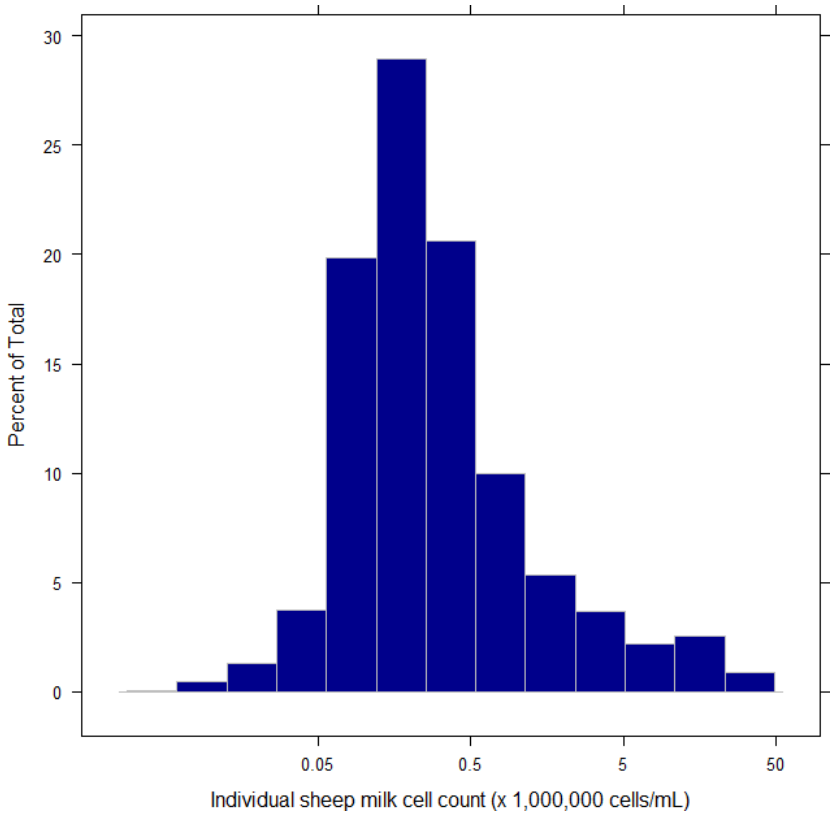


Figure 7 Frequency histogram of log [individual sheep cell count]

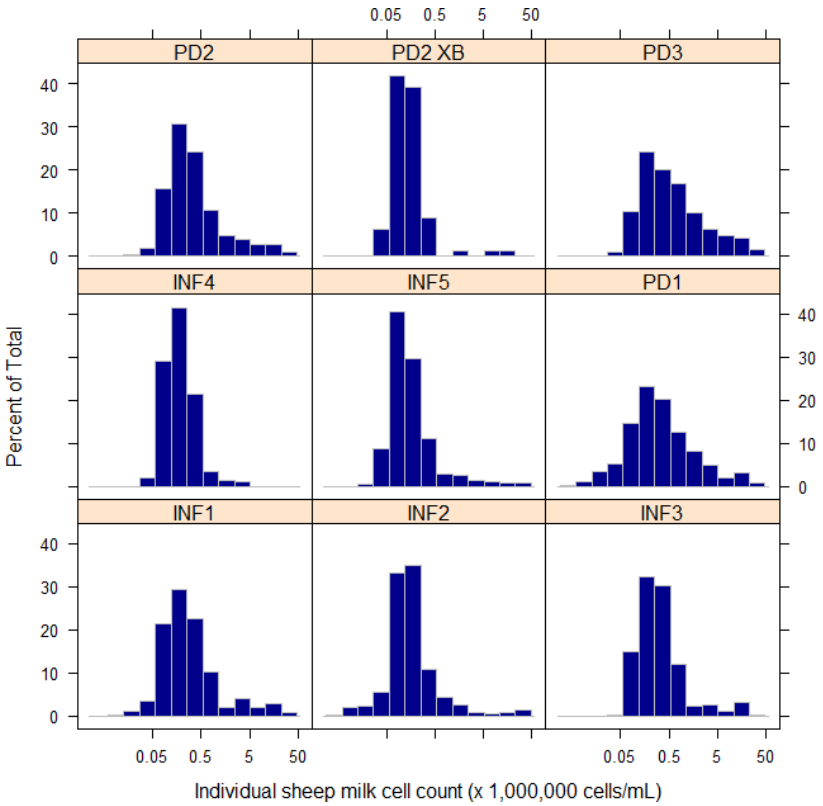


Figure 8 Frequency histogram of log [individual sheep cell count] by flock

4.2 Collection of milk samples from Poll Dorset flocks

4.2.1 Flock numbers

Three Poll Dorset flocks were successfully identified to participate in this project. These flocks had a range of clinical mastitis in their flocks prior to participating in this study. One property had recently experienced an outbreak of mastitis in their ewes (Omaleki *et al.* 2016), but had a history of generally less than 2-3% clinical mastitis; one had a history of clinical mastitis ranging from 2% up to 10% of the flock with selection against clinical and sub-clinical mastitis in the past four years and another with generally low levels of clinical mastitis with up to a few percent of ewes affected each year. Two studs only ran Poll Dorset stud animals on the property, whereas the third managed some maternal breed ewes on the property as well. All three flocks had over 250 Poll Dorset breeding ewes and had been involved in collecting data for genetic analysis for over twenty years. All three flocks had used more than a single link sire across the three properties in the past 20 years with all flocks using some artificial insemination in the previous five years.

4.2.2 Collection of samples

A total of 3900 individual samples of milk were successfully collected from 3500 ewes on Poll Dorset properties (Table 2). This included some milk from commercial ewes on PD2. In August 2013 on PD1 and October/November 2013 on PD2 milk from both teats was collected separately, rather than the samples from all other visits which were a combination of milk from both sides of the udder. This milk was collected separately to assess several on-farm milk testing products (see section 3.9). In general, milk was more easily obtained from PD ewes, than maternal ewes and Merino ewes although significant variation existed

between each breed and varied with time since lambs were weaned and diet quality at time of weaning.

Table 2 Collection of milk from Poll Dorset (PD) properties

Property name	Date	Ewes assessed	Samples collected	Clinical mastitis	SCC >500,000
PD1	12-14/8/2012	286	286	23 (8%)	114 (40%)
PD2	26/9/2012	66	65	5 (8%)	31 (48%)
PD2	20/10/2012	180	175	15 (8%)	45 (26%)
PD2	30/10/2012	164	164	18 (11%)	60 (37%)
PD3	8/11/2012	37	37	10 (27%)	30 (81%)
PD2	3-4/12/2012	250	238	13 (5%)	52 (22%)
PD1	7/12/2013	81	81	6 (7%)	51 (63%)
PD1*	26-28/8/13	267	534	16 (6%)	78 (15%)
PD2	3/10/2013	55	54	6 (11%)	24 (44%)
PD2	17/10/2013	151	150	13 (9%)	34 (23%)
PD3	18/10/2013	92	92	4 (4%)	58 (63%)
PD2*	25/10/2013	40	80	5 (13%)	24 (30%)
PD2*	7/11/2013	129	250	14 (11%)	56 (22%)
PD1	29/11/2013	78	75	6 (8%)	42 (56%)
PD2#	3-5/12/2013	416	405	19 (5%)	70 (17%)
PD2	11/12/2013	64	64	2 (3%)	11 (17%)
PD3	7/8/2014	185	185	19 (10%)	70 (38%)
PD1	9-10/9/2014	332	319	33 (10%)	143 (45%)
PD2	2/10/2014	68	68	11 (16%)	32 (47%)
PD2	20/10/2014	187	186	21 (11%)	54 (29%)
PD3	29/10/2014	97	97	4 (4%)	39 (40%)
PD2	20/11/2014	150	148	21 (14%)	45 (30%)
PD1	5/12/2014	77	147	2 (1%)	42 (29%)
	TOTAL	**3452**	**3900**	**286 (8%)**	**1205 (31%)**
	Average ewes per day	**111**	**126**		

*Milk collected from individual udder halves for assessment.

#Milk collected from some maternal (crossbred) ewes and also Poll Dorset ewes.

4.2.3 Physical assessment of clinical mastitis in PD flocks

Each ewe was assessed for clinical mastitis. This assessment included mammary abnormalities, lymph node abnormalities and teat abnormalities. It did not include ewes that had a dry half of the udder without further abnormality. On Poll Dorset properties clinical mastitis ranged from 1% to a maximum of 27% of ewes and an average across all flocks of 8% (Table 2). The figure of 27% was obtained when only a small sample of ewes was assessed. The level of clinical mastitis in ewes was significantly higher than anticipated at weaning and this was not obvious until the udder was examined closely.

4.2.4 Assessment of subclinical mastitis on PD properties

The percentage of sub-clinical mastitis in PD flocks varied significantly between flocks and times of the year from a low of 15% on PD1 to a maximum of 81% on PD3. The average percentage of PD ewes with cell counts higher than 500,000 cells/mL was 31%. For each ewe observed with clinical mastitis there were 4.2 ewes with cell count greater than 500,000 cells per mL with this figure varying between flocks from 2.6 to 21 ewes with counts greater

than 500,000 for every ewe with clinical mastitis. These figures are close to double the clinical and subclinical mastitis rates observed on INF properties with Merino and maternal breed ewes.

4.3 Milk chemistry

Each milk sample was assessed for percentage of fat, protein, lactose and solids-non-fat (SNF) in milk. Figure 9 to Figure 12 show regression curves for each of these milk components across all milk samples that were collected on PD and INF properties relative to individual sheep cell counts obtained.

As cell count increased beyond 500,000 cells per mL milk fat decreased marginally. Milk protein increased with cell counts greater than 500,000 per mL as reported by other authors (Revilla *et al.* 2009). Levels of lactose were also reduced with cell counts greater than 500,000 per mL while overall SNF reduced marginally with increases in cell count. Reductions in lactose have been observed in dairy sheep systems and were associated with significantly reduced milk volume (Marti De Olives *et al.* 2013).

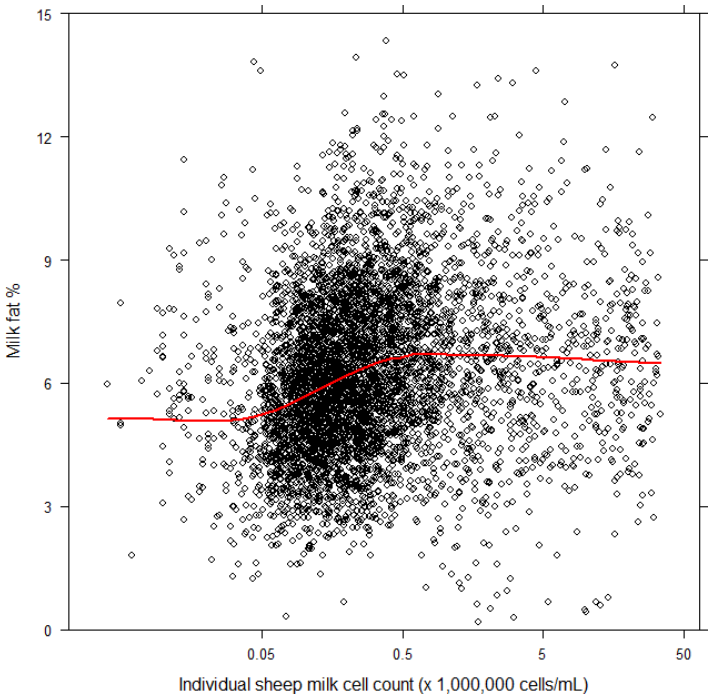


Figure 9: Scatterplot showing the association between log individual milk cell counts (× 1,000,000 cells/mL) and milk fat. A non-parametric (loess) regression curve superimposed. Pearson's correlation coefficient for BMCC > 0.5 million cells/mL: -0.04 (95% CI -0.09 to 0.01).

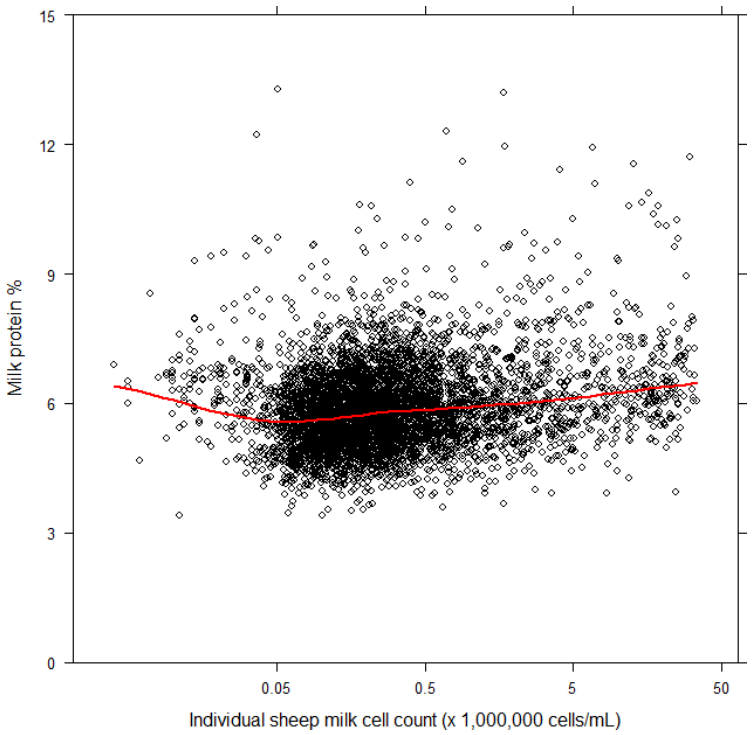


Figure 10 Scatterplot showing the association between log individual milk cell count (× 1,000,000 cells/mL) and milk protein. A non-parametric (loess) regression curve superimposed. Pearson's correlation coefficient for BMCC > 0.5 million cells/mL: 0.2 (95% CI 0.15 to 0.25).

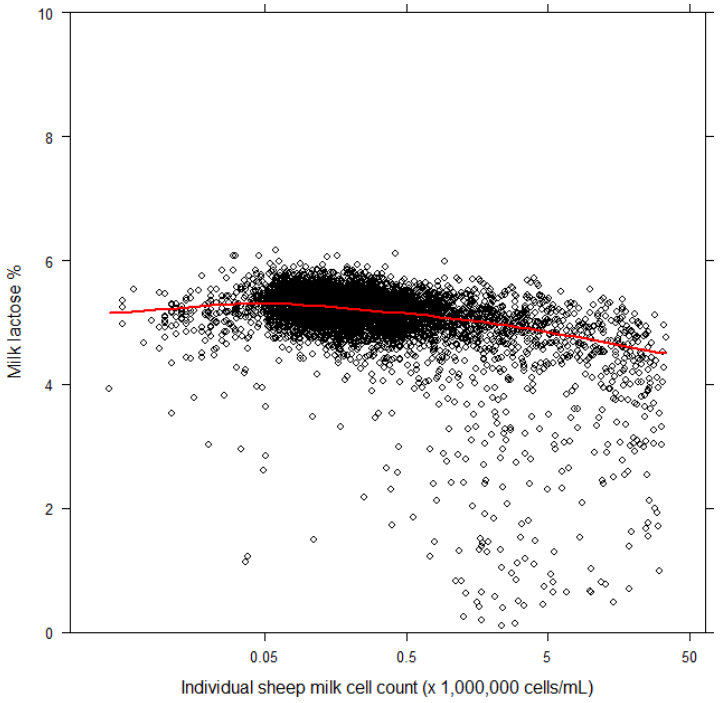


Figure 11: Scatterplot showing the association between log individual milk cell counts (× 1,000,000 cells/mL) and milk lactose. A non-parametric (loess) regression curve superimposed. Pearson's correlation coefficient for BMCC > 0.5 million cells/mL: -0.30 (95% CI -0.34 to -0.25).

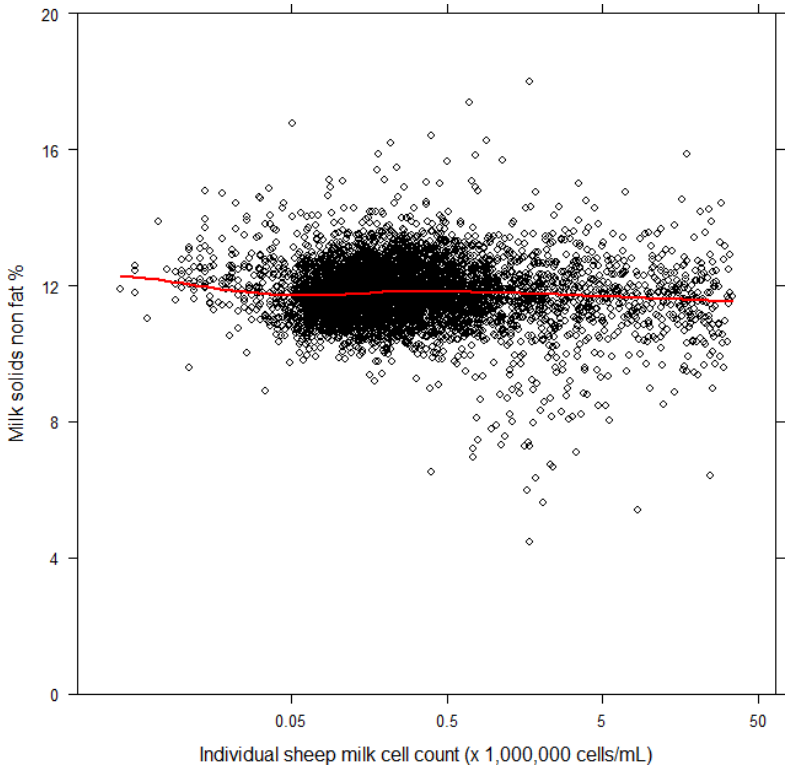


Figure 12: Scatterplot showing the association between log individual milk cell count (×1,000,000 cells/mL) and milk solids non-fat. A non-parametric (loess) regression curve superimposed. Pearson's correlation coefficient for BMCC > 0.5 million cells/mL: -0.05 (95% CI -0.10 to 0.00).

4.4 Udder phenotype data from INF and PD flocks

Four udder phenotype measures were collected from ewes on all flocks. Each phenotypic measure consisted of a 9 point linear scoring system. These phenotype measures were as described in **Error! Reference source not found.**.

The first of these measures is teat placement (TP) that describes the location of the teat on the udder with lower numbers indicating a teat located at the base of the udder and generally pointing vertically. Higher numbers indicate a teat placed on the lateral aspect of the udder and pointing in a more horizontal direction. Figure 13 shows the teat placement distribution for all ewes that were assessed. We did not assess the cranial/caudal direction or placement of the teat as this did not appear to vary dramatically in ewes. This figure demonstrates that if a ewe has teats placed more laterally (closer to the horizontal) then she is significantly more likely to have a higher milk cell count at the end of lactation.

The second measure of udder depth (UD) describes the depth of the udder. This measures the distance between the udder cleft and the abdominal wall, using the line joining the hocks as a reference point. A lower number describes a deeper udder whereas a high number is associated with an udder of smaller volume. Figure 14 shows the udder depth distribution for all ewes that were assessed. Ewes with udders of smaller volume tended to have higher cell counts at the end of lactation compared to ewes with udders of larger volume. In part, this finding may be due to a dilution impact with low volume udders producing lower volume so

any increase in cells is more likely to elevate cell count. In general, younger ewes tended to have smaller volume udders.

The third phenotypic measure used was degree of separation (DS). This measure scores the strength of the median ligament. A score of 1 shows no separation between left and right halves of the udder while a score of 9 demonstrates a large degree of separation. Figure 15 shows the degree of separation for all ewes that were assessed. Ewes with greater udder half separation had higher cell counts.

