BACTERIAL ENTERITIS IN YOUNG MERINO SHEEP.

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Thesis submitted to the University of Melbourne in total fulfilment of the requirements of the degree of Doctor of Philosophy

January 2017

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DEDICATION

This thesis is dedicated to my Dad, Alan. The man who taught me to question why, seek answers and love life. I miss you terribly, but I got here because of you.

To my Mum Jackie, for the unwavering support and utmost belief in me throughout the whole process. Thank you for your patience and your unconditional love.

To my brother Ross, for the advice, the laughs and always putting life back into perspective.
ABSTRACT

Diarrhoea during winter is a common problem of sheep in the high rainfall regions of south-eastern Australia. Gastrointestinal nematodes are the primary cause of this problem, especially in young Merino sheep (those <16 months old). However, a syndrome of persistent diarrhoea (‘winter scouring’) is also relatively common in young sheep with low or moderate nematode burdens, with outbreaks usually occurring between June and October. Affected animals have profuse, watery diarrhoea, and often show signs of abdominal discomfort. Before this study, the cause of this syndrome and the epidemiology and risk factors for outbreaks and faecal shedding of the causative organisms, were not well understood.

To determine the primary cause of the syndrome, 45 outbreaks of ‘winter scouring’ were investigated on 24 farms across south-eastern Australia. Necropsies were conducted and faecal samples were collected from animals in affected flocks. Faecal and tissue samples were cultured for *Yersinia*, *Salmonella* and *Campylobacter* species, and faecal WECs conducted to assess nematode parasitism. The sensitivity of *Yersinia* isolates to four common antimicrobial compounds was also assessed.

*Yersinia pseudotuberculosis* serotype III and virulent *Y. enterocolitica* were the most frequently isolated bacteria, with outbreaks typically occurring when more than 10% of the mob were shedding either of these organisms. *Campylobacter* and *Salmonella* did not play a role in any of the outbreaks investigated. Over 64% of all *Yersinia* isolates were resistant to sulphonamides, but 98% were susceptible to oxytetracycline.

These results highlighted the need to investigate the epidemiology and risk factors of yersiniosis in more detail to support the development of more effective control strategies. As a first stage of this process, a longitudinal study was conducted on four farms to evaluate the patterns of faecal shedding of *Yersinia* and assess some potential risk factors for outbreaks of yersiniosis, including live-weight, average daily weight gain and WEC. Faecal shedding of both species occurred predominantly during the winter, but shedding of
**Y. enterocolitica** also occurred at other times. Therefore, in addition to scouring in winter, **Y. enterocolitica** can also cause diarrhoea at other times.

In contrast to the outbreak investigations, the prevalence of faecal shedding of *Yersinia* species was often very high (>30% of animals within a mob) without any clinical disease. This suggests that complex interactions between risk factors contribute to yersiniosis, and that simply monitoring the prevalence of faecal shedding of *Yersinia* species will not be a reliable predictor of an outbreak of yersiniosis.

High WECs (>300 eggs per gram) were associated with an increased risk of shedding each *Yersinia* species, and so effectively controlling gastrointestinal nematodes should also decrease the risk of yersiniosis. Animals that gained weight were less likely to shed *Y. enterocolitica*, but more likely to shed *Y. pseudotuberculosis*. Despite this, the current strategy of ensuring weaner sheep (those 3-12 month of age) continue to gain weight during autumn and winter is still recommended.

The effect of an autogenous bacterin against *Y. pseudotuberculosis* serotype III was assessed on two farms that consistently had severe outbreaks of yersiniosis. There was no outbreak in the year of the study (2014), and vaccinated weaners had similar live weight, growth, faecal shedding of both *Yersinia* spp. and antibody titres to *Yersinia* outer membrane proteins to unvaccinated controls. Consequently, further studies to evaluate the efficacy of vaccines during an outbreak of yersiniosis are required.
PUBLICATIONS


DECLARATION

I declare that this is my original work and that it complies with the University of Melbourne requirement being fewer than 100,000 words in length.

The work described in this thesis was performed by me, except where indicated in the text.

Acknowledgement has been given to any assistance received, either through the formal acknowledgment at the start of this thesis or in the text of the relevant sections.

I certify that the work presented in this thesis has not been submitted for any other degree or qualification at any other university.

Kelly Jane Stanger
January 2017
ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisors Dr. Helen McGregor, Associate Professor John Larsen and Marc Marenda for their advice, guidance and belief in my ability. In particular, I would like to thank Helen for her friendship, sense of humour and her ‘early weaning’ strategy. Thank you for taking such an interest in my professional and personal development - the road was bumpy at times, but I have achieved more than I thought was possible.

I am truly grateful to the staff of the Mackinnon group, and to Di Rees in particular, you are worth your weight in gold. The field trials involved endless hours in the field and the laboratory. Thank you for your time, but more importantly, for the laughs we had along the way. I have thoroughly enjoyed the last three-something years, I have been so fortunate to be surrounded by people wanting me to succeed.

I wish to thank the producers, particularly Brendan Finnigan, Mark Ritchie and James Tehan, who generously donated their time, sheep and facilities for this study. This work received financial support from Meat and Livestock Australia (MLA) and I received a top-up scholarship from the Mackinnon Group Trust.

Thank you to John Morton (Jemora Pty Ltd) for his epidemiological expertise and guidance during the statistical analysis. I would also like to thank Rhys Bushell, Dr. Nadeeka Wawegama, Denise O’Rourke and Barbra Ilievski from the University of Melbourne, for their technical assistance and training in microbiology and molecular techniques throughout the study.
## CONTENTS

**DEDICATION** 

**ABSTRACT** 

**PUBLICATIONS** 

**DECLARATION** 

**ACKNOWLEDGEMENTS** 

**CONTENTS** 

**LIST OF TABLES** 

**LIST OF FIGURES** 

**CHAPTER 1  LITERATURE REVIEW** 

1.1. Sheep production in south-eastern Australia.................................6

1.2. Winter scours syndrome in young Merino sheep.............................7

1.3. The significance of the winter scours in young sheep ......................8

1.4. Diarrhoea in ruminants.....................................................................9
   1.4.1. Pathogenesis of diarrhoea.....................................................9
   1.4.2. Consequences of diarrhoea ...............................................11

1.5. Causes of diarrhoea in young sheep..............................................12
   1.5.1. Non-infectious causes of diarrhoea ......................................12
   1.5.2. Infectious causes of diarrhoea..............................................15
1.6. Conclusions from literature review ................................................................. 28

CHAPTER 2 MATERIALS AND METHODS .......................................................... 30

2.1. Description of farms ......................................................................................... 30
  2.1.1. Farm A ........................................................................................................ 30
  2.1.2. Farm B ........................................................................................................ 31
  2.1.3. Farm C ........................................................................................................ 31
  2.1.4. Farm D ........................................................................................................ 32

2.2. Observations .................................................................................................... 32
  2.2.1. Live-weight ................................................................................................ 32
  2.2.2. Faecal sample collection ......................................................................... 33

2.3. Blood collection ............................................................................................... 34

2.4. Necropsies ........................................................................................................ 34

2.5. Laboratory methods .......................................................................................... 35
  2.5.1. Parasitology ............................................................................................... 35
  2.5.2. Faecal consistency score ......................................................................... 36
  2.5.3. Microbiology ............................................................................................ 36
  2.5.4. Serotyping of Y. pseudotuberculosis ......................................................... 38
  2.5.5. Molecular testing ...................................................................................... 39
  2.5.6. Antibiotic susceptibility testing ................................................................. 41
CHAPTER 3  AN INVESTIGATION OF OUTBREAKS OF THE ‘WINTER WEANER SCOURS’ SYNDROME IN YOUNG MERINO WEANERS 44

3.1. Abstract ......................................................................................................................... 44

3.2. Introduction .................................................................................................................... 45

3.3. Materials and Methods .................................................................................................. 47
  3.3.1. Study design ............................................................................................................... 47
  3.3.2. Microbiology ............................................................................................................ 47
  3.3.3. Parasitology ............................................................................................................. 49
  3.3.4. Post mortem examinations ....................................................................................... 49
  3.3.5. Antimicrobial susceptibility testing ........................................................................ 50