The final phenotypic measure is degree of suspension of the udder (SU), this describes the ratio of udder attachment width and udder height. Figure 16 shows the degree of suspension distribution for all ewes that were assessed plotted against individual sheep milk cell count. Ewes with poor udder suspension also had higher cell counts.

Along with these phenotypic measures measurements of teat parameters were also collected. This included teat length measured in millimetres from the base of the teat at its attachment to the udder to its tip. Further measurements collected included the width of the teat at the point of attachment to the udder and the width of the teat measured half way between teat attachment and teat end. Results of these measures are shown in Figure 17 to Figure 19 plotted against individual sheep cell count.

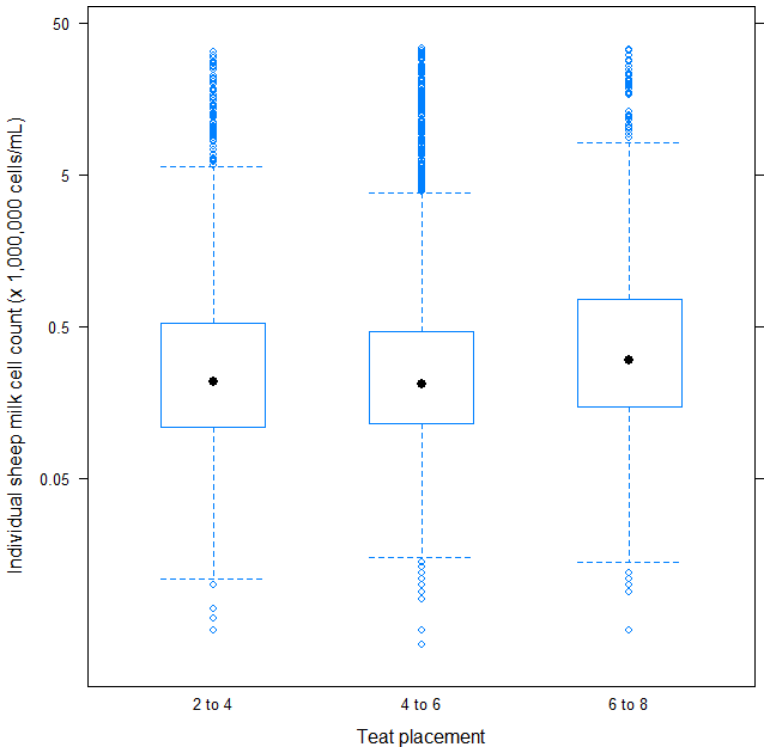


Figure 13: Box and whisker plot showing the distribution of individual milk cell count (× 1,000,000 cells/mL) for three categories of teat placement ($F_{2,5425}$ 5.02; P < 0.01).

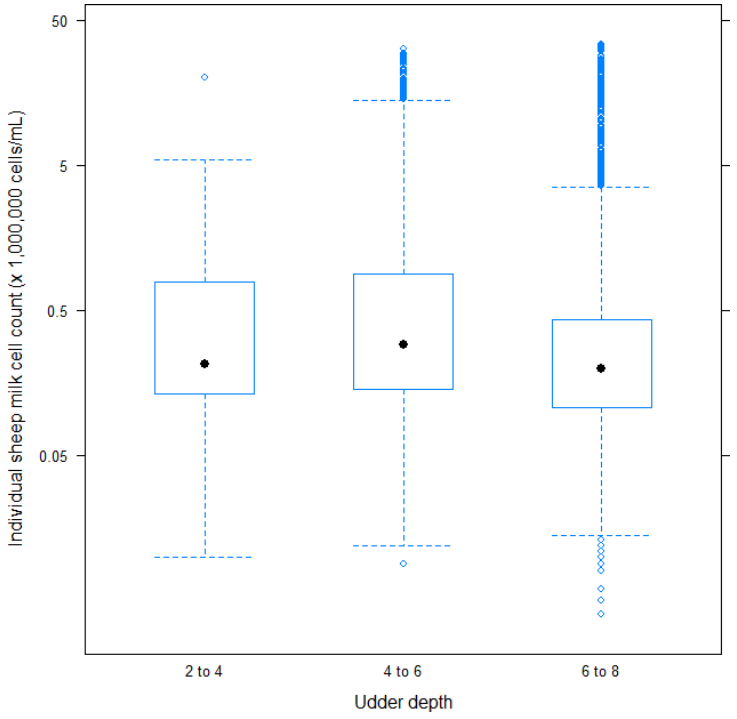


Figure 14: Box and whisker plot showing the distribution of individual milk cell count (× 1,000,000 cells/mL) for three categories of udder depth ($F_{2,5379}$ 23.41; P < 0.01).

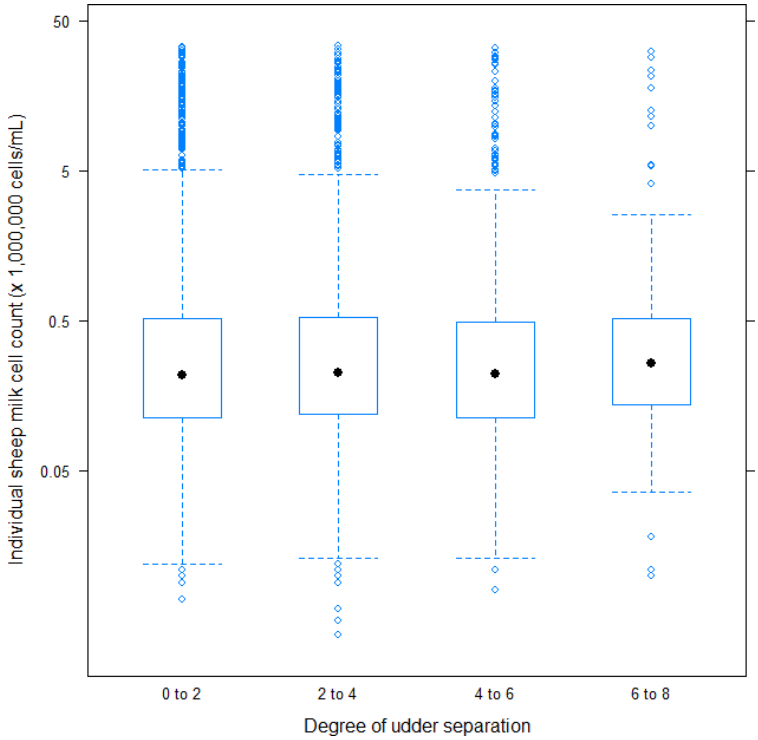


Figure 15: Box and whisker plot showing the distribution of individual milk cell count (× 1,000,000 cells/mL) for four categories of udder separation ($F_{3,4811}$ 3.08; P = 0.02).

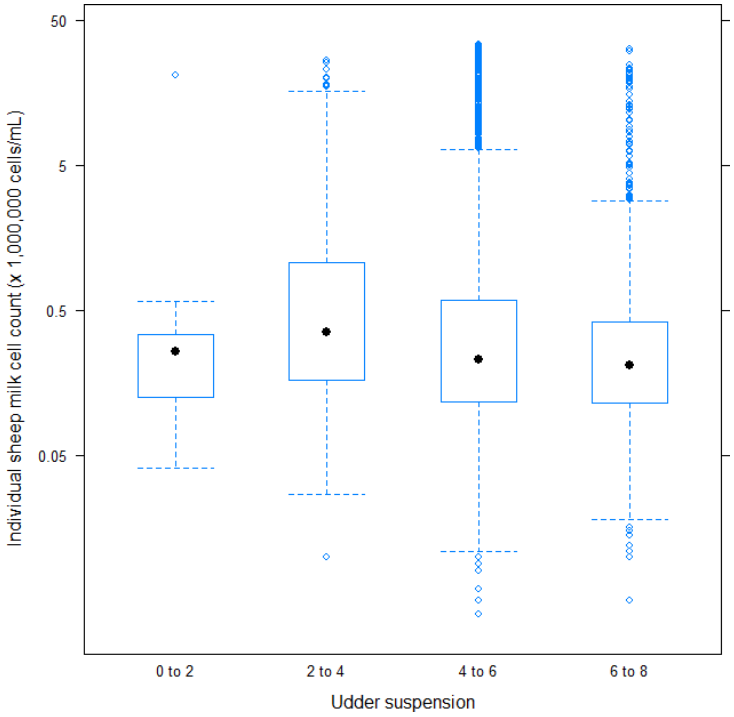


Figure 16: Box and whisker plot showing the distribution of individual milk cell count (× 1,000,000 cells/mL) for four categories of udder suspension ($F_{3,5427}$ 12.77; P < 0.01).

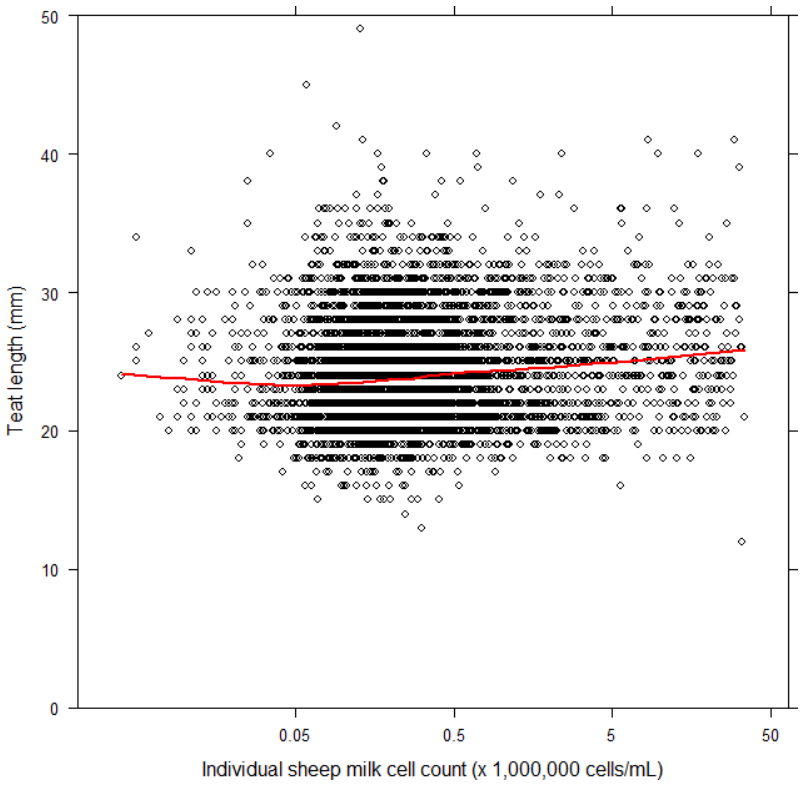


Figure 17: Scatterplot showing the association between log individual milk cell count (× 1,000,000 cells/mL) and teat length. A non-parametric (loess) regression curve superimposed. Pearson's correlation coefficient for BMCC > 0.5 million cells/mL: 0.11 (95% CI 0.05 to 0.16).

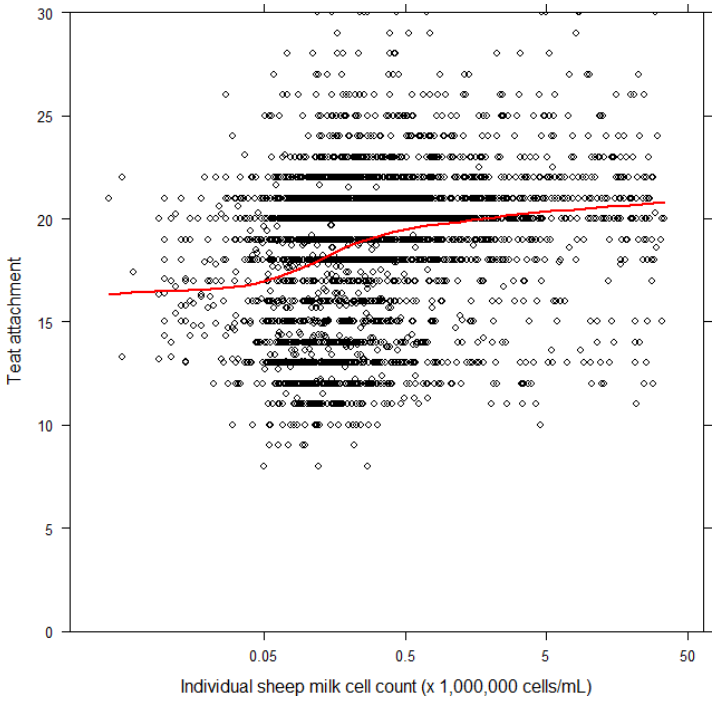


Figure 18: Scatterplot showing the association between log bulked milk cell count (× 1,000,000 cells/mL) and teat attachment. A non-parametric (loess) regression curve superimposed. Pearson's correlation coefficient for BMCC > 0.5 million cells/mL: 0.05 (95% CI -0.02 to 0.11).

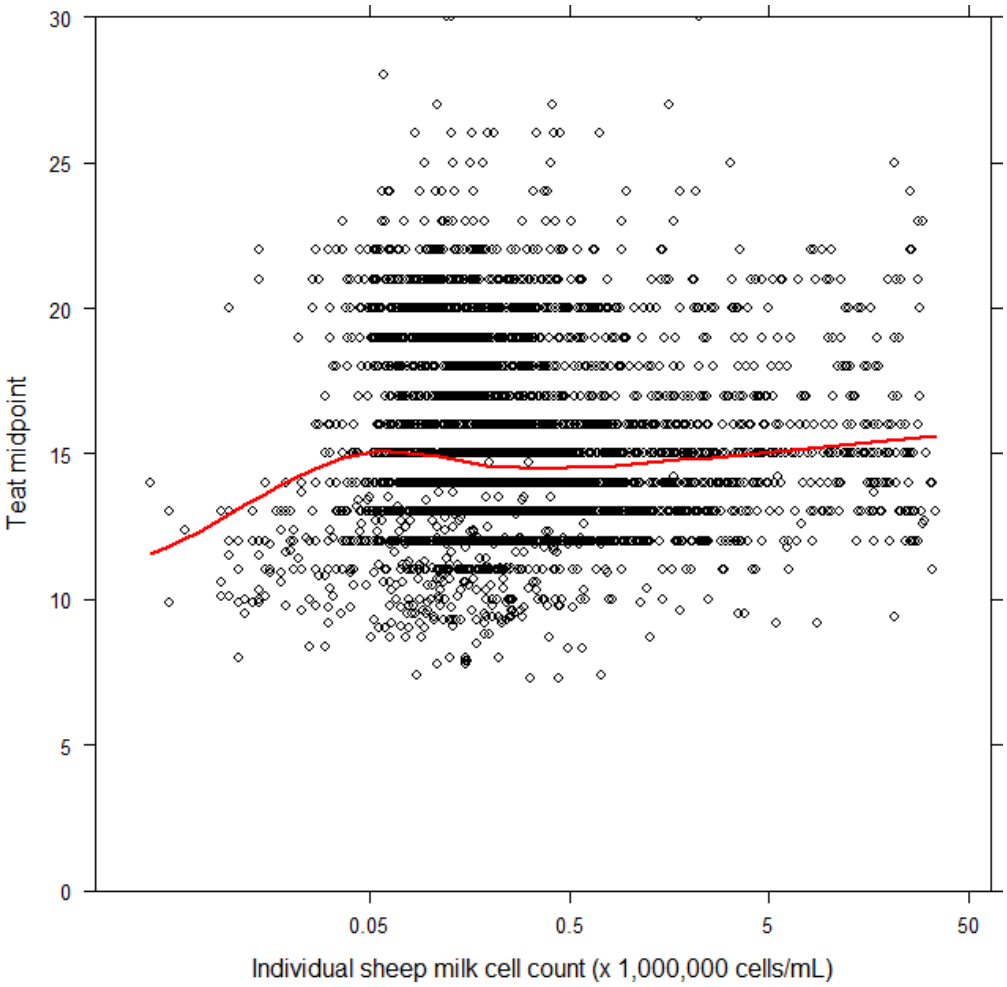


Figure 19: Scatterplot showing the association between log bulked milk cell count (× 1,000,000 cells/mL) and teat midpoint. A non-parametric (loess) regression curve superimposed. Pearson's correlation coefficient for BMCC > 0.5 million cells/mL: 0.08 (95% CI 0.02 to 0.14).

Increases in teat length, teat base width and teat mid-point width were associated with increased cell count. It is important to note that as ewes age these values also tend to increase over time.

4.5 Impact of sub-clinical mastitis on growth rate

The frequency histograms for lamb growth rate across all flocks from birth to weaning and weaning weight are shown in Figure 20 and Figure 21 respectively.

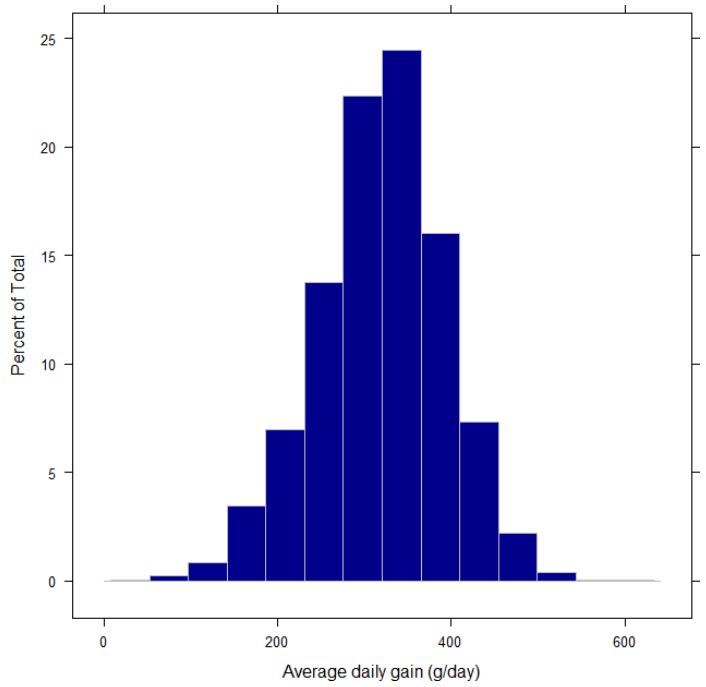


Figure 20 Frequency histogram of average daily gain (grams per day) for all flocks

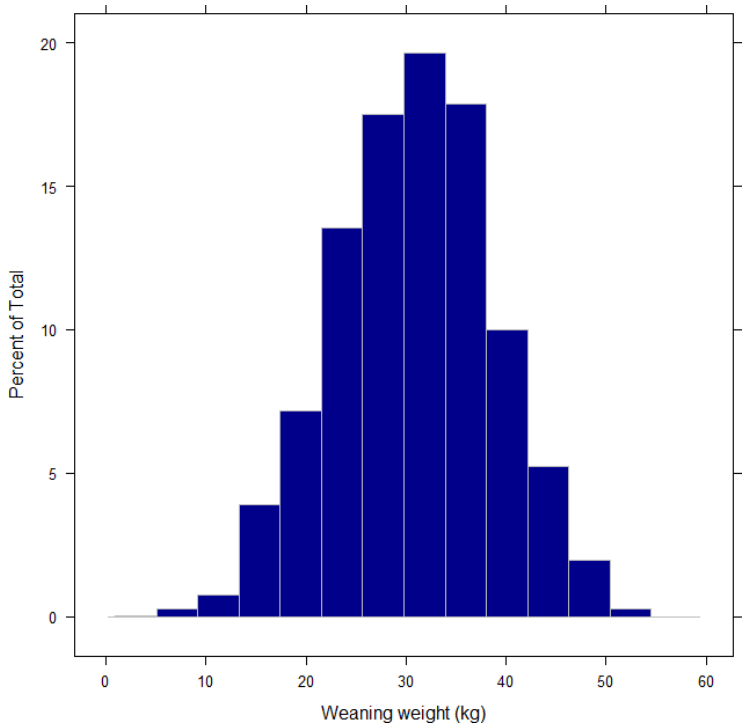


Figure 21: Frequency histogram of weaning weight in kilograms for all flocks

Figure 22 and Figure 23 show individual property lamb growth rate and weaning weight respectively.