3.4. Results .......................................................................................................................... 50
  3.4.1. Field investigations ................................................................................................. 50
  3.4.2. Identification of bacterial species ........................................................................... 51
  3.4.3. Detection of Yersinia species .................................................................................. 51
  3.4.4. Weather conditions ............................................................................................... 52
  3.4.5. Parasitology ........................................................................................................... 53
  3.4.6. Post mortem investigations ..................................................................................... 53
  3.4.7. Antimicrobial sensitivity testing of yersinia species ............................................. 54
3.5. Discussion .................................................................................................................. 58

3.6. Conclusions ................................................................................................................ 63

CHAPTER 4  A LONGITUDINAL STUDY OF FAECAL SHEDDING OF Y. ENTEROCOLITICA AND Y. PSEUDOTUBERCULOSIS BY MERINO WEANERS IN SOUTH-EAST AUSTRALIA

4.1. Abstract ....................................................................................................................... 64

4.2. Introduction .................................................................................................................. 64

4.3. Materials and methods .............................................................................................. 67
    4.3.1. Study design ........................................................................................................ 67
    4.3.2. Bacteriology ........................................................................................................ 68
    4.3.3. Parasitology ......................................................................................................... 69
    4.3.4. Statistical analyses .............................................................................................. 69

4.4. Results ......................................................................................................................... 73
    4.4.1. Number of sheep-samplings, deaths and overall bacteriology results 73
    4.4.2. Live-weight ......................................................................................................... 73
    4.4.3. Bacteriology ........................................................................................................ 74
    4.4.4. Multivariable GEE models .................................................................................. 79
    4.4.5. Faecal consistency ............................................................................................... 80
4.5. Discussion .................................................................................................................... 86

4.6. Conclusions .................................................................................................................. 89

CHAPTER 5  THE EFFICACY OF AN AUTOGENOUS VACCINE AGAINST Y. PSEUDOTUBERCULOSIS 91

5.1. Abstract ....................................................................................................................... 91

5.2. Introduction ................................................................................................................ 91

5.3. Materials and methods ............................................................................................... 92
   5.3.1. Vaccine development .......................................................................................... 93
   5.3.2. Study design .................................................................................................... 95
   5.3.3. Sampling procedure ......................................................................................... 98
   5.3.4. The assessment of the innate immune response to vaccination and natural exposure to Y. pseudotuberculosis ................................................................. 100
   5.3.5. Statistical analysis ............................................................................................ 101

5.4. Results ......................................................................................................................... 103
   5.4.1. Number of sheep samplings, deaths and overall bacteriology results .......... 103
   5.4.2. Outbreak investigation ...................................................................................... 104
   5.4.3. Live-weight and average daily weight gains .................................................. 104
   5.4.4. Worm egg counts ............................................................................................. 105
   5.4.5. The effects of vaccination (EDTA-Vax) compared with no vaccination (CONT) on animal growth and faecal shedding of Yersinia species ............................ 107
   5.4.6. A comparison of the effects of EDTA-vax and STAND-Vax on animal growth and faecal shedding of Yersinia species ......................................................... 110
   5.4.7. The serological response to vaccination and natural exposure to Yersinia species ................................................................. 111
5.5. Discussion .............................................................................................................. 114

5.6. Conclusion ........................................................................................................... 118

CHAPTER 6  CONCLUSIONS  119

REFERENCES  126
LIST OF TABLES

Table 2.1. Location, farm characteristics and regional weather conditions for the four Victorian farms involved in the epidemiology and autogenous vaccine studies. .................. 30

Table 2.2. Optimised conditions of four polymerase chain reaction assays used to identify pathogenic Yersinia species isolated from faecal samples......................................................... 40

Table 3.1. A summary of *Yersinia* species isolated from investigations of outbreaks of diarrhoea in weaned Merino lambs, on 24 farms in South-eastern Australia in 2012 and 2013. .......................................................................................................................... 55

Table 3.2. A summary of the number of investigations and faecal samples, worm egg counts (WEC) and number (%) of samples positive for each *Yersinia* species during outbreaks of winter scours during winter and spring in 2012 and 2013*. .............................................................. 56

Table 3.3. The proportion (%) of *Y. enterocolitica* and *Y. pseudotuberculosis* isolates, collected during outbreak investigations of winter scouring, that are resistant to ampicillin, tetracycline, trimethoprim and sulphafurazole. ................................................................. 57

Table 4.1. Minimal sufficient adjustment sets for estimation of total and direct effects of each exposure variable on the shedding of *Yersinia* species (these sets were based on the directed acyclic graph shown in Figure 4.1). ......................................................................................... 72

Table 4.2. Live weight and average daily weight gain (ADG) since last sampling for Merino lambs on Victorian farms in 2012 (2 farms) and 2013 (4 farms). ............................................................. 74

Table 4.3. Estimated odds ratios for the total effects of exposure variables on the risk of shedding *Y. enterocolitica* by Merino sheep from flocks in Victoria, Australia in 2012 and 2013, adjusted for minimal sufficient adjustment sets detailed in Figure 4.1. Results for direct effects are also shown in brackets if these were largely different from the total effects................................................................. 81
Table 4.4. Estimated odds ratios for the total effects of exposure variables on the risk of heavy shedding of *Y. enterocolitica* by Merino sheep from flocks in Victoria, Australia in 2013, adjusted for minimal sufficient adjustment sets detailed in Figure 4.1. ........................ 82

Table 4.5. Estimated odds ratios for the total effects of exposure variables on the risk of shedding *Y. pseudotuberculosis* by Merino sheep from flocks in Victoria, Australia in 2012 and 2013, adjusted for minimal sufficient adjustment sets detailed in Figure 4.1............. 83

Table 4.6. Estimated odds ratios for the total effects of exposure variables on the risk of heavy shedding of *Y. pseudotuberculosis* by Merino sheep from flocks in Victoria in 2013, Australia, adjusted for minimal sufficient adjustment sets detailed in Figure 4.1............. 84

Table 4.7. Numbers of sheep-samplings with heavy shedding of *Yersinia enterocolitica* and *Y. pseudotuberculosis* and proportions of these that had faecal consistency scores greater than 3 out of 5 or equal to 5 out of 5 (i.e. diagnostic sensitivity of these faecal consistency categories for detection of heavy shedding of *Yersinia* species)................. 85

Table 5.1 Pre-trial animal health evaluation form that was used by producers to assess an animal’s response to a double dose of an autogenous vaccine against *Y. pseudotuberculosis* serotype III. ................................................................................................................................. 96

Table 5.2. A summary of the timing of activities and samples collected from sheep enrolled in an autogenous vaccine trial against *Yersinia pseudotuberculosis* on two farms in Victoria in 2014. .................................................................................................................................................. 99

Table 5.3. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III (EDTA-vax) compared with no treatment (CONT) on the live-weight of weaned Merino lambs from two Victorian flocks in 2014. ..................................................................................................................... 108

Table 5.4. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III (EDTA-vax) compared with no treatment (CONT) on the average daily weight gain of weaned Merino lambs from two Victorian flocks in 2014. ............................................................. 108
Table 5.5. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III (EDTA-vax) compared with no treatment (CONT) on the faecal shedding and heavy shedding of *Yersinia enterocolitica* by weaned Merino lambs from two Victorian flocks in 2014. .................................................................................................................................................. 109

Table 5.6. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III (EDTA-vax) compared with no treatment (CONT) on the faecal shedding and heavy shedding of *Yersinia pseudotuberculosis* by weaned Merino lambs from two Victorian flocks in 2014.................................................................................................................................................. 110

Table 5.7. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III manufactured in a low-calcium culture (EDTA-vax) compared with one manufactured using standard culture methods (STAND-vax) on the live-weight of weaned Merino lambs from two Victorian flocks in 2014.................................................................................................................................................. 112

Table 5.8. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III manufactured in a low-calcium culture (EDTA-vax) compared with one manufactured using standard culture methods (STAND-vax) on the average daily weight gain of weaned Merino lambs from two Victorian flocks in 2014. ........................................................................................................................................... 112

Table 5.9. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III manufactured in a low-calcium culture (EDTA-vax) compared with one manufactured using standard culture methods (STAND-vax) on the faecal shedding and heavy shedding of *Y. enterocolitica* by weaned Merino lambs from two Victorian flocks in 2014. .......... 113

Table 5.10. The proportion of seropositive weaned Merino lambs following vaccination for *Yersinia pseudotuberculosis* serotype III (EDTA-vax) and natural exposure to *Yersinia* species compared with untreated lambs animals (CONT)........................................................................................................................................... 113
LIST OF FIGURES

Figure 2.1. Method of inoculation of agar plates with faecal samples for phenotypic identification and culture growth scoring. ............................................................................................................. 37

Figure 2.2. Muller Hinton agar plates used for agar dilution antibiotic sensitivity testing of Yersinia species to sulphafurazole. From left to right, top to bottom; Muller hinton plate with; no sulphafurazole, 64µg/ml, 128 µg/ml, 256 µg/ml, 512 µg/ml and 1024 µg/ml or sulphafurazole. Twenty-six samples were tested. .......................................................................................... 43

Figure 4.1. Directed acyclic graph indicating postulated causal pathways between putative risk factors (farm, day of year, worm egg count (WEC), average daily gain (ADG), live-weight and gender) and from these to the occurrence of faecal shedding Yersinia species in Merino lambs. ........................................................................................................................................ 71

Figure 4.2. Prevalence of faecal shedding (proportion of mob shedding) of Yersinia enterocolitica (orange bars) and Yersinia pseudotuberculosis (blue bars) by Merino sheep from two flocks in south-eastern Australia in 2012. The same sheep were sampled at each sampling other than those that were culled or died during the study, with from 40 to 94 sheep sampled on each farm at each sampling (bars indicate exact binomial 95% confidence intervals and a red arrow indicates an outbreak of yersiniosis in the trial mob). ........................................................................................................................................ 76

Figure 4.3. Prevalences of light (CGS 1-2; orange sections) and heavy (CGS ≥3; blue sections) faecal shedding of Yersinia enterocolitica by Merino lambs from four flocks in Victoria, Australia, in 2013. The same sheep were sampled at each sampling other than those that were culled or died during the study with 74 to 94 sheep were sampled on each farm at each sampling (bars indicate exact binomial 95% confidence intervals for total shedding of Yersinia species and a red arrow indicates an outbreak of yersiniosis in the trial mob). ........................................................................................................................................ 77
**Figure 4.4.** Prevalences of light (CGS 1-2; orange sections) and heavy (CGS ≥3; blue sections) faecal shedding of *Yersinia pseudotuberculosis* by Merino lambs from four flocks in Victoria, Australia, in 2013. The same sheep were sampled at each sampling other than those that were culled or died during the study with 74 to 94 sheep were sampled on each farm at each sampling (bars indicate exact binomial 95% confidence intervals for total shedding of *Yersinia* species and a hatched arrow indicates an outbreak of yersiniosis in a non-trial mob)........................................................................................................................................ 78

**Figure 5.1.** The number of sheep in treatment groups within 3 mobs of Merino weaners on each of 2 farms. These were part of a study of the safety and efficacy of an autogenous vaccine against *Yersinia pseudotuberculosis*; ‘CONT’ denotes the control group (no treatment), ‘EDTA-Vax’ and ‘STAND-Vax’ were vaccinated with bacterins manufactured in low calcium and standard cultures, respectively........................................................................................................................................ 97

**Figure 5.2.** a) Prevalence of faecal shedding of *Yersinia enterocolitica* (orange sections) or *Y. pseudotuberculosis* (blue sections) by Merino lambs grazing in mobs enrolled in an autogenous vaccine trial for *Y. pseudotuberculosis* serotype III (error bars indicate exact binomial 95% confidence intervals). b) The proportion of non-vaccinated (CONT) and vaccinated (EDTA-Vax) animals with antibodies to *Y. pseudotuberculosis* from two flocks in Victoria, Australia in 2014 (Solid dot represents CONT group, Cross indicates EDTA-Vax group). A black arrow indicates when vaccine was administered and a red arrow indicates a suspected outbreak of yersiniosis in the trial mob. .................................................................................. 106
CHAPTER 1  LITERATURE REVIEW

1.1. SHEEP PRODUCTION IN SOUTH-EASTERN AUSTRALIA

There are almost 69 million sheep in Australia, approximately 50% of these are Merinos and 37% of the national flock are located in the high rainfall regions (>600mm/year) of south-eastern Australia which includes Victoria, New South Wales and Tasmania (Lane et al., 2015; Anonymous, 2016a). In these regions, sheep are usually managed extensively on improved pastures. In wool producing flocks, lambing typically occurs between late July and August, because the objective is to match the pasture growth curve with the periods of highest nutritional demand (i.e. pregnancy and lactation) and minimise the need to supplement the diet with grain. Studies have shown that although lambing at this time is more profitable than lambing in autumn (March to May) animals require far more intensive management during the first 12 months of their life. During this time, young Merino sheep need to gain a minimum of 1kg per month to reduce the risk of mortality, and many producers have considerable difficulty achieving these growth rates in summer and early autumn (Lean et al., 1997; Campbell et al., 2014).

In the high rainfall regions of Victoria, average temperatures during winter (June to August) range from 3-14°C and winter rainfall averages 180-230mm, with approximately 30 rainfall days during this period (Bureau of meteorology sites at Hamilton (090173), Ballarat (089002) and Mansfield (083010)(Anonymous, 2016b). Young sheep are often exposed to these harsh conditions with minimal shelter and mortalities can be high. In Victoria, the average mortality of young sheep from weaning to 12 months of age is 14.3%, with many of these deaths occurring during winter (range 4.5-26.8%)(Campbell et al., 2009). In addition to these mortalities, poor nutrition, gastrointestinal parasitism and infectious disease also affect lamb productivity (Glastonbury, 1990; Williams and Palmer, 2012).

Diarrhoea (‘scouring’) in young Merino sheep has major health, welfare and economic effects for flocks in the high rainfall regions of south-eastern Australia. In addition to mortalities, diarrhoea may directly contribute to reduced productivity through weight loss and ill-thrift, but also indirectly by fleece and carcass devaluation due to faecal soiling of
wool (‘dag’) around the breech (Glastonbury, 1990; Williams and Palmer, 2012). Gastrointestinal parasitism is the most common cause of diarrhoea during winter for all classes of sheep, but diarrhoea in winter that is not responsive to anthelmintic treatment has been identified in young Merino sheep in this region, and is the focus of this review.

1.2. WINTER SCOURS SYNDROME IN YOUNG MERINO SHEEP

During winter in the high rainfall regions of south-eastern Australia, outbreaks of diarrhoea of 8-16 month old Merino lambs are often observed (‘winter scours syndrome’). Affected animals have persistent diarrhoea, become systemically ill and are lethargic. These animals typically have low burdens of gastrointestinal nematodes and so the diarrhoea does not respond to an anthelmintic treatment, but does tend to improve following treatment with an antibiotic. This syndrome has been reported in other sheep breeds, but young, Merino sheep (those from 3-12 months of age) appear to be overrepresented. Further, this syndrome is well recognised in Australia, but has also been reported to affect sheep, cattle and deer in New Zealand, the USA and the United Kingdom (Hubbert, 1972; Mackintosh and Henderson, 1984a; Gill, 1996).

During an outbreak, a significant proportion of sheep can be affected (up to 90%) and mortalities can approach 20% in affected mobs. Affected animals have profuse, watery diarrhoea, are ill-thrifty, lethargic and may exhibit signs of abdominal discomfort, including teeth grinding (Slee and Button, 1990a; Slee and Button, 1990b). Disease typically develops over a number of days, with early, subtle signs, including an increasing proportion of ill-thrifty animals (‘tail’) in the mob, often overlooked. As the syndrome progresses, diarrhoea and faecal staining of the perineum and hocks occurs, which is commonly the first sign detected by the producer (Slee and Button, 1990a; Philbey et al., 1991). Primary nematodiasis is often excluded because of low worm egg counts (WEC), plus a failure to respond to an effective anthelmintic treatment but clinical improvement following treatment with antibiotics such as sulphonamides or tetracyclines (Slee and Button, 1990c; Spier, 1990).