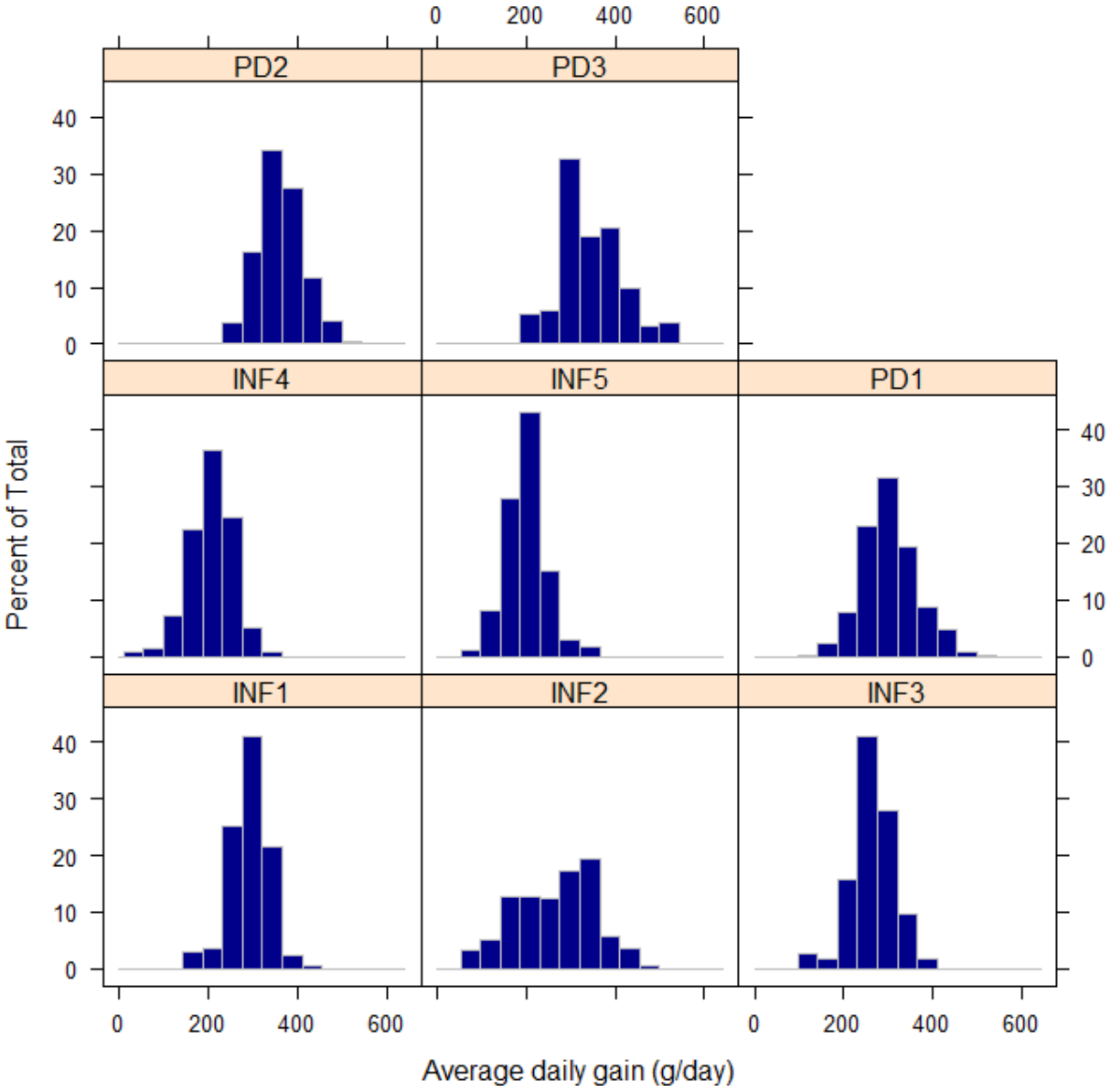


Figure 22: Frequency histogram of average daily gain (grams per day) for each flock

Large differences were observed between lamb growth across properties as shown by lamb growth rates in Figure 22. Significant variation was also observed in growth rate on individual properties. Data shown are from progeny of all ewes assessed on the property so for INF flocks this includes Merino and maternal breed progeny across two years with different lambing dates and different rainfall in the two years. Only stud PD and INF data are shown in this figure as any commercial lamb data are missing due to a lack of parentage and birth weight information. Figure 24 to Figure 30 show the impact of cell count on lamb growth rate and body weight and the impact of dam age on lamb growth rate.

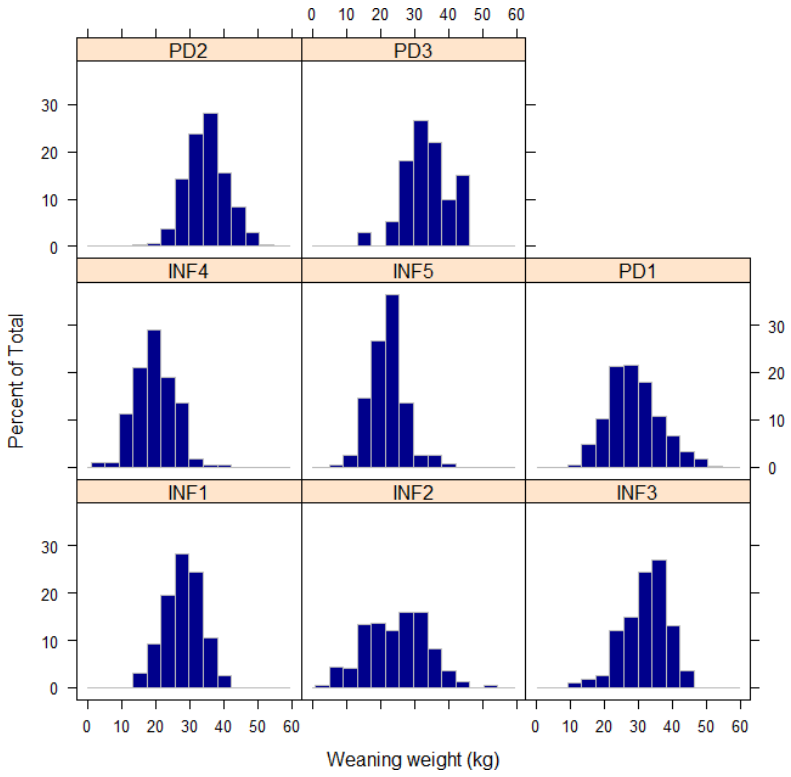


Figure 23: Frequency histogram of weaning weight (kilograms) for each flock

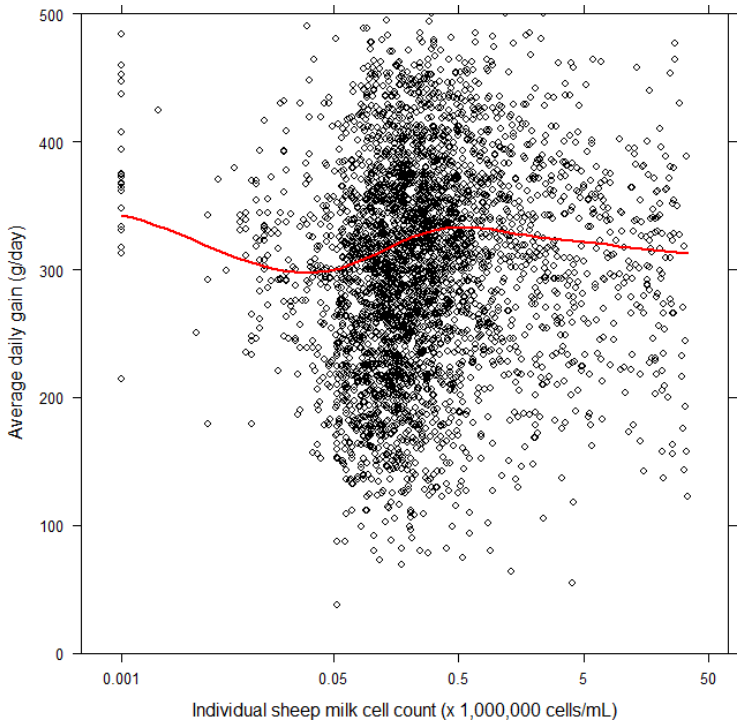


Figure 24: Scatterplot showing the association between log bulked milk cell count (× 1,000,000 cells/mL) and average daily gain (grams per day). A non-parametric (loess) regression curve superimposed. Pearson's correlation coefficient for BMCC > 0.5 million cells/mL: -0.10 (95% CI -0.20 to 0.01).

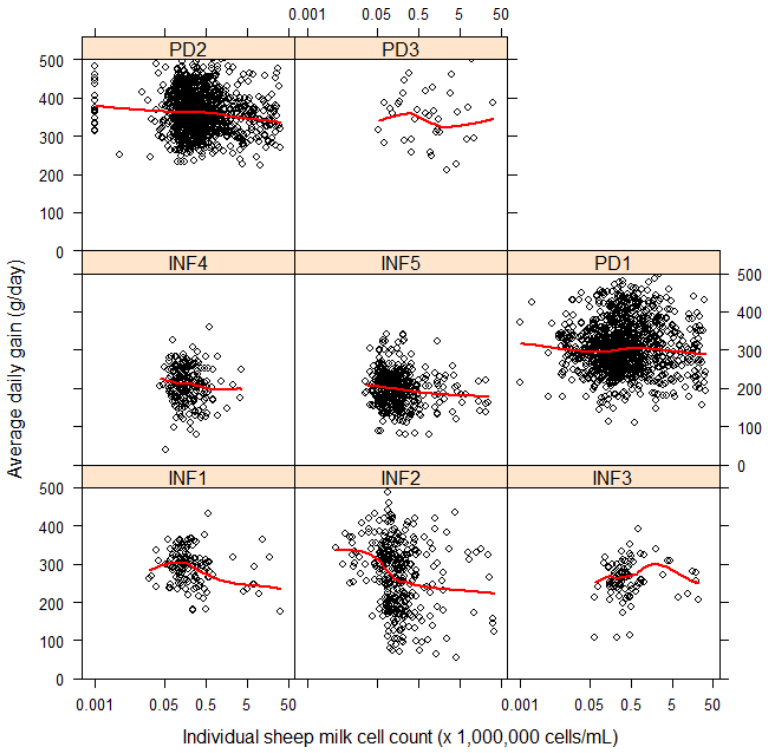


Figure 25: Scatterplot showing the association between log sheep milk cell count (in millions) and average daily gain (grams per day), by flock. A non-parametric (loess) regression curve superimposed.

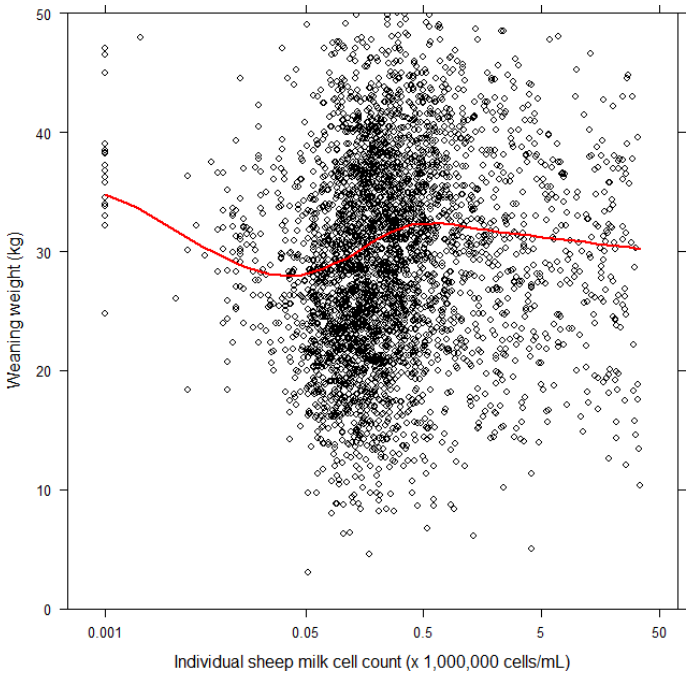


Figure 26 Scatterplot showing the association between log bulked milk cell count (× 1,000,000 cells/mL) and weaning weight (in kilograms). A non-parametric (loess) regression curve superimposed. Pearson's correlation coefficient for BMCC > 0.5 million cells/mL: -0.14 (95% CI -0.24 to -0.03).

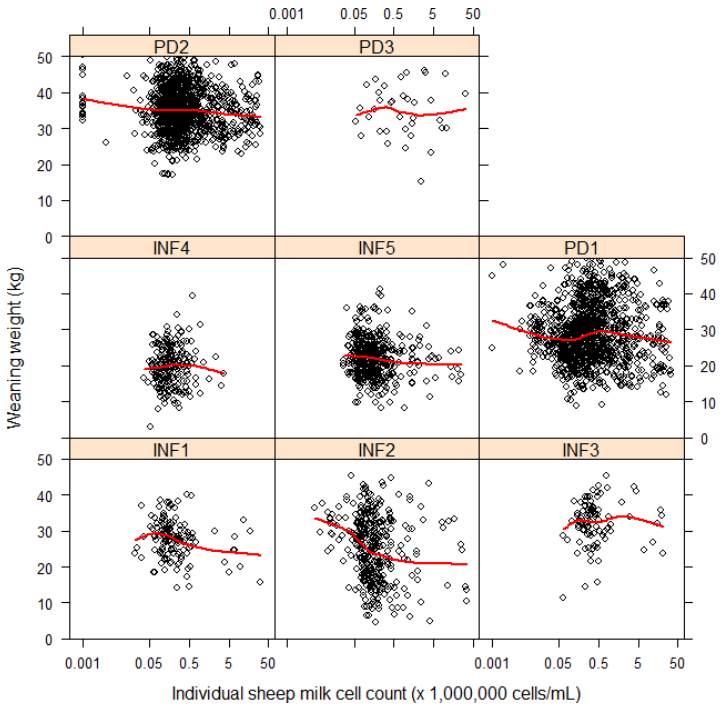


Figure 27: Scatterplot showing the association between log bulked milk cell count (in millions) and weaning weight (in kilograms), by flock. A non-parametric (loess) regression curve superimposed.

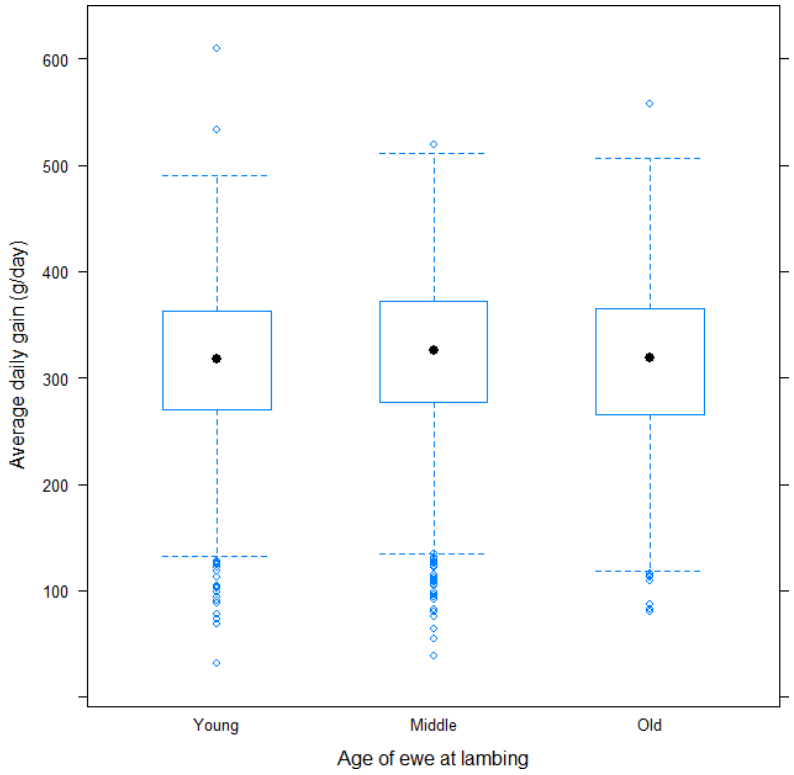


Figure 28: Box and whisker plot showing the association between ewe age at lambing and average daily gain ($F_{2,1159}$ 20.8; $P < 0.01$).

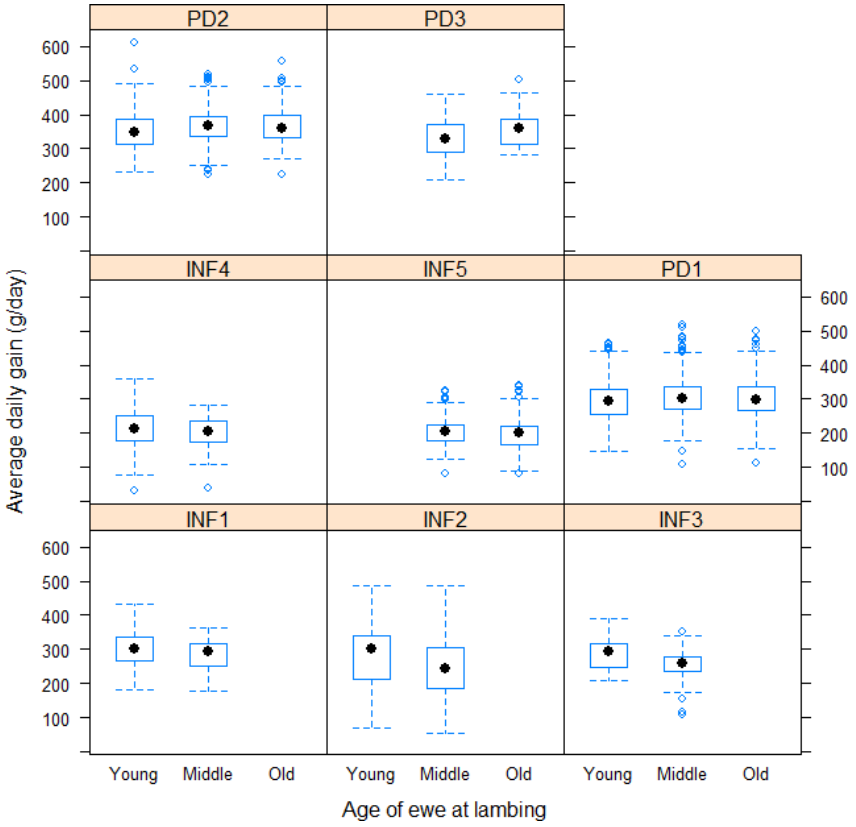


Figure 29: Box and whisker plot showing the association between ewe age at lambing and average daily gain of lambs at weaning, by flock.