Outbreaks of this syndrome have been reported sporadically in most domestic livestock species, however sheep, goats and deer are more likely to develop disease, with
outbreaks occurring annually on some farms (Callinan et al., 1988; Slee and Button, 1990b; Gill, 1996). It has been hypothesised that several risk factors for this syndrome, including inclement weather, poor nutrition and routine management activities such as weaning or shearing, precipitate an outbreak because disease in weaned sheep is often associated with high rainfall, low temperatures are sub-optimal pasture availability (Glastonbury, 1990; Slee and Skilbeck, 1992). The relative importance of these risk factors has not been established, but disease is thought to occur as a result of a complex interaction between them.

The winter scours syndrome is considered endemic in Merino flocks in the high rainfall regions of south-eastern Australia, and it is common for producers to report more than one outbreak during a ‘typical’ winter. In addition, recent observations from sheep veterinary consultants in this region are that the frequency and severity of these outbreaks are increasing, especially on farms with high stocking rates. Consequently, further investigation of the winter scours syndrome is needed to identify the causative agents and risk factors for disease, and develop practical management and preventive strategies.

1.3. THE SIGNIFICANCE OF THE WINTER SCOURS IN YOUNG SHEEP

Estimating the cost of winter scours to the Australian sheep industry is difficult because diarrhoea is rarely caused by a single agent. However, in 2006, based on knowledge of the regional and national distribution of diseases, a panel of sheep and beef experts identified the key endemic diseases affecting these animals and modelled the economic effects of each (Sackett and Holmes, 2006). Modelling assumptions were based on a review of published literature and estimates were designed to quantify the direct and indirect costs of endemic diseases. Cost of treatment, lost productivity, deaths and secondary disease were included in these models. This report concluded that gastrointestinal parasitism, flystrike, lice and post weaning mortality were the four most important endemic diseases affecting the Australian sheep industry, costing $369 million, $280 million, $123 million and $76 million, respectively.

Bacterial enteritis, thought to be one of the components of the winter scours syndrome in young sheep in this region, was ranked the seventh most costly disease for the Australian
sheep industry. It was estimated to cost $29 million per annum and so was more important than arthritis, footrot and ovine Johne’s disease. A more recent analysis, using a similar modelling strategy, found that this may have been an overestimate (Lane et al., 2015). Nevertheless, this study still ranked bacterial enteritis 18th of 24 endemic diseases modelled, with an estimated annual cost of $10 million. The differences in value between these studies may be partly attributable to a declining Merino sheep population. However, due to limited evidence there was also a low level of confidence in the prevalence and distribution of this disease, its causes and the efficacy of various treatments.

In addition to costs that could be assigned a monetary value, the increased risk and prevalence of breech strike, reallocation of labour resources, the administration of ineffective anthelmintic and antibiotic treatments, the development of chemical resistance and suboptimal animal welfare are difficult to value, and so were not included in models. These factors have serious long term implications for the Australian sheep industry and, given the uncertainty around prevalence and other assumptions used for this modelling, it is reasonable to suggest that these cost estimates may be low.

Although the monetary value of bacterial enteritis to the Australian sheep industry is lower than for several other endemic diseases, the cost to individual farms can be quite high. For example, on high producing farms more than one outbreak per year can often occur. This alone would considerably increase the estimates for morbidity and mortality above those used in the models, and so increases the estimate of the total cost to industry. Nevertheless, the 2015 study still estimated that losses associated with bacterial enteritis were $4.57/head. Thus, for a typical, self-replacing Merino flock with 1000 ewes, two outbreaks per year would cost around $10,000, even when relatively conservative estimates regarding morbidity (40%) and mortality (3%) are used.

1.4. DIARRHOEA IN Ruminants

1.4.1. Pathogenesis of Diarrhoea

Diarrhoea occurs when the amount of fluid within the gastrointestinal lumen exceeds the absorptive capacity of the alimentary tract, resulting in a net loss of fluid and electrolytes (McGavin and Zachary, 2007). It is defined as abnormally fluid faeces that are
accompanied by an increased volume and frequency of defecation (McGavin and Zachary, 2007). Diarrhoea associated with small intestinal disorders is more common, and typically more severe than that associated with the disease of the large intestine. However, the large intestine is responsible for 75-80% of fluid resorption, and so diarrhoea originating within this region can also lead to rapid dehydration and death (Klein and Cunningham, 2013).

There are four major types of diarrhoea; exudative, osmotic, secretory and diarrhoea associated with intestinal hypermotility.

In ruminants, exudative diarrhoea is the most common form and is typically caused by bacterial, fungal, parasitic or chemical agents. These agents stimulate an acute or chronic inflammatory response which may cause necrosis of the intestinal mucosa. Consequently, fluid is lost due to excess production of inflammatory products and serum proteins, and a reduced capacity of the intestines to resorb fluid and electrolytes. Salmonellosis is a classic example of an agent that causes exudative diarrhoea (McGavin and Zachary, 2007).

Malabsorption leads to the development of osmotic diarrhoea, and is primarily associated with disease of the small intestine. Increased osmotic pressure within the intestine lumen causes excess fluid to move into the lumen at a rate that exceeds the absorptive capacity of the intestines. This is often associated with villous atrophy characterised by a reduction in villous height and the production of brush border enzymes (Jubb and Grant, 2016). This can be complicated further if bile salt or fatty acid resorption in the colon is compromised which may result in additional fluid secretion from the large intestine (McGavin and Zachary, 2007; Radostits et al., 2007). Many viruses (e.g. rotavirus, coronavirus) induce osmotic diarrhoea by selectively destroying villous absorptive cells which results in atrophy, malabsorption and diarrhoea. However, compensatory crypt hyperplasia often occurs, enabling recovery (Radostits et al., 2007).

The third type, secretory diarrhoea, occurs when there in an imbalance between cellular secretion and the absorptive function of cells in the absence of cellular damage (Jubb and
Enterotoxigenic *Escherichia coli* infection is an example of this, with toxins inducing a host cell hypersecretory diarrhoea (Radostits et al., 2007). Although cellular damage is typically minimal, fluid and electrolyte loss can be severe and even cause death.

Diarrhoea associated with hypermotility is not typically a primary mechanism, but is generally a component of each type of diarrhoea (Radostits et al., 2007). It is a consequence of an increased rate, frequency and intensity of peristalsis, and is thought to be stimulated by increased intraluminal pressure associated with fluid accumulation. It can occur in the absence of concomitant disease, typically in response to stress or transportation, and is characterised by otherwise normal gastrointestinal function (McGavin and Zachary, 2007; Radostits et al., 2007).

The agents responsible for the winter scours syndrome in young sheep have not yet been clearly defined, hence the types of diarrhoea involved have not been established. However, bacterial enteritis is thought to play a major role in this disease, with affected animals developing profuse, watery diarrhoea, and so hypersecretion or exudative diarrhoea are likely to be major components of the pathophysiology of diarrhoea for this syndrome.

### 1.4.2. CONSEQUENCES OF DIARRHOEA

Gastrointestinal infection, inflammation or irritation can lead to acute diarrhoea, resulting in rapid fluid and electrolyte loss. Infectious agents and inflammatory mediators can affect ion flux within the intestine, resulting in crypt damage and the net loss of water, sodium, potassium and bicarbonate (Jubb and Grant, 2016). The resultant hypovolaemia, hyponatremia, hypokalaemia and metabolic acidosis can cause death.

When the acute inflammatory response continues, the animal develops chronic diarrhoea, which may result in thickening of the intestine wall and a subsequent reduction in its absorptive capacity. These changes greatly reduce the amount of protein and nutrient an animal can absorb, leading to weight loss and hypoproteinaemia. An increased burden of gastrointestinal parasites and ovine Johne’s disease are examples of chronic protein-
losing enteropathies. The winter weaner scours syndrome typically presents as an outbreak of acute enteritis, but a chronic scouring condition has also been reported.

1.5. CAUSES OF DIARRHOEA IN YOUNG SHEEP

There are many non-infectious and infectious causes of diarrhoea (‘scouring’) in young sheep.

1.5.1. NON-INFECTIONOUS CAUSES OF DIARRHOEA

The rapid introduction of stock onto cereal grains, grazing of stubbles that have spilled grain, a move to lush, green pasture or the ingestion of toxic plants, such as those containing nitrates, can cause scouring in young sheep (Watts and Perry, 1975; Jacobson, 2006; Radostits et al., 2007). The mineral, carbohydrate and water content of feed have also been hypothesised to contribute to scouring, but their exact role is unclear.

1.5.1.1. Cereal grains

During the summer and autumn in south-eastern Australia, supplementary feeding of sheep with cereal grains such as wheat, barley or oats, is commonly required. These supplements are highly fermentable and therefore must be introduced to stock slowly, usually by increments of 50 grams per head, per day, every few days (Caple, 1994). If the acclimation period is too short, or individual animals take longer to adapt to the supplement, acidosis and diarrhoea may result (Radostits et al., 2007). This can also occur with ‘shy feeders’, sheep which are denied access the feed trail by more dominant stock, and then ingest a large amount of grain when the ration is increased before their rumen has time to adapt.

The fermentation of cereal grains, which contain high concentrations of carbohydrate, alters the balance of the rumen microflora. This change can result in proliferation of bacteria that produce lactic acid, including *Streptococcus bovis*. Consequently, rumen pH decreases from around 6.5 to as low as 4.0, producing a condition known as rumen acidosis (Radostits et al., 2007; Xu and Ding, 2011). Affected animals are anorexic, have decreased rumen motility and profuse, light coloured, sweet smelling diarrhoea. These animals may die unless promptly treated.
1.5.1.2. Composition of pasture

It has been hypothesised that the carbohydrate, mineral and water content of pastures can differ markedly between seasons as a result of variable growth rates and the dominant plant species present. These compositional changes are thought to cause diarrhoea and affected animals often scour and have dag on the breech area, but remain bright, alert and continue to maintain or gain weight (Glastonbury, 1990). Diagnosis of this type of scouring is difficult, as it typically requires the exclusion of all other common causes of diarrhoea of healthy, pasture fed sheep (Larsen, 1997; Jacobson, 2006).

It was thought that during winter and spring, rapidly growing, lush, green pastures had a high water and soluble carbohydrate content, which increased the rate that food passed through the gastrointestinal tract causing diarrhoea (Watts et al., 1979; Glastonbury, 1990). However, a study that investigated how the carbohydrate composition and nutritional value of five pasture species in south-western Victoria changed between seasons, refuted this proposal (Walsh and Birrell, 1987). This study showed that the soluble carbohydrate content of pastures was lowest during the winter, which was consistent with earlier research (Couchman, 1959). Additionally, in a review conducted by Larsen et al (1997) it was concluded that there was no clear association between diarrhoea of adult sheep and the grazing of lush pasture in the winter rainfall regions of Australia.

In addition to the soluble carbohydrate content of feed, non-starch polysaccharides, including cellulose, are important sources of fibre and are thought to influence the incidence of scouring. The importance of fibre composition and length for stimulation of salivation and rumination has been well described in dairy cattle, but has not been investigated in any detail in sheep. However, cattle that do not have an adequate supply of neutral detergent fibre can develop diarrhoea (Parkinson et al., 2010).

In addition to carbohydrate content, an association between the sodium, potassium and magnesium concentration in pasture, and the incidence of diarrhoea, has been proposed (Walsh and Birrell, 1987). These elements play an important role in fluid and electrolyte balance in both the small and large intestine, with changes in their intake hypothesized to
induce osmotic diarrhoea. However, this study showed that the mineral composition of five pastures species did not change significantly between seasons.

1.5.1.3. Toxic plants

In summer and early autumn, when conditions are typically dry and pasture quality is poor, grazing livestock will preferentially consume any available green feed, including weeds and toxic plants. Seventy toxic plants are thought to cause gastroenteritis in sheep, but only the silver leaf nightshade (*Solanum elaeagnifolium*) has been definitively proven to cause diarrhoea (Glastonbury, 1990). Although disease associated with the ingestion of toxic plants is relatively uncommon, many of the species thought likely to cause gastroenteritis are found in the high rainfall regions of south-eastern Australia including Bathurst burr (*Xanthium spinosum*), capeweed (*Arctotheca calendula*) and bracken fern (*Pteridium esculentum*) (Glastonbury, 1990).

Of these, the ingestion of capeweed has been most commonly implicated as a cause of diarrhoea in sheep (Napthine, 1988). The concentration of nitrate in plants is often highest after rain that occurs immediately following a drought. During a drought, the concentration of nitrate in soil can increase due to lack of leaching, a reduction in nitrate uptake by plants and due to the decomposition of organic matter. Nitrate is not always toxic to sheep because the rumen microbes convert nitrate to nitrite and then to ammonia, but toxicity occurs when the intake exceeds the capacity to metabolise it. During its rapid growth phase, the concentration of nitrate in capeweed can reach 2-4% dry matter (Harris and Rhodes, 1969). Concentrations that exceed 1.5% dry matter are considered lethal, with values below this believed to have a caustic effect on gastric mucosa, causing irritation and diarrhoea (Everist, 1981).

A study that compared the incidence of diarrhoea in sheep that were fed freshly cut capeweed with animals grazing pastures free of capeweed or animals fed lucerne hay, found no association between the consumption of cape weed and diarrhoea (Pethick and Chapman, 1991). However, the nitrate content of the cape weed was not measured in this study, and so the association between nitrate concentration and diarrhoea remains unclear.
1.5.1.4. Improved pasture species

In addition to toxic plants, scouring syndromes associated with improved pastures have also been described. Perennial ryegrass (*Lolium perenne*) is the most dominant, sown pasture species in the high rainfall regions of Victoria. It is estimated that 90% of these plants are infected with a fungal endophyte (*Neotyphodium lolii*) which produces a number of secondary metabolites including lolitrems and ergopeptides. The concentration of these endophyte toxins is highest during late spring and autumn when ryegrass is seeding (Fletcher et al., 1999).

The primary metabolites produced by *Neotyphodium lolii* are lolitrem A and B which predominately cause neurological disease. These toxins cause a ‘staggers’ syndrome which can affect a high proportion of animals within a mob grazing toxic pasture. Diarrhoea is not a feature of this toxicity and classic signs include staggering, a high stepping gait and in some cases, convulsion and death. Weight loss, poor productivity, scouring and hyperthermia have also been described in association with consumption of infected ryegrass, but ergopeptides including ergovaline are thought to be the cause of the syndrome (Reed, 1999; Menna et al., 2012).

1.5.2. Infectious Causes of Diarrhoea

1.5.2.1. Gastrointestinal parasites

Gastrointestinal nematodes are a major cause of diarrhoea in sheep that graze pasture in the high rainfall regions of Australia (Anderson, 1972; Larsen et al., 1994; Williams and Palmer, 2012). In sheep, *Trichostrongylus*, *Teladorsagia* (formerly *Ostertagia*), *Cooperia* and *Nematodirus* are the most common nematode genera isolated from the gastrointestinal tract (Radostits et al., 2007). Of these, *Teladorsagia circumcincta*, *Trichostrongylus vitrinus* and *Trichostrongylus colubriformis*, are the primary species associated with disease and productivity loss of Merino sheep in these areas, but invariably present as mixed infections (Anderson, 1972; Anderson, 1973).