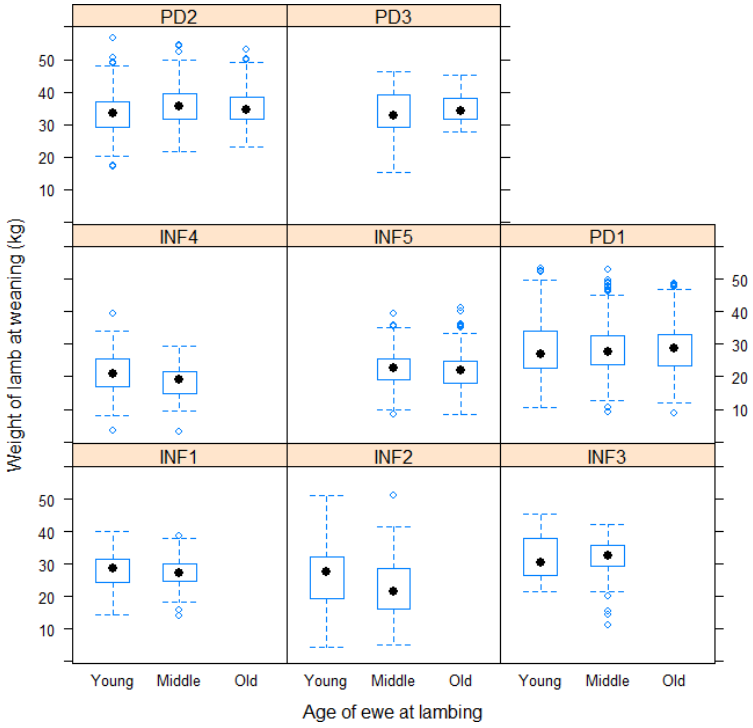


Figure 30: Box and whisker plot showing the association between ewe age at lambing and weight of lamb at weaning, by flock.

Table 3: Descriptive statistics for all flocks

Variable	Mean (SD)	Median (Q1, Q3)	Min, max	Missing
BMCC (millions cells/mL)	1.28 (3.84)	0.22 (0.12, 0.53)	0, 34.26	31
Fat (%)	6.28 (2.03)	6.12 (4.86, 7.58)	0.19, 17.86	0
Protein (%)	5.9 (1.01)	5.79 (5.26, 6.41)	3.41, 18.06	0
Lactose (%)	5.06 (0.64)	5.18 (4.96, 5.37)	0, 6.17	0
SNF (%)	11.78 (0.94)	11.76 (11.28, 12.32)	4.47, 20.15	0
Teat placement	5.31 (1.15)	5 (4, 6)	2, 9	20
Udder depth	6.81 (0.79)	7 (6, 7)	1, 9	20
Separation	2.93 (1.56)	2 (2, 4)	1, 8	636
Suspension	6.09 (0.84)	6 (6, 7)	1, 9	22
Teat length	24.23 (3.92)	24 (21, 26)	12, 51	312
Teat attachment	18.57 (3.7)	19.6 (16, 21)	8, 40	1709
Teat midpoint	15.06 (3.22)	14 (13, 17)	7.3, 30	1660
ADG (g/day)	319.72 (72.94)	323 (273, 369)	31, 610	192
Weaning weight (kg)	30.81 (7.88)	31 (25.3, 36.35)	3.05, 56.5	192

Total ewes assessed for data in Table 3 = 5444. Data from 10,473 lamb birthweights and weaning weights.

Table 4: Multivariable regression analysis of factors associated with average daily gain (grams) from birth to weaning in INF sheep flocks.

Explanatory variable	Coefficient (SE)	t value	P	95% CI
Intercept	266.3452 (6.7068)	39.713	< 0.01	253 to 280
Flock:				
INF 1	Reference	-	-	
INF 2	13.2332 (4.7502)	2.786	< 0.01	4 to 23
INF 3	-14.048 (6.2544)	-2.246	0.02	-26 to -2
INF 4	-62.1293 (5.2596)	-11.813	< 0.01	-72 to -52
INF 5	-72.1774 (5.92)	-12.192	< 0.01	-84 to -61
Cell count (× 1,000,000 cells/mL)	-0.9948 (0.4183)	-2.378	0.02	-2 to 0
Number of lambs reared:				
One	Reference	-	-	-
Two or more [a]	-85.8953 (4.3158)	-19.902	<	-94 to -77
Birthweight (kg) [b]	7.8666 (0.9479)	8.299	2.44E-16	6 to 10
Age of ewe at lambing (years)				
1 – 2	Reference	-	-	
3 - 4	-9.5826 (3.3347)	-2.874	0.00412	-16 to -3
> 4	-12.1156 (5.7066)	-2.123	0.03392	-23 to -1

[a] Interpretation: Compared with single lamb litters and adjusting for the confounding effect of flock, individual sheep milk cell count, birthweight and age of the ewe at lambing, average daily gain for lambs from litters of two or more was reduced by 86 (95% CI 77 to 94) grams per day.

[b] Interpretation: After adjusting for the confounding effects of flock, number of lambs born, birth weight and age of ewe at lambing, one kilogram increases in birthweight increased average daily gain by 8 (95% CI 6 to 10) grams per day.

Table 5: Multivariable regression analysis of factors associated with average daily gain (grams) from birth to weaning in PD sheep flocks.

Explanatory variable	Coefficient (SE)	t value	P	95% CI
Intercept	292.0451 (2.2805)	128.061	<0.01	288 to 297
Flock:				
PD 1	Reference	-	-	
PD 2	66.7365 (1.0722)	62.241	<0.01	65 to 69
PD 3	22.2116 (4.3591)	5.095	<0.01	14 to 31
Cell count (× 1,000,000 cells/mL)	-0.7053 (0.1314)	-5.367	<0.01	-1 to 0
Number of lambs reared:				
One	Reference	-	-	
Two or more [a]	-53.9522 (1.6812)	-32.091	<0.01	-57 to -51
Birthweight (kg) [b]	1.5892 (0.3254)	4.884	<0.01	1 to 2
Age of ewe at lambing (years)				
1 – 2	Reference	-	-	
3 - 4	11.0901 (1.2571)	8.822	<0.01	9 to 14
> 4	8.8147 (1.547)	5.698	<0.01	6 to 12

[a] Interpretation: Compared with single lamb litters and adjusting for the confounding effect of flock, individual sheep milk cell count, birthweight and age of the ewe at lambing, average daily gain for lambs from litters of two or more was reduced by 53 (95% CI 51 to 57) grams per day.

[b] Interpretation: After adjusting for the confounding effects of flock, number of lambs born, birth weight and age of ewe at lambing, one kilogram increases in birthweight increased average daily gain by 2 (95% CI 1 to 2) grams per day.

Table 4 and Table 5 demonstrate that mastitis present at the time of weaning reduced Merino or maternal lamb growth rate by 1.0 g for each increase in dam milk cell count of 1,000,000 cells per mL. The corresponding figure for PD ewes was 0.7 grams per 1,000,000 cells per mL. Lamb growth was also reduced by increased number of lambs or reduced birth weight. A multivariable regression analysis comparing all ewes and lambs in the study is available in the Appendix, Table 21.

4.6 Clinical mastitis in Australian sheep flocks

4.6.1 Number of samples sent to producers

Over 1,500 sample containers were sent to 50 producers either by mail via request or during presentations to producer group meetings or contacts (Bestwool/BestLamb presentation in Bendigo, Birchip Cropping Group and South West Victoria Prime Lamb Group meeting).

4.6.2 Number of samples received from producers

The return rate of samples during this project was lower than anticipated and previously achieved during PIRD V06 where each property was visited on an individual basis and samples were collected from the property by a second visit. Samples were received from 6 properties with a total of 49 samples submitted.

4.6.3 Bacteria isolated from cases of clinical mastitis during lactation

The bacteria isolated from cases of mastitis throughout lactation on six different properties are listed in Table 6. All of these samples were collected during lactation and then mailed to the laboratory and plated onto Sheep Blood Agar plates on arrival. Only 55% of the submitted samples produced viable bacteria. This may in part be due to the samples being frozen for up to 4 months, due to potentially fastidious nature of some organisms that did not grow under standard culture conditions or if bacteria were not present in the milk sample at the time of collection. This culture rate is similar to previous studies using frozen milk from cases of sheep mastitis in our laboratory.

The most common bacteria isolated from cases of mastitis were *Mannheimia* species. The next most common were *Bacillus* species and then coagulase negative Staphylococci and *Staphylococcus aureus*. These results are similar to results from PIRD V4 2006, “Reducing mastitis in sheep”. In this report 154 samples were submitted and 77 grew bacteria. Of samples that grew 52% were Mannheimia, 27% were Staphylococcus species, 10% Streptococcus, 6% *E. coli* and 2% *Pasteurella multocida* or *Bacillus*. The increased amount of *Bacillus* in this group of clinical samples may reflect the very wet year in 2013 and samples delivered from a property in western Victoria where some paddocks were underwater for several days.

Table 6: Bacteria isolated from clinical mastitis during lactation

Flock	Year Collected	Samples submitted	Samples grown	Bacterial species	Location
A	2014	1	1	*1 * Staphylococcus aureus*	Gippsland, Victoria
B	2014	5	1	1 * *Staphylococcus devriesei*	SW Victoria
C*	2014	2	1	1 * *Mannheimia*	NW Victoria
D	2012	1	1	1 **Staphylococcus chromogenes*	Victoria
C*	2012	8	4	2 * *Mannheimia*, 1 * *Corynebacterium mastitidis*, 1 * *Streptococcus suis*	NW Victoria
E	2012	2	2	2 * Staphylococcus simulans	NE Victoria
F	2013	30	17	8 * *Mannheimia sp*, 4 * *Bacillus sp*, 1 * *Leucobacter*, 1 * *Paenibacillus sp,* 3 * mixed (*Bacillus & Staphylococcus epidermidis, Pseudomonas & Staphylococcus aureus*, *Staphylococcus* sp, *Bacillus sp*.	W Victoria
TOTAL		49	27		

*Property C= PD3. Samples collected throughout year, not at weaning.

4.6.4 Bacteria isolated from cases of clinical mastitis at weaning

At the time of weaning in INF and PD properties in this study the udder was assessed for any signs of clinical mastitis and milk was collected for bacterial culture in all flocks. This milk was kept at 4 degrees Celsius until plated, usually within 3 days of collection, except for samples from INF5 which was stored for up to 7 days. This is in contrast to clinical samples from other flocks where samples were received frozen. The result of Gram stains from bacteria grown on SBA from cases of clinical mastitis at weaning are shown in Table 7.

Table 7: Gram test result for bacterial isolates from clinical mastitis at the time of weaning

	2012 lambing		2013 lambing		2014 lambing	
	Gram positive	Gram negative	Gram positive	Gram negative	Gram positive	Gram negative
PD flocks	42	6	21	13	46	14
INF	1	3	6	7	n/a	n/a
TOTAL	**43**	**9**	**27**	**20**	**46**	**14**

In 2012 there were two bacteria with variable Gram stain and in 2013 one yeast.

There were more cases of clinical mastitis overall caused by Gram positive than Gram negative bacteria as shown in Table 7 although the relative percentage varied each year significantly and between breeds of sheep, with INF sheep having numerically greater Gram negative infection. The species identified are shown in Table 8.

Table 8: Identification of bacteria isolated from cases of clinical mastitis at weaning 2012-2014 (species with >5% are shown in bold)

2012		2013		2014	
TOTAL (%)	**Bacteria**	**TOTAL (%)**	**Bacteria**	**TOTAL (%)**	**Bacteria**
5 (9.3%)	***Mannheimia sp***	**6 (12.5%)**	***Mannheimia sp***	**12 (20%)**	***Mannheimia sp***
1 (1.9%)	*Enterobacter hormaechei*	1 (2.1%)	*Escherichia coli*	1 (1.7%)	*Escherichia coli*
1 (1.9%)	*Morganella morganii*	1 (2.1%)	*Pseudomonas fluorescens*	1 (1.7%)	*Pseudomonas aeruginosa*
2 (3.7%)	Unidentified Gram negative	**12 (25%)**	**Unidentified Gram negative**		
1 (1.9%)	*Arthrobacter sp*			1 (1.7%)	*Pantoea sp*
1 (1.9%)	*Bacillus sp*				
1 (1.9%)	*Enterococcus sp*	2 (4.2%)	*Enterococcus*	2 (3.3%)	*Enterococcus sp*
1 (1.9%)	*Microbacterium sp*				
6 (11.1%)	***Staphylococcus sp***	1 (2.1%)	*Staphylococcus sp.*	1 (1.7%)	*Staphylococcus capitis*
2 (3.7%)	*Staphylococcus aureus*			**4 (6.7%)**	***Staphylococcus aureus***
7 (13.0%)	***Staphylococcus chromogenes***	**4 (8.3%)**	***Staphylococcus chromogenes***	**11 (18.3%)**	***Staphylococcus chromogenes***
3 (5.6%)	***Staphylococcus devriesei***			**5 (8.3%)**	***Staphylococcus devriesei***
1 (1.9%)	*Staphylococcus haemolyticus*			1 (1.7%)	*Staphylococcus warneri*
7 (13.0%)	***Staphylococcus simulans***	**3 (6.3%)**	***Staphylococcus simulans***	**13 (21.7%)**	***Staphylococcus simulans***
1 (1.9%)	*Streptococcus pluranimalium*	1 (2.1%)	*Streptococcus pluranimalium*	**3 (5%)**	***Streptococcus pluranimalium***
2 (3.7%)	*Streptococcus suis*	1 (2.1%)	*Streptococcus suis*		
1 (1.9%)	*Trueperella pyogenes*			1 (1.7%)	*Streptococcus entericus*
		1 (2.1%)	*Streptococcus ovis*		
				2 (3.3%)	*Streptococcus uberis*
9 (16.7%)	**Unidentified Gram positive**	**14 (29.2%)**	**Unidentified Gram positive**	2 (3.3%)	Unidentified Gram positive
2 (3.7%)	Unidentified variable Gram stain	1 (2.1%)	Yeast		

Almost all bacteria were identified in 2014, whereas approximately 20% of the clinical samples collected in 2012 and 55% in 2013 were not definitively identified to species level.

The most common Gram negative pathogen causing clinical mastitis in the 8 flocks in this study were *Mannheimia* species. This includes *Mannheimia haemolytica*, *Mannheimia glucosida* and *Mannheimia ruminalis* (Omaleki *et al.* 2012). This finding is in agreement with the clinical data from the other farms in this study and previous PIRD V04. Where a Gram negative organism was grown from a milk sample it was more likely to have come from a ewe with clinical mastitis as shown in Table 9. This table demonstrates that Gram negative isolates in general were more likely to be detected by a standard clinical exam, although at best this would identify one in two sheep with mastitis and as low as one in ten.

Table 9: Percentage of bacterial isolates at weaning from PD and INF properties due to clinical mastitis

	2012 lambing		2013 lambing		2014 lambing	
	Gram positive	Gram negative	Gram positive	Gram negative	Gram positive	Gram negative
PD flocks	21%	29%	10%	22%	21%	52%
INF	7%	19%	4%	8%		
TOTAL	20%	24%	7%	14%	21%	52%

The most common Gram positive pathogen causing clinical mastitis across the flocks at weaning were coagulase negative Staphylococci, including *Staphylococcus chromogenes*, *Staphylococcus simulans* and *Staphylococcus devriesei*.

4.7 Development of a sheep model for measuring mammary lymphatic drainage

Material from this section has been published in BMC Veterinary Research: Development of an ovine efferent mammary lymphatic cannulation model with minimal tissue damage. Authors: Hung-Hsun Yen, Elizabeth Washington, Wayne Kimpton, Evan Hallein, Joanne Allen, Silk Yu Lin, Stuart Barber: Yen et al. BMC Veterinary Research (2016).

4.7.1 Development of a novel mammary lymphatic cannulation model

4.7.1.1 Baseline milk parameters of ewes before and after mammary cannulation

For the first six days after arrival in the animal house prior to surgery, the average half-day milk production for a single gland from the ewes ranged from 51.5 to 180 millilitres.

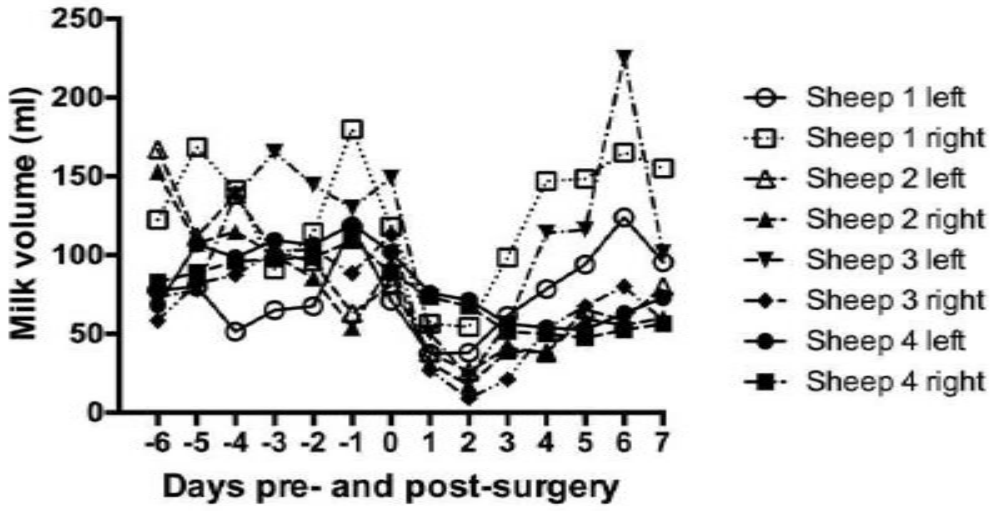


Figure 31 Milk volume collected per 12 hours pre and post lymphatic cannulation

Milk was not collected from ewes in the evening following surgery. After surgery, the average half-day milk volume had an obvious drop in the first 3-4 days post-surgery in all sheep and then started to increase. At 4-7 days post-surgery, the milk production levels of two ewes returned to similar quantities of that before surgery, but the milk production of the other two ewes remained at lower levels. The quantities of milk collected were however adequate for milk quality tests.

The amounts of fats, proteins, lactose and SNF in the milk before and after surgery were also monitored. Daily fluctuations and individual differences on the percentages of these components in the milk were observed, but no obvious changes were noticed post-surgery as shown in Table 10.

Table 10: Range in milk component value before and after lymphatic cannulation surgery.

Milk component	Pre surgery %	Post surgery %
Fat	6.54-10.02	7.04-10.13
Protein	5.86-8.40	5.92-8.57
Lactose	4.36-5.10	4.53-5.17
Solids non fat	12.03-13.38	11.72-13.96

4.7.1.2 Results of lymph collection

Lymph was collected from all eight cannulated mammary ducts and the rate of lymph flow was measured, the cell concentrations and the cell output per hour were also calculated as shown inFigure 32 A & C respectively. Three ducts had slow, but continuous flow rates ranging from 0.09-1.07 mL/hr. The left side duct of sheep 2 became blocked at day one post-surgery. More than 97% of cells in the lymph samples were small lymphocytes, with the remainder large or blast-like lymphocytes. The phenotypes of small lymphocytes in mammary efferent lymph are shown in Table 11. Mammary lymph was composed of 86% T cells with CD4+ T cells comprising 60% of the total T cells in mammary lymph (Table 11).

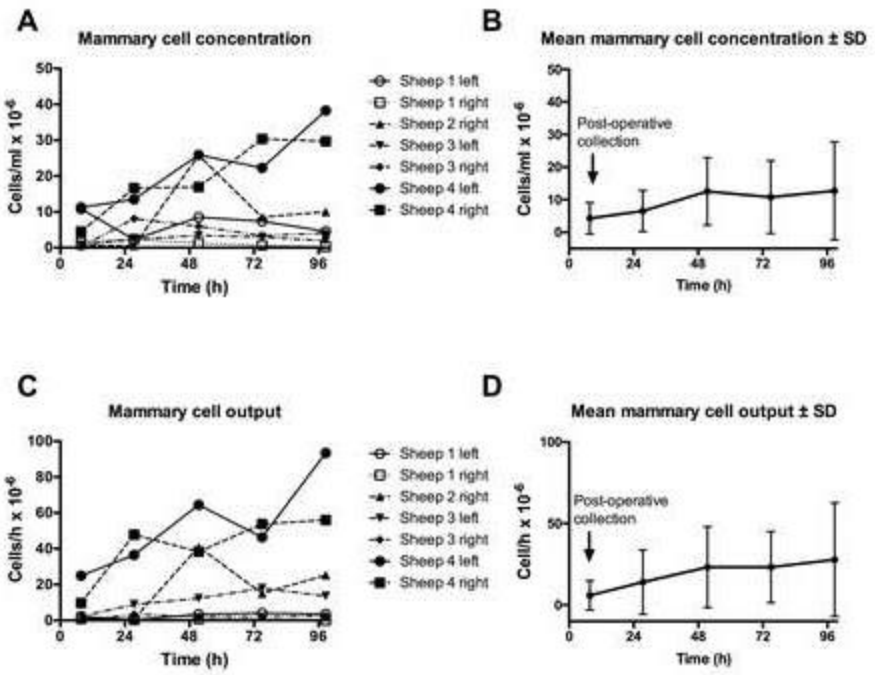


Figure 32 Cell concentration and output from the mammary lymph node

The presence of a low number of red blood cells was noted in the lymph samples for up to 4-5 days post-surgery. Lymph flowed well in four ducts with the flow rate ranging between 1.58-5.72 mL/hr (daily volume, 37.8-205.7 mL) and the individual cell concentrations and outputs are shown in Table 11.

Table 11 Phenotypes of lymphocytes in mammary efferent lymph

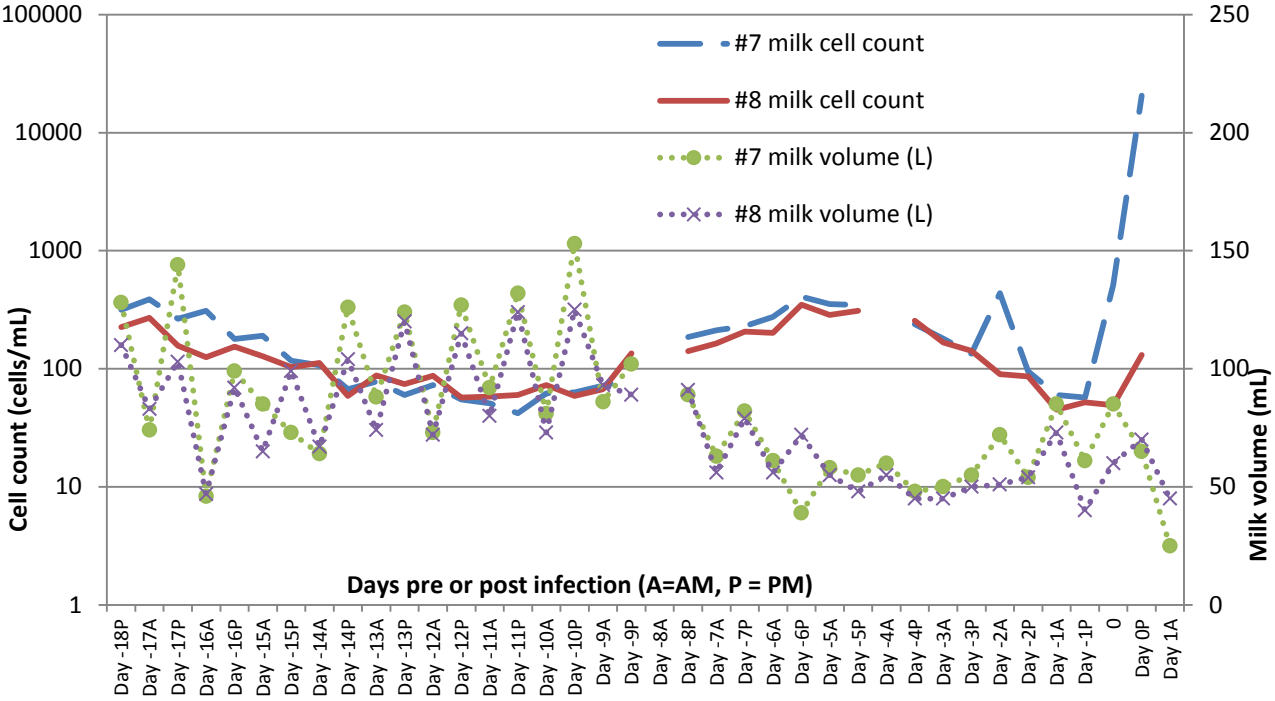
Subset	Mammary efferent lymph Mean % ± SD
CD4	51 ± 14
CD8	15 ± 2
□□ TCR	20 ± 9

4.7.2 Response to mastitis within mammary milk and lymph

Figure 33 shows milk production and milk cell count before and after administration of *M. haemolytica* (Day 0) in an individual sheep. Figure 34 demonstrates the dramatic reduction in milk yield following infection with *M. haemolytica* in the gland infected, but also an impact on the non-infected gland with reductions of 56% and 29% respectively in the 24 hours post infection across the four ewes in the study.

Glands that were infected with *M. haemolytica* demonstrated an elevation in milk cell count within 12 hours of infection with an average seventy four-fold increase on preinfection levels as illustrated by the ewe in Figure 33. This increase ranged from two to 190 fold increase with cell counts ranging from 3,014,000 to 24,495,000 cells per millilitre. By 12 hours post infection ewes were also showing clinical signs of mastitis with anorexia and elevated temperatures. Immediately prior to infection the ewes temperatures averaged 39.0 (SD: 0.3)

degrees Celsius and this did not change significantly by 5.5 hours post infection with an average of 39.1 degrees Celsius (SD: 0.3). By 12 hours post infection the average temperature was 40.5 degrees Celsius (SD: 1.0) and this increased further by 26 hours post infection to 40.7 (SD: 0.8) degrees Celsius. A Milkcheck test performed at 12 hours post infection showed an average difference between the two halves of each ewe of 1.3, ranging from zero difference up to 2.7.



#7 gland was infected with *Mannheimia haemolytica, #8 gland was not infected*

Figure 33 Milk yield versus milk cell count before and after infection with M. haemolytica

Following infection with *Mannheimia haemolytica* there was a significant increase in flow rate of lymph through the mammary lymph node as shown in Figure 35. There was a smaller increase in total cells per hour. It was possible to collect lymph as frequently as desired as the location of the lymph collection bottle was easy to access.

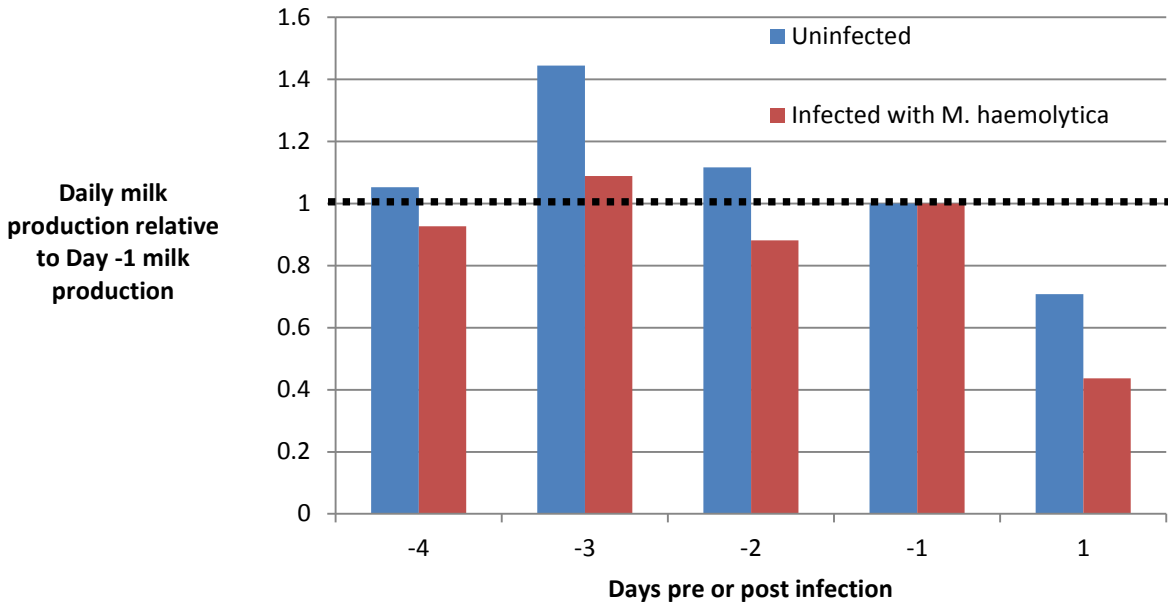


Figure 34: Milk yield for all ewes relative to milk yield immediately prior to infection

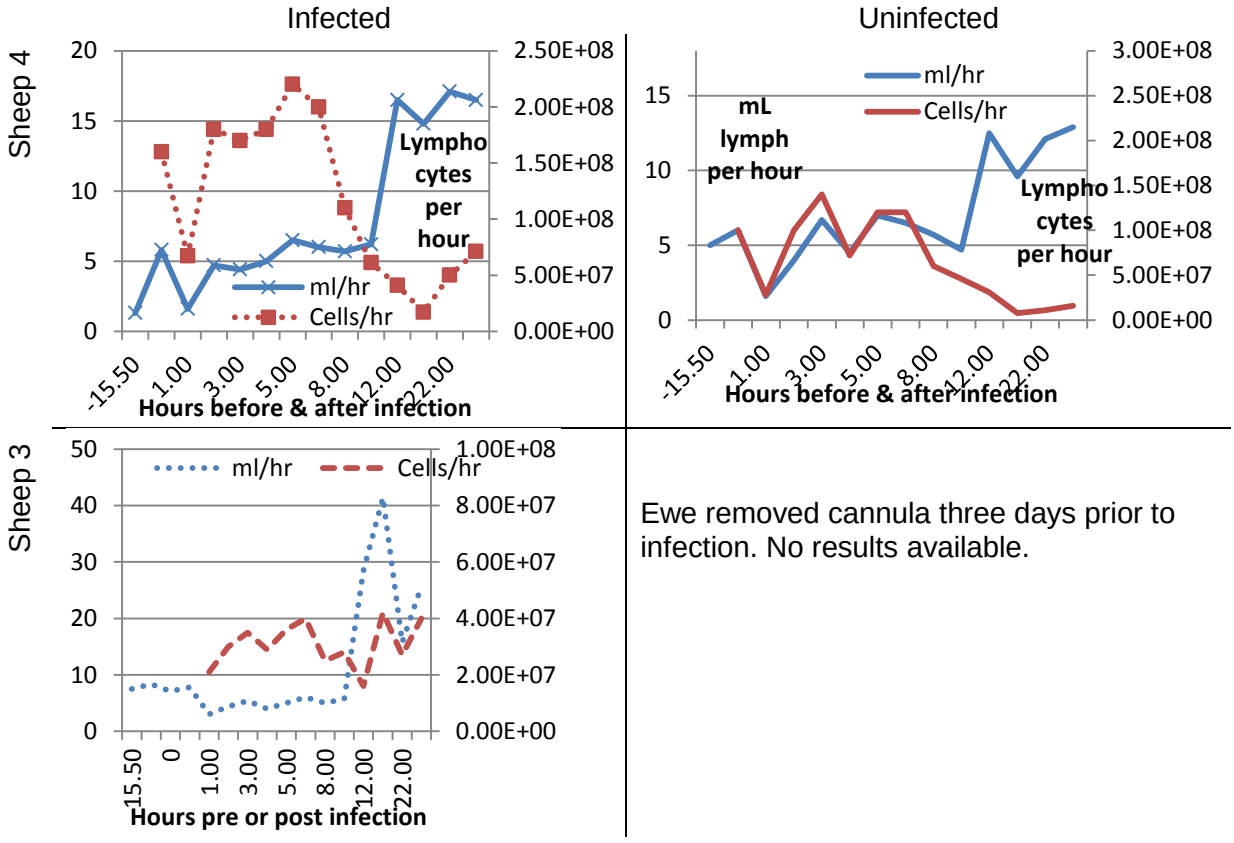


Figure 35: Example of lymph flow per hour and total lymphocytes per hour before and after infection with *Mannheimia haemolytica* in two ewes

4.7.2.1 Other samples collected

Ewes were euthanased at completion of this trial and samples from left and right udder, lymph nodes, teats and non-local lymph nodes were collected and stored in formalin for haematoxylin and eosin staining and another section frozen at -80 degrees Celsius for future immunostaining. Surgical sites on all ewes were healing well.

4.8 Incidence and causes of mastitis in eight pasture based sheep flocks

4.8.1 Merino and Maternal flocks (INF)

The percentage of gram negative and gram positive infections on INF properties is listed in Table 12: a breakdown of catalase and oxidase reactions in Table 13 and where species identification was achieved this is detailed in Table 15.

Table 12: Result of milk culture and Gram stains from bacterial colonies grown on SBA at time of weaning from INF flocks

Property/Year	Gram positive		Gram negative		No growth		Yeast/ Uncertain
2012	Number	%	Number	%	Number	%	Number (%)
INF1	4	2%	0	0%	158	97%	1 (1%)
INF2	5	3%	7	4%	185	94%	0 (0%)
INF3	3	2%	9	6%	141	92%	1 (1%)
INF4	2	1%	0	0%	150	96%	4 (3%)
Total	14		16		634		6
Average 2012		**2%**		**2%**		**95%**	**1%**
INF1	2	9%	5	4%	116	85%	4 (3%)
INF2	7	5%	2	1%	135	94%	0 (0%)
INF3	13	12%	10	9%	81	76%	2 (2%)
INF4	9	18%	3	6%	37	76%	0 (0%)
INF5	106	17%	65	10%	471	73%	12 (2%)
TOTAL	137		85		840		18
Average 2013		**13%**		**8%**		**77%**	**2%**
TOTAL (2012-2013)	**151**	**9%**	**101**	**6%**	**1474**	**84%**	**24 (1%)**

Table 12: shows the percentage of Gram positive versus Gram negative cultures grown from milk from Merino and maternal breed ewes at the time of weaning on INF properties. In 2012, these properties were all in Victoria or South Australia and all consisted of ewes on their first or second lactations. The maximum number of samples that yielded bacteria in that year was 8%. In the following year where INF5 was included in collections with a mixed age flock the overall rate of mastitis was considerably higher at 27% on that flock. It was however also higher on the other INF properties despite most samples still coming from ewes on first or second lactation. This demonstrates the challenges obtaining reliable reference data for mastitis if only a single year is used for this purpose given the inherent variation due to the organism, animal and environment interaction changing regularly.

Table 13: Percentage distribution of Gram positive and negative isolates by catalase and oxidase across breeds and year (oxi = oxidase, cat = catalase, incomplete = data missing for one or more tests)

	Gram positive					Gram negative				
Flock	Oxi + Cat -	Oxi + Cat +	Oxi - Cat -	Oxi - Cat +	Incomplete	Oxi + Cat -	Oxi + Cat +	Oxi - Cat -	Oxi - Cat +	Incomplete
2012 INF	3		7	30	7		13	3	17	20
2013 INF		6	8	45	3		15	1	20	
2012 PD	1	2	19	64	3		4		5	2
2013 PD			9	34	35		6	3	4	8
2014 PD		2	16	72			6	1	4	

4.8.2 Poll Dorset flocks

The percentage of Gram negative and Gram positive infections in PD properties are listed in Table 14 and where species identification was achieved this is detailed in Table 15.

Table 14: Result of milk culture and Gram stains from bacterial colonies grown on sheep blood agar at the time of weaning from Poll Dorset (PD) flocks

Property/Year	Gram positive		Gram negative		No growth		Yeast/ Uncertain
2012	Number	%	Number	%	Number	%	Number (%)
PD1	73	20%	6	2%	284	77%	8 (2%)
PD2	114	18%	11	2%	514	79%	8 (1%)
PD3	10	18%	4	7%	43	75%	0 (0%)
Sum 2012	197		21		841		16
Average 2012		**18%**		**2%**		**78%**	**1%**
PD1	75	12%	22	4%	511	83%	4 (1%)
PD2	171	17%	33	3%	805	79%	16 (2%)
PD3	8	9%	4	4%	79	86%	1 (1%)
Sum 2013	254		59		1395		21
Average 2013		**15%**		**3%**		**81%**	**1%**
PD1	83	17%	10	2%	394	81%	0 (0%)
PD2	79	19%	8	2%	324	79%	0 (0%)
PD3	54	19%	9	3%	220	78%	0 (0%)
Sum 2014	216		27		938		0
Average 2014		**18%**		**2%**		**79%**	**0%**
TOTAL (2012-2014)	**667**	**17%**	**107**	**3%**	**3174**	**80%**	**37 (1%)**

Table 14 shows the percentage of Gram positive versus Gram negative cultures grown from milk from Poll Dorset ewes at the time of weaning on PD properties. The percentage of Gram positive samples was almost double that of INF properties and Gram negative half that of INF. Overall 20% of milk samples collected from Poll Dorset ewes were positive for bacteria where a positive was considered counting more than one colony grown from a 10 µl loop of milk. Merino and maternal breed ewes were lower than this with 16% positive for bacterial growth. These results provide a contrast to cell count where PD flocks had 31% of ewes with cell counts greater than 500,000 cells per millilitre compared to 15% in INF properties and clinical mastitis rates in PD flocks were double that of INF (Table 1 and Table 2).

4.8.3 Identification of causes of mastitis at the time of weaning

At the commencement of this project it was projected that approximately 10% of samples overall would be culture positive. The increased number of samples that were culture positive meant that not all culture identifications were completed with 50% of samples identified to species level in 2012 and 25% in 2013. The overall ratio of samples based on Gram stain, catalase and oxidase tests however stayed relatively consistent over these years within PD and INF properties (Table 13). On all properties Gram positive, oxidase negative, catalase positive isolates were the most commonly identified group

Table 15: Bacterial identification from milk at the time of weaning. Total bacteria cultured from milk at weaning in 2012 = 248, 2013 = 545, 2014 = 243. Approximately half the bacterial samples were identified to species level in 2012, one quarter in 2013 and approximately 90% identified in 2014.