Nematodes have a direct lifecycle which involves a free-living environmental stage, whereby non-infective larvae (L1 and L2) moult and become infective (L3). The L3 retain their sheath, making them more resistant to desiccation which facilitates survival on
pasture. Once the L3 are ingested they undergo two additional molts within the gastrointestinal tract (L4 and L5). During maturation, L4 and L5s can cause physical damage to mucosal cells and induce an inflammatory response, which may result in appetite suppression, elevated abomasal pH, protein loss and reduced protein metabolism (Coop and Sykes, 1976; Taylor and Pearson, 1979; Coop and Kyriazakis, 1999).

Specifically, *T. circumcincta* L3 inhabit the gastric glands of the abomasum and produce nodular lesions on the mucosal surface, which are of little consequence to the host. Approximately 18-21d after infection, adult parasites emerge from these glands, and stimulate hyperplasia of neighbouring cells, which renders them non-functional. There is a net loss in parietal cells which corresponds with an elevation of abomasal pH, favouring the survival of opportunistic bacteria and protozoa. The altered pH also interferes with pepsinogen production, reducing the host’s ability to digest protein (Radostits et al., 2007). In contrast, *T. vitrinus* and *T. colubriformis* inhabit the anterior third of the small intestine, where they induce an inflammatory reaction causing mucosal hyperplasia and villous atrophy. *Trichostrongylus vitrinus* is more pathogenic than *T. colubriformis*, however severity of infection is dependent on worm density (Beveridge et al., 1989). High burdens have been associated with anorexia, productivity loss and hypoproteinaemia associated with protein leakage from damaged cells. The pathology associated with gastrointestinal parasitism in young sheep depends on the number and species of nematodes present, but clinical signs typically include diarrhoea, inappetence, depression, faecal staining of the breech and ill-thrift (Taylor et al., 2015a).

### 1.5.2.2. Hypersensitivity to gastrointestinal parasites

An age-related immunity to gastrointestinal nematodes has also been described, whereby young sheep (3-6 months) ingest L3 but do not become infected (Anderson, 1973; Pullman et al., 1991). This has been demonstrated by a reduction in parasite numbers, independent of anthelmintic treatment, decreased survival of parasites and by a reduction in the fecundity of female nematodes (Radostits et al., 2007). Where immunity has developed, animals may still develop diarrhoea despite having a low WEC (<100epg), a phenomenon now referred to as ‘hypersensitivity scours’ (Larsen et al., 1994; Larsen and
Anderson, 2000). In such cases, otherwise healthy animals scour, and are consequently at an increased risk of breech strike (Morley et al., 1976).

It is believed that this diarrhoea develops in association with the hosts’ hypersensitivity reaction to ingested Trichostrongylid larvae. Cellular infiltrates typically include an increased population of eosinophils which produce potent inflammatory mediators. These mediators assist with the rejection of worms by increasing the permeability of vascular tissue, enhancing mucus production and stimulating smooth muscle contractions, which results in diarrhoea (Jones and Emery, 1991; Larsen et al., 1994). Recent studies have shown that this hypersensitivity response, as measured by WEC and faecal consistency, is moderately heritable and animals that are selected for a low WEC are more likely to scour (Pollott et al., 2004; Williams and Palmer, 2012). Similar observations have been made with Romney sheep in New Zealand, with the estimates of heritability of dag score in Romneys (0.24±0.8) (Bisset et al., 1992) similar to those for 9 month-old Merinos (0.27±0.1) (Pollott et al., 2004).

In the high rainfall regions of south-eastern Australia, young sheep are inevitably exposed to substantial populations of gastrointestinal nematode larvae during winter. The availability of L3 is determined by timing of the autumn rains and the relative success of gastrointestinal parasite control programs (Anderson, 1983, 1990).

Although gastrointestinal parasites are an important component of diarrhoea during the winter, the ‘winter scours’ syndrome described in young Merino sheep is typically not responsive to short-acting anthelmintic treatments, and so other infectious agents are likely to be the primary cause.

1.5.2.3. Protozoal infections

1.5.2.3.1. Cryptosporidiosis

Cryptosporidium spp. can be detected in the faeces of young sheep that do not have diarrhoea, but the significance of this organism in causing diarrhoea is unclear. In Australia, detection of C. xiaoi is most common, followed by C. ubiquitum and C. parvum (Yang et al., 2014a). Outbreaks of Cryptosporidiosis organism typically compound the
effects of other infectious agents, such as enteric bacteria which cause more severe intestinal damage and diarrhoea (Radostits et al., 2007). Infection with Cryptosporidium can cause damage to the enterocytes of the jejunum and ileum and loss of the brush border, resulting in villous atrophy, mucosal erosion and hypersecretion (Radostits et al., 2007; Lefevrem et al., 2010).

The disease is most commonly seen in calves rather than lambs, but when outbreaks occur in sheep, animals less than 2 weeks of age are typically affected. The importance of this organism as a cause of diarrhoea in animals >12 weeks is not clear, however they have been associated with reduced carcass productivity and are potentially zoonotic (Sweeny et al., 2011; Yang et al., 2015a). Infected animals often exhibit few clinical signs, but acute infection may result in watery diarrhoea, depression, anorexia and a high case mortality rate, which can be exacerbated by cold conditions (Tzipori et al., 1981; West et al., 2009). Although the disease does not occur in adult animals, the prevalence of oocyst in their faeces can reach 100%, and a peri-parturient rise in oocyst excretion by ewes, one week prior to lambing, is reported (Xiao and Herd, 1994; Lefevrem et al., 2010). Although the absolute numbers of oocysts excreted during this time are relatively low, pregnant ewes may be an important source of environmental contamination and therefore increase the likelihood that neonates are exposed to infection. A diagnosis of primary cryptosporidiosis is usually based on the detection of a large number of oocysts in faeces (10^5-10^7 oocysts/ml faeces), faecal flotation, post-mortem examination and more recently, qPCR (Yang et al., 2015a).

Improving hygiene is the most effective strategy to prevent this disease, and antibiotic treatment is largely ineffective. However, paromomycin sulphate has been used to treat lambs during the first 7-12 days of life, when outbreaks are most likely (Ridler, 2008). Typically, infection resolves without treatment and a strong age-related immunity develops in lambs, independent of previous infection (Radostits et al., 2007; West et al., 2009).

1.5.2.3.2. Giardiasis

Giardia duodenalis is one of the most common protozoal organisms that cause enteritis in humans and animals (Caccio and Ryan, 2008). There is considerable genetic variation
within this species and the major assemblages are genetically distinct and appear to be host specific. Genotypes A and B typically affect humans and genotype E affects cattle, sheep and pigs, but sheep can also carry the zoonotic assemblages A and B and are thought to play a role in disease transmission (Yang et al., 2015a). Although *Giardia* spp. are primarily of public health importance, infections in sheep are typically reported in lambs less than 2 months of age (O'Handley and Olson, 2006; Yang et al., 2009).

Excretion of cysts in faeces is highest in lambs less than six weeks of age, but lower numbers may be excreted intermittently into adulthood. In contrast to many agents transmitted by the oral-faecal route, housing conditions, water contamination and season do not appear to influence the risk of transmitting infection (Radostits et al., 2007).

Animals become infected by ingesting cysts from contaminated pastures. These adhere to the small intestinal villi and replicate, causing local infiltration with inflammatory cells, villus atrophy and a reduction of brush border enzymes, which causes a malabsorptive diarrhoea (Jubb and Grant, 2016). Faecal shedding of *Giardia* is rarely associated with clinical signs in sheep, although semi-fluid, pasty faeces may persist for up to 6 weeks in some cases, and reduced growth rates can occur despite a normal appetite (Radostits et al., 2007). No clear relationship between the presence of *Giardia* and disease in livestock has been found, and so it is not considered an important pathogen for production animals. Some livestock can carry species of *Giardia* that are pathogenic to humans, and so may play an important role in the zoonotic transmission of these organisms, but sheep typically carry assemblages that are not considered to be zoonotic (Yang et al., 2015b).