Bacterial Species	2012	(%)	2013	(%)	2014	(%)
Aerococcus viridans			**2**	1%		
Arcanobacterium pyogenes	1	1%				
Arthrobacter spp.	1	1%	5	3%		
Bacillus spp.	1	1%	4	3%		
Brachybacterium faecium			1	1%		
Brebibacterium spp.			1	1%		
Cellulomonas spp	1	1%				
Corynebacterium spp.	1	1%	5	3%	2	1%
Curtobacterium spp.			1	1%		
Dietzia			1	1%		
Enterococcus faecalis					2	1%
Enterococcus hirae					6	3%
Enterococcus spp.	4	3%	1	1%		
Exiguobacterium spp.			2	1%		
Jeotgalicoccus psychrophilus			1	1%		
Kocuria spp.			**8**	**6**%	1	0%
Luteimonas marinus			1	1%		
Mannheimia spp	**4**	**3%**	**9**	**6**%	**23**	**11%**
Microbacterium spp			1	1%		
Pantoea spp					2	1%
Pasteurella multocida			1	1%		
Planomicrobiium			1	1%		
Pseudomonas spp					1	0%
Pseudomonas spp (*)	2	2%	6	4%		
Serratia sp						
Staphylococcus aureus	3	2%	**10**	**7**%	5	2%
Staphylococcus capitis					1	0%
Staphylococcus chromogenes	**40**	**31%**	**18**	**13**%	**65**	**30%**
Staphylococcus devriesei	2	2%	4	3%	**11**	**5%**
Staphylococcus equorum			2	1%		
Staphylococcus haemolyticus			2	1%	2	1%
Staphylococcus pseudointermedius	1	1%			3	1%
Staphylococcus simulans	**51**	**40%**	**26**	**18**%	**56**	**26%**
Staphylococcus sp.			5	3%		

Bacterial Species	2012	(%)	2013	(%)	2014	(%)
Staphylococcus vitulinus			1	1%		
Staphylococcus warnerii			5	3%	2	1%
Staphylococcus xylosus					1	0%
Stenotrophomonas sp					1	0%
Streptococcus entericus					3	1%
Streptococcus gallolyticus			1	1%		
Streptococcus macedonicus			1	1%		
Streptococcus ovis	1	1%	5	3%	2	1%
Streptococcus parauberis					1	0%
Streptococcus pluranimalium	**8**	**6%**	**8**	**6%**	**18**	**8%**
Streptococcus suis	4	3%	4	3%	4	2%
Streptococcus uberis	2	2%	1	1%	2	1%
TOTAL IDENTIFIED	**127**		**144**		**214**	

Note that data included in this table includes clinical samples at weaning as per Table 8

The initial methodology of assessing coagulase negative Staphylococci by the ID 32 Staph API system (BioMerieux) was changed as there was significant disparity in correctly identifying the species using the API system, particularly for some of the novel genotypes found in this study. This required that molecular diagnostic methods be developed to achieve species confirmation. Genotypic characterisation has been a reliable method for identification of pathogens in milk and was therefore considered the optimal solution for further analysis of the isolates (Onni *et al.* 2010).

4.8.4 *In vitro* bacterial resistance to commonly used antibiotics

A Pseudomonas species was resistant to all antibiotics apart from Ceftiofur and Tetracycline. Table 16 demonstrates that the antibiotic with the highest level of *in vitro* resistance was penicillin and this is also the most commonly used antibiotic against sheep mastitis in many areas.

Table 16: Percentage of bacterial isolates susceptible to tested antibiotics

Antibiotic disk	% susceptible
Amoxicillin	97%
Cephalothin	97%
Erythromycin	98%
Penicillin G	90%
Tetracycline	100%
Trimethoprim/Sulphonamide	99%
Total samples tested	**242**

Not all resistant bacteria have been identified to species level however those that were included:

Enterococcus – resistant to Ceftiofur and Penicillin
Mannheimia – resistant to Penicillin
Staphylococcus simulans – resistant to Amoxicillin
Staphylococcus haemolyticus – resistant to Erythromycin
Bacillus sp – resistant to Erythromycin
Pseudomonas sp – resistant to all antibiotics except Cephalothin and Tetracycline.

It is possible that a number of these species may be contaminants, rather than mammary pathogens, particularly given we adopted a conservative approach to describing a gland as infected with 2 colonies per 10 µl of milk as the minimum.

4.9 Review of "on-farm" diagnostic tests for mastitis

Ewes with cell counts greater than 500,000 cells per millilitre were more likely to have bacteria cultured from their milk with 44% of these cultures positive for bacteria compared to 16% of ewes in the group with cell count less than 500,000.

Table 17: Comparison of bacterial growth in ewes with cell counts (ISCC) above and below 500,000 cells per mL

ISCC (10^3)	Number	Bacteria cultured	% Gram positive	% Gram negative	% Other & Mixed
>500	201	89 (44%)	60 (67%)	17 (19%)	12 (14%)
<500	775	123 (16%)	86 (70%)	18 (15%)	19 (15%)
TOTAL	976	212	146	35	31

Table 18: Comparison of "on-farm" tests for ewes with cell count above and below 500,000 cells per mL

ISCC	Total samples	Draminski average	Milkcheck average	Mas-D-Tec average	CMT – number positive	pH paper – colour change	Infrared temperature average
>500	201	381	5.1	1.8	175 (87%)	16%	34.6
<500	775	427	4.1	0.3	241 (31%)	1%	35.0

Table 18 demonstrates that all tests relying on conductivity (Draminski, Milkcheck and Mas-D-Tec) were useful at detecting mastitis, although they were less reliable than ISCC. The CMT correctly identified 87% of ewes with cell count higher than 500,000 with an additional 33% of ewes with cell counts less than 500,000 positive to at least a trace level on the CMT. pH paper was useful at confirming diagnosis of severe mastitis but of less use for identification of subclinical mastitis. Infrared thermography aided diagnosis of clinical mastitis but was less useful in subclinical mastitis.

Table 19: Bacterial cultures from ewes with cell count from 0 to 200,000 and 200,000 to 500,000 cells per mL

ISCC (10^3)	Total animals	Bacteria cultured	% Gram positive	% Gram negative	% Other & Mixed
200-500	251	41 (16%)	29 (71%)	8 (20%)	4 (9%)
0-200	523	83 (16%)	57 (69%)	10 (12%)	15 (19%)
TOTAL (0-500)	774	124	86	18	19

Table 20: "On-farm" milk tests from ewes with cell counts ranging from 0 to 200,000 and 200,000 to 500,000 cells per mL

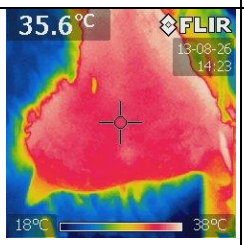
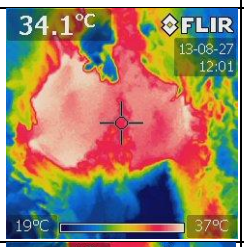
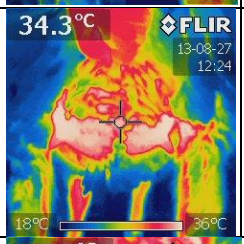
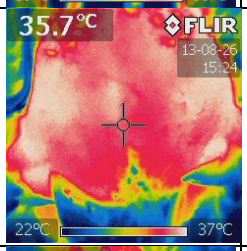
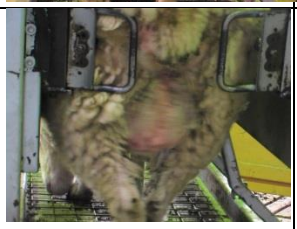
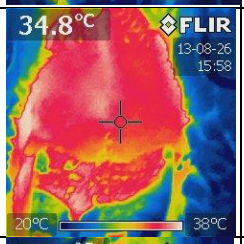
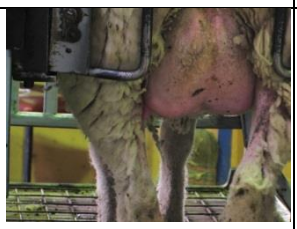
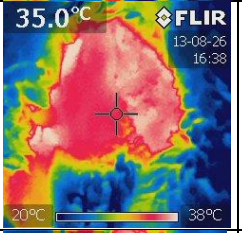
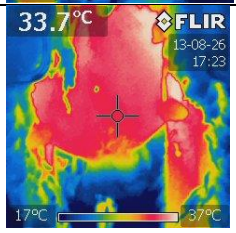
ISCC	Total samples	Draminski average	Milkcheck average	Mas-D-Tec average	CMT – number positive	pH paper – colour change	Infrared temperature average
200-500	251	444	4.1	0.4	133 (47%)	0%	34.9
0-200	524	420	4.1	0.2	400 (24%)	2%	35.1

Table 19 and Table 20 demonstrate that as cell count reduces below 500,000 cells per millilitre of milk there is a relatively even distribution of gram positive bacteria in samples although a lower percentage of Gram negative isolates. In the zero to 200,000 cells per millilitre group there was an increase in mixed infection, probably relating to some samples being contaminated. Table 20 demonstrates that the CMT is more able to pick up infection than conductivity measures, however may also result in some ewes providing positive results with low cell count and no apparent bacterial infection from culture results.

Figure 36 illustrates some examples of individual sheep udder images with their associated readings from conductivity, CMT, pH and infrared assessment. This illustrates the variability observed between animals with how effective varying tests are. The primary reason for using ISCC as the base measure for infection is that a substantial number of ewes may be infected with bacteria but not produce a positive milk culture and ISCC has been shown to be reliable in sheep (Paape *et al.* 2001; Winter *et al.* 2003)

Infrared imaging of the udder as shown in Figure 36, Animal 226 was difficult in some sheep due to the amount of wool on the udder. The presence of wool significantly dropped the temperature of the scan. Infrared imaging was useful in some ewes to demonstrate clinical mastitis as shown in animal 105 with the left mammary gland having a temperature of 36 degrees Celsius compared to the right of 35.2 degrees Celsius. However for sheep 447 there was only 0.1 degrees Celsius variation between the two halves. This variation in temperature of the udder may depend on the duration of the infection and damage to udder tissue as it may be higher or lower than expected.

Figure 36: Examples of individual sheep "on-farm" tests compared to individual sheep cell count (ISCC)

Animal ID & (Photo)	Infrared image (FLIR camera)	Standard photograph	ISCC (L; R)	Milk test results (Left,Right) (Draminski, Milkcheck, Mas-D-Tec, CMT, pH)
325 (38, 32)			31,000; 5,000	**Test** **Left** **Right** Dram 340 340 Milkch 5 4.8 Mas D 0 0 CMT 0 0 pH n n
488 (145, 136)			64,000; 88,000	**Test** **Left** **Right** Dram 410 400 Milkch 3.8 3.8 Mas-D 0 0 CMT 0 0 pH n n
226 (151 , 143)			497,000; 586,000	**Test** **Left** **Right** Dram 400 420 Milkch 4.1 3.8 Mas-D 0 0 CMT 0 t pH n n
447 (47, 39)			3,829,000; 39,000	**Test** **Left** **Right** Dram 410 390 Milkch 3.8 3.8 Mas-D 0 0 CMT 1 0 pH n n
420 (58,48)			1,229,000; 31,000	**Test** **Left** **Right** Dram 420 450 Milkch 3.8 3.4 Mas-D 0 0 CMT 1 0 pH n n
105 (68, 59)			588,000; 7,245,000	**Test** **Left** **Right** Dram 370 300 Milkch 4.5 5.3 Mas-D 0 2 CMT 0 1 pH n x
507 (75,66)			417,000; 6,167,000	**Test** **Left** **Right** Dram 400 400 Milkch 3.8 4.1 Mas-D 0 0 CMT 0 t pH n n

4.9.1 "On-farm" cell counting using the De Laval DCC

The De Laval DCC uses a small bench mounted unit to count cells in milk suspended inside a proprietary cartridge http://www.delaval-us.com/-/Product-Information1/Milking/Products/Milk-test--treatment/DeLaval-Cell-Counter/DCC/). This unit was used to compare results between the De Laval DCC and the Fossomatic Cell Count through a commercial laboratory (DTF Food Laboratories, North Melbourne). Immediately prior to sending samples for assessment at the laboratory milk was tested in the DCC and the results comparisons are shown in Figure 37.

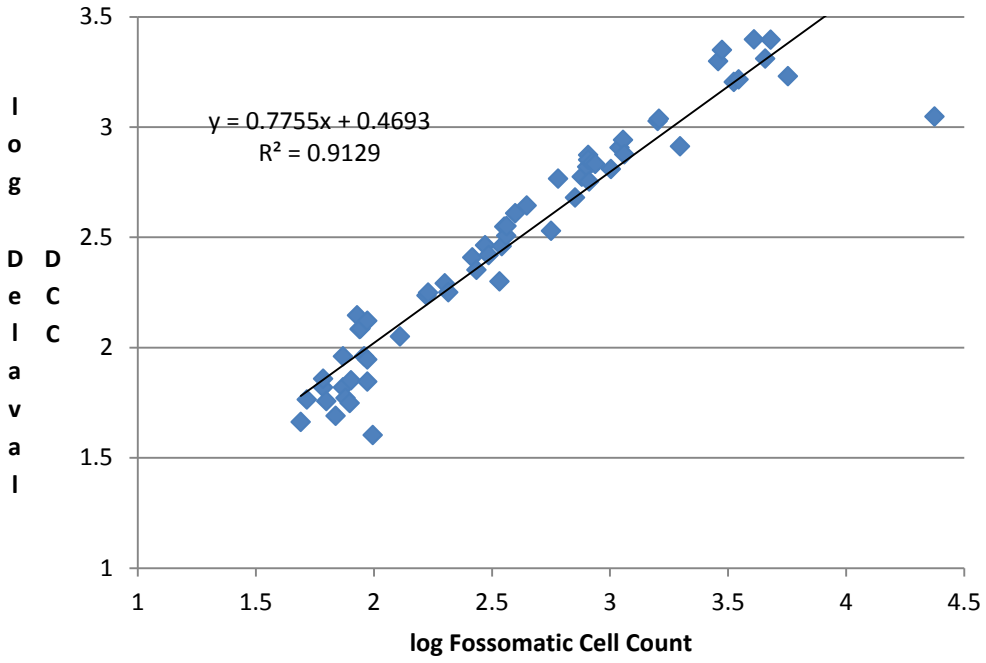


Figure 37: Comparison of Fossomatic Cell count versus De Laval DCC

A total of 61 samples were available for assessment with Fossomatic and De Laval DCC test. The Fossomatic cell count values ranged from 48,000 to 23,704,000 cells per millilitre of milk, with a median of 356,000 and average of 1,290,000 cells per millilitre of milk. The corresponding figures for the De Laval DCC were a minimum of 39,000 and maximum of 2,493,000 with median of 320,000 and average of 575,000 cells per millilitre of milk.

Results from Fossomatic and De Laval DCC were similar for milk samples with counts up to 200,000 cells per millilitre. Once counts exceeded 200,000 cells per millilitre the De Laval DCC produced lower cell counts than the Fossomatic and this was more pronounced when Fossomatic counts exceeded one million. This result could potentially be improved by diluting milk for use in the De Laval DCC to enable more accurate counting. A greater number of samples is required to further evaluate the use of this equipment on farm. The primary benefits of the equipment are very rapid turnaround times on results with less risk in transporting samples and the ability to retest samples if required. The volume of milk is also significantly smaller than the 20 mL required for Fossomatic testing, which also requires good mixing of the milk prior to collection of the sample in the cartridge.

5 Discussion

5.1 Meeting project objectives

This section covers achievement of the individual project objectives with individual project objectives written in *italics*.

1) *The primary objective of this proposal is to give sheep breeders and veterinarians a better understanding of the causes and importance of mastitis in the Australian sheep industry.*

5.1.1 Improvement in producer and veterinary knowledge of ovine mastitis

This project has substantially increased knowledge about the causes and extent of both clinical and sub-clinical mastitis of Merino, Poll Dorset and maternal lines of ewes in south-east Australia. Results from this project show for the first time that there is a direct cost in reduced weight gain attributable to mastitis measured at weaning in the grass based sheep systems assessed in this study. This reduction in weight gain was marginally greater in the progeny of Merino and maternal ewes (1.0 g/d per 1,000,000 cells/mL) compared to Poll Dorset progeny (0.7 g/d per 1,000,000 cells/mL), possibly due to the higher volume of milk that Poll Dorset ewes generally produce compared to Merino, particularly in the first 4-6 weeks post parturition. On the eight flocks in this study, higher rates of clinical mastitis than producers anticipated were observed during udder observation and palpation at weaning, illustrating the importance of ewe assessment at weaning We have identified novel bacterial causes of subclinical infection as well as confirming that in general the bacterial species in sheep and meat production systems are similar to those seen in sheep dairying with the predominant cause of subclinical mastitis being coagulase negative Staphylococci. It has also shown that clinical mastitis only identified a small part of the production loss in a flock due to the relatively higher number of ewes infected by subclinical disease. In many ways this is not dissimilar to what is observed with clinical and sub-clinical parasitosis, with similar skewed distributions of cell count and faecal egg count graphs. This information has been shared at the Australian Veterinary Association conference, Best wool/Best lamb conference and a range of other producer meetings. Further extension and education about the impact of mastitis on sheep flocks would further enhance the benefits of this study. .

2) *The project will also lay the foundation for some tools that may assist producers in reducing the incidence of mastitis using selection.*

5.1.2 Foundation for tools to assist in mastitis reduction using selection

The collection of milk samples from ewes on both INF and PD properties demonstrated a significant variation in cell count between ewes in each flock as shown in

Figure 8 with a skewed cell count distribution. Previous studies have shown that selection against elevated cell count in this type of population can reduce both sub-clinical and clinical mastitis in sheep dairy flocks (Rupp *et al.* 2009). Data from this study may be used to assess the total number of ewes required to produce an EBV for mastitis or numbers of ewes required for development of genomic breeding values in future studies as the variation in cell count across flocks had not previously been measured in grass based, extensive production systems.

The methodology for milk collection is now defined so that further samples can be collected in similar ways if more samples are required for genetic assessment. Devising satisfactory handling systems for milk collection from ewes is one of the most important parts of milk testing if it is to become common practice on properties. There are a number of

commercially available sheep handling systems that perform a similar role to the “bush-tiger” milker as they are capable of holding the ewe still while milk is collected without placing strain on an operator. Any producer wishing to integrate cell count into their selection criteria may also utilise the methodology used in this study to collect milk from ewes and integrate cell count into a current breeding value index.

3) *Collect over 3100 milk samples from sheep from INF and terminal sire flocks and analyse using Fossomatic cell counting and standard bacterial culture.*

5.1.3 Sample analysis and collection

This project successfully collected 3,900 milk samples from 3 Poll Dorset flocks and 1,648 milk samples from 5 Information Nucleus Flocks consisting of Merino and Maternal breed ewes for a combined total of 5,548 milk samples over south-eastern Australia. It was not possible to collect further samples from INF properties in 2014 as ewes were moved from Victorian and South Australian INF properties following the 2013 breeding season to flocks where weaning did not occur until 2015 when sampling was complete. The lack of older ewes in INF1, INF2, INF3 and INF4 may potentially bias the results from this study compared to average commercial flocks as some studies demonstrate increased clinical and sub-clinical infection in older ewes (Arsenault *et al.* 2008). Outside of the sheep milking industry this is the largest study of its kind in sheep primarily bred for producing wool and/or meat with significant geographic and temporal diversity. It would however be useful to collect milk from further flocks in different areas over a longer time to compare an increased number of sheep flocks and demonstrate impact at a local or regional level.

In common with many observational studies of health and production outcomes of livestock (see (Stevenson 2000) for an example) it is likely that the productive performance of the flocks that took part in this study was, in general, as good or better than in the general population of Australian sheep flocks. This said, with respect to the analyses identifying characteristics associated with average daily gain (Table 21), the important issue is not whether the individual ewes who were studied were ‘typical’ or representative of the source population, but whether the association between average daily gain and each of the exposures observed for the study participants are likely to apply to other groups. In general, the difficulties of applying results from one group of subjects to another is minimal for investigations of physiological associations as it was for this particular study (Elwood 2007).

Fossomatic cell counting was successfully used to assign cell counts to almost all ewes with the exception of ewes with clinical mastitis where milk clotted and could not be assayed using this technique. Standard bacterial culture was useful to identify bacteria to genus level however molecular methods were required to definitively identify a number of bacterial species. The use of some standard bacterial identification kits may result in a non-definitive bacterial diagnosis due to lack of some bacterial species from sheep in the relevant database/s, which are in some cases based primarily on human pathogens. Identification of milk samples to the species level thus is more expensive but in most cases only required for particular epidemiological investigation. Due to the increased number of bacterial samples collected during this trial and increased time and cost for diagnosis, not all samples were identified beyond basic Gram staining and biochemical testing. There was minimal antibiotic resistance in subclinical mastitis pathogens although 10% of samples showed resistance to penicillin, which is the most common used antibiotic against mastitis. All bacterial samples have been stored at -80 degrees Celsius for future reference.

4) *Collect all submitted clinical mastitis samples from across Australia provided by sheep producers and assess using standard plating techniques – likely to be over 200 samples (this is highly seasonal and difficult to predict).*

5.1.4 Clinical mastitis samples

In a number of previous studies it has been challenging to access large numbers of clinical mastitis isolates. Telephone conversations with producers across the width and breadth of Australia indicated that significant problems existed in some flocks during the project, however it was difficult to get samples submitted to the laboratory as these problems were often identified quite late in the disease outbreak. In a previous study we visited each property on multiple occasions to aid the collection of milk from ewes with mastitis. In the current study we used a mail out and handover of sample collection vials at meetings and conferences with more than 1,500 sample containers sent (20-30 vials per property). A total of 49 samples were returned from which 27 samples were grown. This demonstrates the challenge of identifying mastitis pathogens. Results from this limited return however were similar to previous work in PIRD V04 with the most common pathogens being *Mannheimia* sp, *Staphylococci* and a range of other pathogens. This also agrees with historical data from NSW DPI laboratories where *Mannheimia* were the most commonly identified bacteria (pers. Comm. Dr J Plant). If we were to attempt to collect clinical samples again we would do so via visiting properties as sample return rate was significantly better than in this project.

5) *Provide MLA with guidelines to assist producers in treating clinical mastitis in their flocks to maximise chances of therapeutic success.*

5.1.5 Guidelines for treatment of clinical mastitis

The primary guidelines for treatment of clinical mastitis are:

- Identify mastitis as early as possible and commence appropriate treatment based on any pre-existing knowledge of mastitis pathogens on property in conjunction with your local veterinarian
- Immediately prior to treatment collect a milk sample from infected gland for culture and sensitivity – this can be frozen if only one or two ewes are infected and then used for testing if more ewes show signs of mastitis. It is important to be aware that some sheep mastitis pathogens can be zoonotic hence care is required in the collection and handling process. Gloves should be worn during milk collection and treatment.
- There is limited resistance to penicillin in *S. aureus* however in some flocks there may be penicillin resistance. Identification of the pathogen and its resistance to commonly used antibiotics can aid treatment and also identify potential zoonoses issues on-farm.
- Stock with clinical mastitis and their young should ideally be removed from the flock into a hospital area where possible. Ewes should be milked out and treated as rapidly as possible. Lambs will often refuse to drink from infected glands hence ewes require at least twice daily milking to remove this milk. Lambs may require supplementary feed due to the reduction in milk quantity and quality. While lambs can be fostered this does increase the risk of bacterial transfer to the foster dam, particularly if there is any teat injury.
- Matching feed to ewe requirements can avoid over/underproduction of milk and subsequent mastitis. Many examples of mastitis throughout this project were based around supplementary feeding or containment zone feeding. It is often not possible to remove stock from these zones so evaluating feed and water sites and diet may help to identify areas to reduce problems.
- Treatment of ewes via antimicrobial treatment should occur as soon as possible. The only drug routes registered for use in sheep are intravenous, intramuscular or subcutaneous. A veterinarian may choose to supply intramammary products “off-label” with an associated elevated withholding period where appropriate. Treatment should be based on bacterial culture

and sensitivity and should continue for at least 3 days in most instances to resolve infection. During a mastitis outbreak stock should be checked at least daily due to the rapid onset of disease.

6) *Review the impact that sub-clinical mastitis has based on weaning weight and cell count and if any sires produce progeny that are more likely to develop an increased cell count and/or clinical mastitis.*

5.1.6 Impact of sub-clinical mastitis on weaning weight

This project has demonstrated for the first time in Australia that not only does clinical mastitis result in lamb weight reduction, but that subclinical mastitis can also cause a loss of weight over lactation. For each increase in cell count by 1,000,000 cells per ml in this study there was an associated reduction in lamb growth rate of one gram per day i.e. For a Merino ewe that had a cell count of 3,000,000 at dry off her lamb would be expected to weigh 3 grams per day less than a lamb from ewe with low cell count – presuming a weaning date 100 days post birth this would equate to 300 grams less than a lamb from a dam with low cell count. At current prices this equates to a loss of approximately $1 per lamb. This figure doesn't take into account the losses of ewes and lambs from clinical mastitis which are likely to be more substantial. All data for the flocks from this trial will be provided to SGA and the CRC to allow potential future use to establish a research ASBV for mastitis and investigate sire impact on mastitis.

7) *Describe the incidence and causes of mastitis in more than 7 representative flocks across Australia to indicate the importance of mastitis to the sheep industry.*

5.1.7 Incidence and causes of mastitis in 8 flocks – how important is mastitis?

The flocks involved in this study are similar to many across Australia with the exception of an increased percentage of British Shortwool animals in the study. Merino and maternal breeds dominate the Australian sheep population hence data from INF animals is applicable to the majority of sheep breeders across Australia. Data from the Poll Dorset flocks is highly valuable to short wool British Breed flocks as anecdotal evidence for decades has suggested they suffer an increased rate of mastitis compared to other breeds. In this study the British Breed flocks consisting of Poll Dorsets had almost double the rate of both clinical and sub-clinical mastitis compared to INF ewes. It is however important to note that there was substantial variation with some INF properties having higher cell counts than PD flocks in different years and ewe age and environmental conditions may be a factor in this. From discussions with a number of producers throughout this study a common link with increased levels of mastitis in ewes was drought feeding. Presuming global warming leads to more variable climatic conditions this is likely to increase, hence further understanding of mastitis epidemiology and preventive strategies are likely to be more important in coming years. In all flocks in this study mastitis impacted on the production and welfare of animals although the impact varied tremendously. The causes of mastitis varied from flock to flock but clinical mastitis was most commonly caused by *Mannheimia* species with a number of other pathogens including *Staphylococcus aureus*, *Bacillus* and a range of coagulase negative Staphylococci. Subclinical mastitis was most commonly caused by coagulase negative Staphylococci or Streptococci, with a range of species involved. The impact of mastitis on all of these flocks was significant, although no financial analysis of the results has as yet been produced. This study did not assess the loss rate of lambs due to clinical mastitis during lactation as it would have required regular monitoring of a smaller research flock. This data would be useful to aid the assessment of overall economic cost of the disease.

5.2 Milk collection

5.2.1 Infrastructure

The time required for milk collection from ewes in the Bush Tiger Milker compared well to previous milk collection techniques; collecting milk from sheep in a race or collecting from ewes on the shearing board. It did not have the associated occupational health and safety issues associated with these methods of collection and presumably reduced sheep stress as each animal was restrained in the handling crate rather than restrained via a person. The ability to have a standard work platform at each property reduced the risk of changes in phenotype measurement due to different handling facilities and also improved staff safety. While there is other sheep handling equipment available for sheep restraint the Bush Tiger Milker is very successful in offering excellent visualisation of the udder with minimal repetitive strain on back muscles. Collecting milk from ewes in the standing position also reduces dust and wool falling into collection tubes which can be difficult to control when collecting milk across a shearing board with the ewe held in standard shearing commencement position. This is particularly important for bacterial culture. The ability to close the sides of the trailer was also highly beneficial in inclement or dusty conditions to reduce contamination of samples. For purposes of future research a standard restraint device would be ideal to reduce variation on phenotypic scoring, given different restraint devices may change udder/teat visualisation and potentially bias results. This is rarely mentioned in papers but an important part of doing milk collection safely and accurately.

5.2.2 Occupational health and safety during milk collection

The collection of milk from sheep has similar occupational health and safety issues as for all repetitive livestock procedures. There is risk involved from direct injury with animals, in regular repetitive movements and also with zoonoses (Franklin 2001). Where large numbers of milk samples are to be collected an appropriate animal handling device should be used to avoid repetitive strain injury. This also reduces the chances of direct trauma from sheep if they are kept in standard handling systems. Gloves should always be worn when milking sheep to avoid transfer of bacteria from human skin onto the sheep and vice versa. While the majority of bacterial samples collected during this trial are of relatively low risk to humans the identification of pathogens such as *Streptococcus suis* that can cause meningitis and sepsis demonstrate the importance of proper personal protection (Wertheim *et al.* 2009; Palmieri *et al.* 2011). Other pathogens such as coagulase negative staphylococci and *Staphylococcus aureus* may also cause serious disease, particularly is they carry bacterial resistance genes and in young, old or immunocompromised individuals (Martins and Cunha Mde 2007; Dahms *et al.* 2014; Blanchard *et al.* 2015).

5.3 Identification of bacteria causing clinical and sub-clinical mastitis

A large range of bacteria are capable of causing clinical and sub-clinical mastitis and this topic has been reviewed extensively (Bergonier *et al.* 2003). The development of new molecular techniques over the past twenty years has however significantly changed the identification of a number of bacteria and led to renaming and redistribution of a number of species such as reclassification of *Pasteurella* to *Mannheimia (Angen et al. 1999)*. An issue identified during the course of this study was that some standard laboratory techniques do not result in good identification of species due to a lack of data contained within human bacteriological databases. For this reason we utilised molecular techniques for identification of bacteria in preference to biochemical. The results from our study for identification of samples due to clinical mastitis were similar to previous reports, although there is significant variation worldwide in the relative importance of *Mannheimia* species compared to

Staphylococcus aureus (Quinlivan 1968b; Watson et al. 1990b; Bergonier et al. 2003; Mork et al. 2007; Arsenault et al. 2008; Omaleki et al. 2010; Omaleki et al. 2016).

Previous studies of ewes in the New England region of Australia and in the Rangitikei, Feilding, Southern Hawke's Bay and Wairarapa districts in NZ have demonstrated that *Staphylococcus aureus* was the most common pathogen involved in clinical mastitis, although these studies occurred in the 1980's and 1960's respectively (Quinlivan 1968b; Watson *et al.* 1990b). In the New England *S. aureus* accounted for 40% of all clinical infections and in NZ it accounted for 16%, with a higher level of *E. coli* infections in the NZ study (16%) compared to New England (0%) and in this report (2%) (Quinlivan 1968b; Watson *et al.* 1990b). (Quinlivan 1968b) noted that there was significant variation in the time of year of milk collection and also with environmental conditions such as extreme cold and rain. Experience in our laboratory is also similar to this with dramatic variation both within and between properties and regions. This is perhaps one of the most important things to understand in managing mastitis; that each property is different and applying a "one size fits all" approach to management is less likely to be unsuccessful. Collection of milk for bacterial culture and sensitivity is required to maximise the outcomes of any treatment or prevention campaign.

The relative distribution of sub-clinical mastitis across different genera in this study was similar to reports in previous reviews of mastitis (Bergonier *et al.* 2003). The most common cause of mastitis was coagulase negative staphylococci with a total of 11 species recorded. The majority of these species had been previously identified, with the exception of *Staphylococcus devriesei* which has been identified in cattle, but not sheep (Supre *et al.* 2010). Similarly, Streptococci were the second most commonly identified cause of elevated cell count with one new species identified, *Streptococcus pluranimalium* (Devriese *et al.* 1999).

Relatively few bacterial isolates were resistant to the antibiotics tested with 10% of isolates in 2012 resistant to penicillin. In general, extensively raised sheep receive very little antibiotic treatment so this finding is not unexpected compared to higher rates in dairy cattle (Pengov and Ceru 2003). It does however suggest that milk samples should be monitored for sensitivity to evaluate change over time and enable optimal treatment of ewes as some pathogens of sheep do demonstrate significant resistance (Sanciu *et al.* 2013).

5.4 Identification of sub-clinical mastitis through cell count

During this study we were able to milk approximately 200 sheep per day as well as record phenotype data. If the only test required for sheep was to collect a cell count then 250-300 sheep may be able to be tested per day, particularly if lambs were removed at least two hours prior to milk collection commenced. It is not possible to remove lambs from ewes the previous day as this will increase the cell count obtained (Tatarczuch *et al.* 2000; Tatarczuch *et al.* 2002).

We decided to utilise individual sheep cell count as our primary method of identifying subclinical mastitis due to repeat sampling of ewes demonstrating the number of ewes that may be infected and yet not produce positive bacterial culture results (Winter *et al.* 2003). Other organisms such as *Mycoplasma* and *Acholeplasma* would also not survive culture conditions from our study design along with anaerobic bacteria. The Fossomatic cell count has previously been identified as an accurate test compared to the direct microscopic cell count (Gonzalo *et al.* 2003). The addition of a Bronopol table to each milk vial allowed an increased time from collection until cell counting while maintaining accuracy, particularly if milk was refrigerated (Gonzalo *et al.* 2003). While not required for our study this may be useful if it is not possible to submit milk samples on a weekly basis.

The cut point of 500,000 cells per millilitre was chosen in this study for subclinical mastitis as this represented the combination of both halves of the mammary gland. At an upper level of 250,000 for uninfected halves this equates to a cell count of 750,000 in the infected half given very few sheep have both halves with elevated cell counts simultaneously. Other authors have used cut points ranging from 200,000 to 1,500,000 cells per millilitre (Bergonier *et al.* 2003).

5.5 Lymphatic cannulation model

Previous studies have assessed response to *Mannheimia haemolytica* infection using post mortem sampling or changes in blood and milk (Fragkou et al. 2008). This requires a relatively large cohort of sheep to be used in the study and also sequential sampling to achieve an understanding of the process of infection and the body's response to it. Lymphatic cannulation of the mammary lymph node allows regular lymph sampling along with milk and blood sampling to monitor changes in the infective process without sacrificing animals. Recent advances in RNAseq allow an assessment in the change in transcript expression over time (E et al. 2016; Suarez-Vega et al. 2016). The lymphatic cannulation model allows assessment of this using blood, milk and also lymph to understand this change over time and improve understanding of the acute phase of infection and over a longer duration, depending on the pathogen used. This can significantly reduce the numbers of experimental animals required for a study and improve on 3R (refine/reduce/replace aversive procedures) animal welfare outcomes.

This sheep model of mastitis has demonstrated that the novel method of cannulation allows collection of lymph for an extended period of time from both sides of the udder so could be used for assessment of acute or chronic infections. This could be used to assess pain relief, antibiotics, vaccines or the impact of other compounds at the level of the udder. This technique has significant potential to improve our understanding of sheep mammary immunobiology and also as a potential model for other animals or people. It may be used to collect milk, blood and lymph (either afferent or efferent) over the same time period to monitor the time course of disease or treatment.

One of the clear outcomes from this trial was the rapid nature of infection with an Australian field isolate of *Mannheimia haemolytica*. Within 12 hours of placing approximately 2,000 cfu 2 mm inside the teat clinical signs of mastitis were present and by 24 hours sheep had all the typical signs of mastitis. This illustrates the importance during outbreak scenarios of producers checking stock regularly if they are to note disease early enough to successfully treat ewes to minimise mammary tissue damage.

While the four ewes in this study were selected from a property using milk culture and on-property cell count, one ewe arrived at the laboratory animal house with a coagulase negative staphylococci infection. This was recognised at the end of the first week of housing when the first group of cell counts were performed; however no bacterial colonies grew on the sheep blood agar plate. For future studies we suggest taking multiple samples to be confident sheep for trials such as this are not infected with bacteria at the time of the study given the importance of each sheep in the study. Previous research has demonstrated the variability in culture of CNS from ewes with mastitis (Winter *et al.* 2003).

5.6 Selection to reduce mastitis in sheep flocks

5.6.1 Phenotypic measures of selection

The phenotypic measures used in this study from (Casu *et al.* 2006a) showed that sheep with placement of their teat at the mid point of the scoring system (4-5 and 7-8 o'clock position on the udder) had significantly lower cell counts than teats with orientation more

towards horizontal and higher on the udder (9 o'clock). One other study overseas has assessed teat conformation using a similar model and found that vertically oriented teats at the 6 o'clock position were more likely to have higher cell count, however this was with sheep in housed conditions hence this may be due to greater pathogen load and ewes resting in soiled areas (Grant *et al.* 2016). Increasing teat length and width were also associated with increased cell count however these are associated with increased ewe age and this has been shown to be associated with increased cell count in some studies (Watkins *et al.* 1991).

5.6.2 Use of genetics and genomics for selection

Assessment of sheep milk cell count in flocks in this study demonstrated a heavily skewed curve. This curve is similar to that observed in egg count of parasites in sheep faeces (McManus *et al.* 2014). Previous selection studies on high and low divergent cell count lines of dairy ewes resulted in a reduction in both subclinical and clinical mastitis and maintenance of milk production (Rupp *et al.* 2009). Previous literature reviews have also recommended sheep mastitis as being amenable to genetic improvement via selection (Davies *et al.* 2009; Riggio *et al.* 2010; Bishop 2015), in a similar way to dairy cattle (Rupp and Boichard 1999; Rupp *et al.* 2000; Rupp and Boichard 2003). To receive an Australian Sheep Breeding Value (ASBV) for parasite worm egg count (WEC) producers must currently submit a WEC value for animal in a group on a particular date. Similarly, producers could provide a milk cell count (MCC) at weaning date for ewes if an ASBV for MCC was developed. This could then become part of a selection index in conjunction with the current range of traits. Data from this study and pre-existing cell count data from PD1 and PD2 will be uploaded to SGA for potential future use in developing a prototypic ASBV. It is however likely that a larger number of milk samples may be required to develop an accurate ASBV and this may be possible via the INF/MLA resource flock or other interested producers. The primary problem with selection against mastitis using ASBVs is that a ram requires progeny to produce a high accuracy breeding value. This means that to obtain a high accuracy a sire needs daughters with at least one recorded lactation. Presuming a ewe first gives birth at two years this means the accuracy of a sire will be relatively low until two and a half to three years after he is first joined and he will probably be mated with ewes for at least two years of lamb production.

The cattle dairy industry has similar issues with genetic selection for resistance to mastitis as already noted. The advent of genomic technology has allowed significant improvements in selection accuracy of dairy bulls for difficult to measure traits, including mastitis (Larkin *et al.* 2012; Hayes *et al.* 2013). Genomic technology is also available in the sheep industry for hard to measure traits including intramuscular fat in meat, meat tenderness and lean meat yield (http://www.sheepcrc.org.au/genetic.php). Data collected from this study from ewes on INF properties have already had SNP data collected and analysed. Most ewes on PD properties also come from sires that have had SNP data collected but have not been blood tested. With an adequate size data set this data could be used to establish prototypic genomic breeding values for sires that could be assessed within a few months of birth. This will require further research and possibly further milk samples but has the potential to improve sire selection for reduced mastitis in the future ewe flock and removal of potential sires that are more likely to increase mastitis in the flock. Data have been returned to the sheep CRC from this study to go towards future analysis.

5.7 On-farm testing

This study produced similar results for on-farm tests of subclinical mastitis to a study in New Zealand (McDougall *et al.* 2001b). California mastitis test proved to be the best indicator of early mastitis although could result in treating some sheep with low cell count and no bacterial growth. If producers were to treat ewes based on any of the conductivity tests then

it was highly likely that ewes were subclinically infected, however these tests missed significant numbers of ewes that had cell counts greater than 500,000 and also were bacterial culture positive. In addition to electrical conductivity measures and California mastitis test we also evaluated use of an infrared camera. Two previous studies have used infrared to assess the ability to diagnose subclinical infection (Martins *et al.* 2013; Castro-Costa *et al.* 2014). The two studies came to differing conclusions as to the merit of using infrared for subclinical testing. In our work we were only able to assess ewes on a single day. The camera was able to distinguish clinical cases easily however it lacked the ability to determine low level sub-clinical infection in the flock it was tested on. Using the camera regularly via an automated walk over imaging system may improve its ability to diagnose mastitis however this would be challenging in an extensive management system. We also compared the De Laval DCC instrument to Fossomatic cell counting and this provided similar results up to 250,000. Further trial work is needed with this device to evaluate its accuracy as we only tested a small number of samples. For producers with minimal experience with mastitis any of these "on-farm" tests are likely to improve diagnostic capability for early clinical mastitis although the Fossomatic cell count remains the more accurate for genetic selection purposes.