The diagnosis of *Giardia* infection can be made by phase contrast microscopy following appropriate floatation techniques, but use of molecular tools such as qPCR are becoming more common (Yang et al., 2014b). Necropsy lesions are non-specific and can include an increased number of intraepithelial lymphocytes, moderate to severe, diffuse inflammation and the presence of trophozoites on the mucosa or in mucosal scrapings (Radostits et al., 2007).
1.5.2.3.3. Coccidiosis

There are 11 species of *Eimeria* that are carried by sheep and an early Australian study have reported than *Eimeria* spp. can be detected in up to 80% of faecal samples collected from of healthy sheep (O’Callaghan et al., 1987). *Eimeria ovinoidalis* is the main species associated with coccidiosis in young sheep, but *E. ovina*, *E. ahsata*, *E. crandallis* and *E. parva* are also capable of causing disease (Radostits et al., 2007; Ridler, 2008; Lefevrem et al., 2010). Although coccidiosis can have serious economic repercussions associated with diarrhoea and lost productivity in intensive sheep production systems such as those employed in Europe, the disease is much less common in Australia due to the extensive, pasture based management systems (O’Callaghan et al., 1987; Foreyt, 1990).

For disease to occur, animals must ingest a large number of sporulated oocytes. Sporulation of oocysts occurs on pasture under moist, cool conditions (12-32ºC). Sporulated oocysts hatch and reproduce asexually within host enterocytes to produce sporozoites, which form schizonts that contain many merozoites. The merozoites produce gamonts, micro- and macro-gametes, that produce oocysts via sexual reproduction. Diarrhoea results from a combination of mucosal invasion during reproduction and elevation of pH in the abomasum, which favours overgrowth of gram negative bacteria (Taylor et al., 2015b).

Primary coccidiosis is rare, but affected animals have acute, profuse watery diarrhoea that may contain blood. Outbreaks of the disease are most often seen in 6-8 week old, intensively reared lambs when hygiene is poor, or in older sheep grazing irrigated pastures at high stocking rates (Ridler, 2008). Under these conditions, the contamination of the pastures or feedlot area with oocysts is high and the severity of disease is directly related to number of oocytes that are ingested (Glastonbury, 1990; Radostits et al., 2007). Stress associated with management activities such as weaning or transport, high stocking rates and moist, warm conditions favour oral-faecal cycling of oocysts and the perpetuation of disease (Radostits et al., 2007; West et al., 2009).

Severe *E. ovinoidalis* infection causes catarrhal enteritis with generalized congestion and haemorrhage of the caecal mucosa, with mucus and fibrin present in the caecal lumen.
and, to a lesser extent, the colon and distal small intestine. Aggregations of oocytes, visible as white spots, nodules or polyps throughout the intestine, are a characteristic of chronic infection (Radostits et al., 2007; Lefevrem et al., 2010). Chronic infections may result in poor growth rate, low grade diarrhoea with or without blood, mild abdominal discomfort, recumbency and in rare cases, death (Radostits et al., 2007).

Although *Eimeria* can induce disease, the oocytes are often present in the faeces of healthy animals and the presence of the organism alone is not sufficient to assume that it is causing disease (McGavin and Zachary, 2007; Lefevrem et al., 2010). Diagnosis therefore requires consideration of the clinical signs, epidemiology of disease and necropsy lesions in addition to the identification of the organism in faeces (Radostits et al., 2007; Lefevrem et al., 2010).

The successful management and prevention of coccidiosis relies on avoiding overstocking, ensuring feed and water is not contaminated with faeces and by frequent rotation of animals onto alternative pastures. In most cases, coccidiosis is a self-limiting disease, but if treatment is required, oral sulphonamides are usually highly effective.

1.5.2.4. **Viral infection**

There are few viral agents that cause diarrhoea in weaned sheep, but pesti- and rotaviruses can cause diarrhoea in lambs less than 10 weeks age (Napthine, 1988; Jacobson, 2006; Radostits et al., 2007). Rinderpest, Peste des pestis ruminants, Rift Valley fever and Nairobi sheep disease can also cause scouring in sheep, but all are exotic to Australia (Radostits et al., 2007; West et al., 2009).

1.5.2.5. **Bacterial infections**

1.5.2.5.1. **Listeria**

Enteric listeriosis is not common in Australia. *Listeria monocytogenes* and *L. ivanovii* are the important pathogenic species that infect ruminants, but large numbers of these organisms are required for the development of disease. *Listeria* spp. are ubiquitous in the environment and survive well in soil, faeces, water and feed. Outbreaks are often associated with feeding silage, as the fermentation reaction and aerobic conditions within
poorly made silage or the surface of wrapped silage are ideal for the proliferation of Listeria (Clarke et al., 2004). Disease has been reported in all age classes of sheep, but rarely occurs in animals less than 6 weeks of age. Outbreaks often occur sporadically during the winter and spring, when silage is provided as a supplementary feed (Clarke et al., 2004; Radostits et al., 2007).

Outbreaks of Listeriosis can be characterised by encephalitis, abortion or septicaemia, but sporadic cases of gastroenteritis in recently weaned sheep have been reported in New Zealand (Clarke et al., 2004). In such cases, the clinical signs resemble those observed with acute Salmonellosis and include anorexia, lethargy, green-brown diarrhoea and sudden death. Necropsy findings generally include reddening, haemorrhage and sometimes ulceration in the abomasum and duodenum, but also the caecum and colon in some cases (Clarke et al., 2004).

1.5.2.5.2. Salmonella

In Australia, healthy sheep can be carriers of pathogenic Salmonella, but enteric disease associated with this organism occurs only sporadically (Vanselow et al., 2007; Yang et al., 2014c). Outbreaks are typically associated with Salmonella enterica subspecies enterica infection, and serotype Typhimurium is most commonly linked to gastroenteritis in sheep (Quinn et al., 2002; Radostits et al., 2007). Outbreaks of disease are most commonly reported in feedlots or during transport by sea and are often exacerbated by a stressor such as high stocking densities, inclement weather or periods of limited nutrient availability (Higgs et al., 1993; Richards et al., 1993). Infections are typically established by oral-faecal cycling in contaminated feed or water, although trans-mucosal infection across the upper respiratory tract has been reported.

Following ingestion, bacteria adhere to enteric mucosal cells, which facilitate the transmembrane passage of these organisms within membrane bound vesicles. The resulting severe inflammation of the lamina propria induces a hypersecretory diarrhoea (Quinn et al., 2002). The bacteria commonly localize in the mucosa of the ileum, caecum and colon, as well as in the mesenteric lymph nodes of infected animals. In most cases, the host immune response effectively clears these bacteria, although in some sheep a
subclinical or latent infection (‘carrier’ state) may be established. In these cases, a small number of organisms may survive within the gall bladder or intestinal tissue without being shed, or are only excreted in very small numbers (Quinn et al., 2002). However, if these ‘carrier’ animals are stressed they can excrete large numbers of *Salmonella* in their faeces, causing environmental contamination which can precipitate an outbreak of salmonellosis (Glastonbury, 1990; West et al., 2009).

Salmonellosis can occur in sheep of any age, but young and debilitated or older animals are more often affected (Quinn et al., 2002). The disease can manifest in a number of ways, ranging from subclinical infection to per-acute, fatal septicaemia or abortion (Radostits et al., 2007; Uzzau, 2013). Outbreaks of acute salmonellosis are typically characterised by high morbidity and mortality rates, with putrid bloody diarrhoea containing mucus and epithelial casts, high fever, anorexia, weakness and severe dehydration (Glastonbury, 1990; Radostits et al., 2007; West et al., 2009). Diarrhoea commonly develops within hours of the onset of illness, and death occurs within 1-5 days (Quinn et al., 2002).

The management history and gross post mortem signs can be highly suggestive of salmonellosis, but a definitive diagnosis must be confirmed by microbiology and histology. Necropsy lesions include focal intraluminal haemorrhage and enlarged lymph nodes, most often in the abomasum and proximal small intestine. In subacute to chronic cases, oedema, haemorrhage, ulceration and necrosis are also reported in the ileum, caecum and proximal colon (Lefevrem et al., 2010).