While there may be potential to use infrared imaging in dairy systems the application of this technology in meat and wool systems appears limited unless stock are regularly walking over platforms where the udder can be visualised adequately. New phone based apps using an additional infrared camera may also enhance useability of this type of device and make it more portable for the paddock as an extra tool for mastitis assessment.

All of the devices used in this study were purchased from commercial providers with a significant range in pricing of the devices. For the inexperienced stockperson all of these devices would add greater certainty to the diagnosis of mastitis to then allow rapid treatment, potentially prior to clinical mastitis being diagnosed on farm. This may significantly enhance the likelihood of successful treatment improving welfare and production of the ewe and her lamb/s. The CMT offers the cheapest and best chance of detecting mastitis early, although it is more open to operator interpretation and may result in increased sheep being treated than the other tests. It also requires the most education to ensure optimal use compared to relatively simple and objective use and results from the other devices.

6 Conclusions/recommendations

6.1 Future research and development

6.1.1 Vaccination research

There are no vaccines currently available in Australia for sheep mastitis. Once an outbreak of mastitis occurs on a property it can be very challenging to manage as sheep must be checked at least every day if treatment is likely to be successful. An effective vaccine may reduce this requirement and be useful insurance in flocks at higher risk such as those lambing under intensive grazing or drought-lots. Vaccines exist for Staphylococcal mastitis overseas and previous work has demonstrated that autogenous *Mannheimia* vaccines can reduce clinical disease. Commercialisation of these, or other novel vaccine antigens, would be of significant benefit to producers with flocks at higher risk of mastitis.

6.1.2 Anti-inflammatory and antibiotic use

There is very little information available to veterinarians or owners of small ruminants as to efficacy of antibiotic and anti-inflammatory use in small ruminant mastitis. The recent registration of anti-inflammatory products for use in small ruminants is useful, but data on

benefits to controlling pain in mastitis may enhance their use and sheep welfare. Similarly, further research and registration of intramammary antibiotics would be useful if these are shown to be beneficial. Further data on pharmacokinetics of commonly used antibiotics for mastitis in sheep may also improve therapeutic outcomes from improved treatment regimes.

6.1.3 Genetics and genomics

The cell count data from milk samples in this study demonstrate a skewed distribution similar to previous reports indicating that selection to reduce the impact from mastitis should be possible (Barillet *et al.* 2001; Conington *et al.* 2008; Davies *et al.* 2009; Riggio *et al.* 2010; Bishop 2015). Heritability of milk cell count in dairy sheep has been reported ranging from 0.04 to 0.12 depending on what stage of lactation is assessed (Barillet *et al.* 2001). Further research is needed to review cell counts against sheep genome data through the sheep CRC or investigation of development of a prototypic ASBV through SGA.

6.2 Application of project outcomes and implications for industry

There are a number of important outcomes and implications for industry from this project:

- Mastitis is a significant disease within the Australian sheep industry with liveweight losses associated with clinical and subclinical disease in lambs
- Although subclinical mastitis is not able to be detected on-farm without further diagnostic aids, it still reduces lamb growth rate.
- For properties with problems with mastitis, the selection of replacement animals from an identified group of low cell count ewes may reduce the incidence of both clinical and subclinical mastitis.
- The impact of mastitis varies significantly from year to year and property to property. This can make management of the disease challenging as it can disappear and then suddenly reappear in an outbreak some years later.
- There is no current vaccination for mastitis in the Australian sheep flock and this is an important area for further research.
- A range of bacteria cause clinical mastitis and some of these are resistant to common antibiotics. Milk from sheep with mastitis should be cultured and sensitivity of bacteria to common antibiotics determined in conjunction with your veterinarian to treat with the optimal antibiotic.
- Bacteria that cause clinical mastitis can cause a sheep become severely ill in less than 24 hours. On properties with an outbreak of disease treatment outcomes can be maximised by treating stock early in the course of disease with antibiotics. Stock need to be checked at least daily to allow early identification and maximise chances of recovery.
- The most common signs of clinical mastitis in ewes are lameness and inappetance. Closer inspection will show abnormalities in the udder and teats and usually changes in the milk.
- Further research and availability of antibiotics and anti-inflammatories may improve outcomes for sheep with clinical mastitis as there are fewer products registered for use in sheep.
- There is significant potential to further evaluate genetic and genomic approaches to reduce the economic and welfare impact of mastitis in sheep flocks. This is a key area for future research.

6.3 Development and adoption activities

6.3.1 Communication throughout project

Throughout this project there has been significant communication with industry at conferences, through media and at the local producer level. This has included presentations at the Australian Veterinary Association conference, Best Wool/Best Lamb conference in Bendigo and SW Prime Lamb producers group. Less formally a number of producers across Australia have requested further information on mastitis, mostly when their flock has been experiencing losses.

6.3.2 Further extension

The production of a number of scientific papers from this work will further enhance extension of information to the veterinary community. Articles describing the projects outcomes in "Feedback" magazine or similar would aid extension of the knowledge generated from this project and ensure producers are aware of what to look for with mastitis and its welfare and financial consequences.

Further collection of milk cell count data in different regions would both enhance epidemiological data and provide valuable extension material for local sheep producers similar to programs for other diseases such as parasitosis.

7 Key messages

7.1 Changes to practice

7.1.1 Wet/Dry

Clinical mastitis is a relatively common disease of sheep, although is mostly observed in a relatively small percentage of a flock. In this study at weaning 8% of Poll Dorset ewes had clinical mastitis at weaning and 4% of Merino or Maternal breeds had clinical mastitis at weaning. Very few of these ewes had obvious clinical mastitis prior to physical examination. Ewes should be assessed at weaning and/or marking for signs of clinical mastitis to allow closer observation and/or treatment of those with mastitis. Historically wet/dry of ewes was a relatively common practice that recently seems to have lost favour. Checking ewes at weaning and/or marking can identify a significant number of ewes that will most probably be unable to rear a lamb or lambs in the following lactation. While removing these ewes will not dramatically improve overall selection within the flock against mastitis due to the large number of ewes with subclinical mastitis, it does reduce the overall welfare impacts of the disease as well as improving production indices in the following season.

7.1.2 Collecting milk from ewes with mastitis

One of the challenges in disease outbreaks is to identify the disease causing agent early in the outbreak and institute an appropriate treatment program. Depending on what pathogens are causing mastitis it may take several samples to identify the primary agent of disease. If producers keep a small supply of sterile collection vials in the vehicle or bike used for stock work, samples can be collected and frozen for analysis if more ewes subsequently become ill. It is important to also carry a small screw top container with 80% alcohol to clean the end of the teat to avoid contaminated samples. This avoids having to wait to establish a diagnosis and also allows the property to do a survey of all causes of mastitis at the end of lactation. While there is a lower success rate for growing bacteria from frozen samples compared to fresh ones, the success rate is far better than if samples are not collected.

7.1.3 Identifying and treating mastitis

A program for treating mastitis should always be undertaken between the producer and their local veterinarian. A few critical elements to treatment however are quite general:

1. Identifying mastitis – ensure people monitoring stock know the typical signs of disease. Where stock are being "trail fed" grain this will often appear as a lame ewe that is unwilling to walk to the grain trail or ewes that appear generally "flat". This can be confused with foot abscess depending on the time of year.
2. Treat early in the disease – don't wait to treat as an apparently healthy sheep can be severely ill or dead within 12 hours with bacteria such as *Staphylococcus aureus* and *Mannheimia haemolytica*. There is some bacterial resistance to penicillin in sheep mastitis but this is not widespread but collecting a sample for testing can
3. Where possible remove ewes and their lambs from other sheep if they have mastitis – particularly if they are in a confinement area or feedlot. Both the ewe and her lamb or lambs are a potential source of contamination and require extra care until the disease is resolved.

7.1.4 Selecting ewes based on phenotype

Evaluation of phenotype during this study has shown that in general ewes that have teats placed on the side of the udder at a "nine-o-clock" position are more likely to have subclinical mastitis than those with teats placed more vertically at a "7-o-clock" position. Selection against breeding replacements and rams from ewes with teats placed horizontally maybe a useful tool to reduce the overall incidence of mastitis. Similarly removing ewes with poor suspensory ligament function will also reduce the overall cell count of the flock and decrease mastitis.

7.1.5 Future potential of genetics and genomics

While it is not yet commercially available, there is significant potential for genetics and genomics to reduce the impact of mastitis on flocks. This will take further research; for flocks which have experienced problems with disease in their ewes assessing dams of potential sires for cell count and utilising sires from low cell count ewes may reduce mastitis in the flock.

7.2 Benefits to producers

The primary benefits to producers from this study is a much greater knowledge of mastitis in sheep in south east Australia. This project identifies that both clinical and sub-clinical mastitis are significant problems in some years for some flocks and common problems for other flocks. Producers and their veterinarians should consider mastitis as a potential disease where they have increased ewe losses during lambing and/or poor weaning rates compared to pregnancy scanning expectations. To maximise outcomes from mastitis investigations producers should work with their veterinarian to collect milk samples from ewes with clinical mastitis for culture and sensitivity of the bacteria.

If sheep management becomes more intensive with challenges of changes in climate it is likely that mastitis will become a greater problem. This project has identified that further work is needed to investigate potential vaccination strategies for control and selection tools to identify rams likely to sire ewes with reduced risk of developing mastitis. Where mastitis has been observed in the flock during lactation it is useful to check udders at weaning to remove ewes that have had clinical mastitis. These ewes are unlikely to effectively rear lambs in future years, particularly twins or triplets hence this will improve animal productivity and welfare.

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9 Acknowledgements

This project would not have been possible without the assistance of a large number of groups and individuals. Firstly, I would like to acknowledge the assistance of all the farm personnel on the eight properties where we collected milk from sheep at the time of weaning. Without the assistance of these staff and their dogs this project would not have been possible. I would also like to thank those producers that provided clinical samples from their ewes for the study and to producers and veterinarians who have contacted me throughout this project to discuss issues surrounding mastitis management on their properties.

I would like to acknowledge the assistance of all the information nucleus flock staff based at Struan, Turretfield, Rutherglen, Hamilton and Armidale. A considerable amount of work was required before any sheep milk collection could occur on each property with a range of administration and coordination. In particular, I would like to acknowledge the assistance of Professor Geoffrey Hinch for coordinating initial interactions with INF staff at each property and then staff on each individual property.

My thanks to the three families of Poll Dorset producers and their staff who were involved in this study. In particular my thanks for extra help in penning up stock and making days of milking large numbers of sheep go a little bit faster.

I would like to acknowledge the assistance of a large team involved in the collection of milk on-property and subsequent work in the laboratory. Firstly to Dr Michelle Sait for assistance in milk collection on-farm over the first year of the project as well as developing initial laboratory methodologies in bacterial identification and processing large numbers of samples. Thank you to Shaiful Islam, Michiko Mirams and Judith Bourne for continuing this bacterial identification and catalogue. Thank you to Dr Indu Panicker for completing bacterial identification for the project and Joanne Allen for supporting all laboratory based bacterial identification and to staff at the Veterinary Clinical Centre Werribee for completing some biochemical tests. A very big thank you to Mr Paul Eason for his assistance in data collection on farm and sharing driving over several thousand kilometres with the “bush tiger” milker. Thank you to Dr Peter Mansell and Mr Evan Hallein for their technical assistance in testing ewe milk for mastitis using “on-farm” tests. Thank you to other members of the Australian Pacific Centre for Animal Health laboratory at the University of Melbourne for a range of assistance throughout the project.

Thank you to the team involved in developing a novel model of lymphatic cannulation which required significant extended work hours over a number of weeks; Dr Hung-Hsun Yen, Dr Elizabeth Washington, Mr Evan Hallein, Ms Joanne Allen and Dr Silk Yu Lin. My thanks also to animal house staff for care of livestock and to the supplier of the ewes.

My thanks to Professor Mark Stevenson for assistance with data analysis for the very large dataset generated from this study. My thanks to Steven Field from Sheep Genetics Australia for assistance with access to data for SGA flocks and to Klint Gore for assistance with data from INF properties.

To Mr Gary Nolan, co-designer and builder of the “Bush-Tiger” milker and trailer, a huge thank you for an excellent design that stood the rigorous demands of the project. Also my thanks for assisting with sample collection on the first property visit.

I would also like to acknowledge the service provided by Dairy Technical Services in North Melbourne for performing milk quality testing for all of our sheep milk samples. Additionally I would like to thank staff at De Laval, Australia for supply of a De Laval cell counter for use in the field.

I would like to acknowledge the significant support of my family during this project with substantial time away both inter and intrastate for the three years of data collection. Finally, my thanks to all Australian sheep producers via MLA for funding this important work to improve animal welfare and productivity in the Australian sheep flock.

10 Appendices

10.1 Letter sent to producers interested in collecting clinical mastitis samples

THE UNIVERSITY OF MELBOURNE

21 May 2013

Dr. Stuart Barber and Dr. Michelle Sait
Faculty of Veterinary Science
The University of Melbourne
Cnr. Park Drive and Flemington Rd.
Parkville. VIC. 3010.

Dear Producer.

We would like to thank you for participating in this study, funded through Meat and Livestock Australia, which aims to give sheep breeders and veterinarians a better understanding of the causes and importance of clinical, and sub-clinical mastitis to the Australian sheep industry.

Your participation in this study will involve you collecting milk samples from ewes that have clinical signs of mastitis and freezing the milk samples until weaning has occurred. Detailed instructions for sample collection are provided. Following weaning, simply send the milk samples back to us in the reply-paid envelope with some general information about the ewe and we will isolate and study the mastitis causing bacteria.

In this pack you will find the following items:

1 x instruction sheet	(guidelines for milk collection)
1 x collection sheet (4 pages)	(to record information about the affected ewe)
30 x 5ml vials with yellow tops	(for milk collection)
1 x plastic ziplock bag	(to store tubes in freezer)
1 x padded postpack	(packing material)
1 x express postpack	(prepaid return shipping)

Once we have received and analyzed the samples, you will be provided with a summary of the bacteria identified in the milk samples you provide. Your participation, farm location and the animals involved in this study will remain anonymous to other participants and to Meat and Livestock Australia.

If you have any questions about the sample collection or would like additional tubes, please contact Dr. Michelle Sait by telephone (03) 9035 3345 or email: michelle.sait@unimelb.edu.au.

Best wishes

Dr. Stuart Barber and Dr. Michelle Sait

10.2 Milk collection guidelines for clinical samples sent to producers

THE UNIVERSITY OF MELBOURNE

GUIDELINES FOR COLLECTION OF MILK

1. ITEMS TO INCLUDE IN YOUR COLLECTION KIT:
   a. In this package you will find 30 sterile 5 ml vials (yellow tops). These are sterile and it is important that when you open them you do not touch any part of the inside (lid or bottle).
   b. Methylated spirits and distilled water
   c. Small plastic jar with screw top (I normally use a 500ml screw top jar)
   d. Packet of cotton wool balls

   You will find the 30 sterile vials in this kit and the rest of the required collection material can be purchased at your local chemist or supermarket.
2. Prior to the commencement of lambing we suggest you make up your milk collection kit. This requires mixing methylated spirits and water in a 7:3 ratio (eg. 700ml methylated spirits to 300 ml water – you probably won't need that much at once so a half or quarter mix is best). Rinse your screw top jar prior to use. Add this mix to your screw top jar that you have filled with cotton wool balls. The mixture should just cover the cotton wool.
3. If you have a "lambing kit" that you take around lambing, add the screw top jar with swabs, a box of wrist length latex gloves and the yellow top tubes provided. This means that you are prepared to take a sample should you find a sick ewe. The kit can stay here until the lambs are weaned.
4. Once you identify a ewe that has got clinical mastitis you need to get a clean sample of milk from within her udder into the sterile tube. To do this you first:
   a. Put on a pair of latex (or similar) gloves to reduce contamination from your own hands. If this is not possible, ensure your hands are clean.
   b. Use the cotton swabs to clean the outside of the teat, concentrating particularly on the end of the teat. Keep swabbing the teat until no more dirt comes off on a fresh, clean swab (this will often take 2-3 swabs).
   c. When the teat is clean, you need to squirt at least one squirt of the milk from that teat onto the ground to remove any bacterial contaminants at the end of the teat.
   d. You then need to squirt milk into the sterile tube. Remove the top of the tube without touching the inner part of the lid or the tube itself – this must remain sterile. Squirt some milk into the tube without touching the tube with the end of the teat, or the hairs that are often on the end of the teat. Approximately half the tube is a good amount of milk, however smaller amounts are ok.
   e. Once you have adequate milk in the tube, close the lid without touching the inside of the lid or the tube.
   f. As soon as possible transfer the tube into a freezer sealed inside a ziplock bag (provided in kit). This reduces the chance of any contamination in the sample.
5. Write down the details of the ewe on the collection sheet so we can then evaluate what age sheep are more likely to get mastitis and how long after they lamb. If you can take the temperature of the sheep this may be a useful indicator of how likely the animal is to live. Note any treatment of the animal and also how successful this was (did the ewe live or die).
6. If you have any questions please let us know.

   Good luck.

10.3 Milk collection sheet sent to producers

Sample ID	Sheep ID	Birth Year	Sire of sheep ID	Lambing date	Single/Tw/ Tr	Date of sample collection	Description	Outcome	Treatment used	Temp (C)	Temp next day
	eg: 234	eg. 2003	eg. 55/2001	eg. 10 Apr 06	eg. Twins	eg. 27 Apr 06	eg. Hot, swollen udder, milk clots	Ewe died/lived, lambs died/lived	eg. Mamyzin	eg. 41	eg. 39.5

Table 21 Multivariable regression analysis of factors associated with average daily gain (grams) from birth to weaning in sheep flocks.

Explanatory variable	Coefficient (SE)	t value	P	95% CI
Intercept	287.8096 (4.4921)	64.071	< 0.01	279 to 297
Flock:				
INF 1	Reference	-	-	
INF 2	-19.7724 (4.7494)	-4.163	< 0.01	-29 to -10
INF 3	-15.4766 (6.3954)	-2.42	0.015	-28 to -3
INF 4	-80.4248 (5.417)	-14.847	< 0.01	-91 to -70
INF 5	-118.4464 (5.017)	-23.609	< 0.01	-128 to -109
PD 1	10.9478 (4.302)	2.545	0.011	3 to 19
PD 2	77.5184 (4.2943)	18.051	< 0.01	69 to 86
PD 3	34.6653 (6.179)	5.61	< 0.01	23 to 47
Individual milk cell count (× 1,000,000 cells/mL)	-0.762 (0.1294)	-5.889	< 0.01	-1.02 to -0.51
Explanatory variable				
Number of lambs reared:				
One	Reference	-	-	-
Two or more [a]	-57.3177 (1.6142)	-35.508	< 0.01	-60 to -54
Birth weight (kg) [b]	2.0239 (0.3171)	6.382	< 0.01	1 to 3
Age of ewe at lambing (years)				
1 – 2	Reference	-	-	
3 - 4	7.3347 (1.2097)	6.063	< 0.01	5 to 10
> 4	6.6913 (1.5384)	4.35	< 0.01	4 to 10

[a] Interpretation: Compared with single lamb litters and adjusting for the confounding effect of flock, bulked milk cell count, birth weight and age of the ewe at lambing, average daily gain for lambs from litters of two or more was reduced by 57 (95% CI 54 to 60) grams per day.

[b] Interpretation: After adjusting for the confounding effects of flock, number of lambs born, birth weight and age of ewe at lambing, one kilogram increases in birth weight increased average daily gain by 2 (95% CI 1 to 3) grams per day.