1.5.2.5.3. *Campylobacter*

*Campylobacter jejuni* and *C. coli* are part of the normal gastrointestinal microflora of most ruminants, but there is currently little evidence to support their role as primary pathogens of sheep (Ivanovic, 2012). Detection of *Campylobacter* species involves anaerobic culture of faecal samples using selective agar plates. However, these organisms have been isolated from the faeces of sheep both with and without diarrhoea, and so interpreting their isolation in association with clinical signs of diarrhoea or other morbidity is difficult.
Nevertheless, both are zoonotic species and so the food safety risk they pose is of major public health importance (Bailey et al., 2003; Radostits et al., 2007; Ivanovic, 2012).

On the few occasions that outbreaks of Campylobacter spp. have been reported in lambs, nutritional stress and close confinement to facilitate supplementary feeding were considered to be key factors contributing to disease (Glastonbury, 1990). Symptoms were typically mild, with soft, watery diarrhoea and breech soiling (‘dag’) (Glastonbury, 1990; Radostits et al., 2007). Although more than 30% of sheep can be affected, deaths are uncommon and a low grade, chronic diarrhoea is the primary feature of these infections.

Outbreaks of diarrhoea in 5-6 month old weaned lambs have occurred during the summer and autumn in Gippsland, a high uniform rainfall area in eastern Victoria. These outbreaks were investigated because the diarrhoea failed to respond to anthelmintic treatment, and were associated with a Campylobacter-like organism. The prevalence of clinical cases varied from 20-75%, but mortalities were usually only about 1% (Stephens, 1983). Subsequently, young cross-bred sheep were experimentally infected with the Campylobacter-like organism isolated from field cases. They did develop diarrhoea, but had no other systemic signs of illness, and so the importance of this organism remains unclear (McOrist, 1985; McOrist et al., 1987). However, the mortality rates are far lower than observed with ‘winter scours’, and so it is unlikely to have a major role in this syndrome.

1.5.2.5.4. Yersinia
Three of the 12 Yersinia species that have been identified are pathogenic to mammals – Yersinia pestis, Y. enterocolitica and Y. pseudotuberculosis (Carniel et al., 2006). Yersinia intermedia, Y. kristensenii and Y. fredricksenii are also commonly isolated from sheep faeces, but are considered to be non-pathogenic (Carniel et al., 2006). Yersinia pestis is the causative agent of the bubonic plague and has been extensively reviewed (Robert and Fetherston, 1997). This disease is exotic to Australia and is currently confined to small areas of Africa, Asia and the Americas (Carniel et al., 2006).
*Yersinia enterocolitica* and *Y. pseudotuberculosis* can cause acute gastroenteritis in humans and livestock, including sheep, cattle, pigs, goats and deer (Pullar, 1931; Slee and Button, 1990b; McNally et al., 2004). Farmed deer are most susceptible to disease, and outbreaks are often associated with high morbidity and mortality. As a consequence, the New Zealand deer industry has implemented routine vaccination as a means of prevention and control of yersiniosis (Mackintosh and Henderson, 1984a; Mackintosh et al., 1990).

Of the *Yersinia* spp., *Yersinia pseudotuberculosis* has been most often isolated during outbreaks of disease in sheep during winter, but cases associated with *Y. enterocolitica* have also been reported (Slee and Button, 1990a; Slee and Button, 1990b; Philbey et al., 1991). Outbreaks of *Y. enterocolitica* can occur throughout the year, but are clinically indistinguishable from those caused by *Y. pseudotuberculosis* (Slee and Skilbeck, 1992). The clinical signs of yersiniosis include an increased proportion of sheep lagging behind the mob when being moved (a ‘tail’ in the mob), profuse, foul smelling diarrhoea, faecal staining of the perineum and hocks (‘dag’), ill-thrift and teeth grinding associated with abdominal discomfort. Severely affected animals become dehydrated and may die within 48 hours of infection (Glastonbury, 1990; Slee and Button, 1990b; Slee and Skilbeck, 1992). Sporadic cases of placentitis, abortion, epididymitis and mastitis have also been recorded (Biberstein, 1999; Juste et al., 2009; Jubb and Grant, 2016).

A similar proportion of sheep are affected during outbreaks of enteric yersiniosis caused by either *Y. pseudotuberculosis* or *Y. enterocolitica*; an average (range) of 18% (0-90%) and 16.5% (2-55%), respectively. However, more deaths are reported with *Y. enterocolitica* infection compared to *Y. pseudotuberculosis*; an average (range) of 4.4% (0.3-16.7%) and 1.8% (0.0-6.7%), respectively (Philbey et al., 1991). Affected animals rapidly improve following the administration of an effective antibiotic, usually recovering within 10-14 days after the first cases have been noticed (McSporran et al., 1984; Spier, 1990).

Observing typical clinical signs and culturing large numbers of *Yersinia* on selective agar is highly indicative of yersiniosis, but histology is required for a definitive diagnosis. Gross post mortem changes include fluid filled intestines with oedema, thickening of the caecum
and colon and generalized congestion. The distal small intestine and colon are often roughened due to small (3-5mm) raised nodules within the mucosa that have depressed, erosive centres (Philbey et al., 1991; Jubb and Grant, 2016). Typical histological changes include microabscesses within the lamina propria and around crypt necks in the distal half of the small intestine (Obwolo, 1977; Fredriksson-Ahomaa et al., 2010; Jubb and Grant, 2016).

Yersiniosis in grazing sheep, particularly associated with \textit{Y. pseudotuberculosis}, is common in young sheep during their first winter. Outbreaks have been linked to periods of limited feed (<400-600 kg dry matter/hectare (DM/ha)) and inclement weather, and are reportedly precipitated by management interventions and time off feed, such as shearing or crutching (J Webb Ware & G Lean, pers. comm.). Anecdotally, lighter animals are more often affected, and a correlation between nematode infection, as indicated by high WECs (>500 eggs per gram), and an increased risk of yersiniosis, has been proposed. However, there is conflicting evidence about this. Early case reports indicated that infected sheep had negligible gastrointestinal nematode burdens (Pullar, 1931), but more recent ones suggest that yersiniosis often occurs secondary to a substantial nematode infestation or larval challenge (Glastonbury, 1990; Anonymous, 1993). Moderate or high burdens of gastrointestinal nematodes can cause a temporary inappetence (Barker, 1973; Beveridge et al., 1989), and so this may also contribute to the increased risk of yersiniosis.

Although pathogenic \textit{Yersinia} species can cause disease, these organisms have also been isolated from the faeces of healthy sheep, cattle, goats and pigs (Hodges and Carman, 1985; Bullians, 1987; McNally et al., 2004). Consequently, it has been hypothesised that a complex interaction between environmental, management and animal factors are necessary for the development of disease (Mackintosh and Henderson, 1984a; Slee and Skilbeck, 1992; Gill, 1996). Furthermore, although \textit{Y. enterocolitica} can sporadically cause disease in livestock, it is considered to play a more important role in human disease and is one of the leading causes of infectious gastroenteritis. Pigs are believed to be the primary reservoir hosts, but the role of ovine strains is not yet clear (Slee and Button, 1990a; Philbey et al., 1991; Rahman et al., 2011).
Of the bacterial species that can cause acute gastroenteritis, disease associated with *Yersinia* species appears to most closely fit the clinical presentation and temporal pattern described during outbreaks of the ‘winter scours’ syndrome. In the high rainfall regions of Victoria, case investigations of diarrhoea in sheep were conducted by government veterinary laboratories in the 1980s and 1990s (Anonymous, 1982, 1985, 1986, 1993). These indicated a strong association between the winter diarrhoea and the presence of *Yersinia* and *Campylobacter* species, but outbreaks of *Campylobacter* were more frequent during summer. Additionally, a survey conducted by the Mackinnon project of producers in the high rainfall regions of Victoria, indicated that 13% of surveyed producers experienced an outbreak of *Yersinia* during winter, with many reporting multiple outbreaks in consecutive years (J. Larsen, pers. comm.). Further, in the past 10 years, key veterinary advisors have observed that the prevalence and severity of the diarrhoea of young sheep in winter has increased, and many report that *Yersinia* species are commonly isolated during investigations of outbreaks of diarrhoea that do not respond to treatment with anthelmintics (J Webb Ware & G Lean, pers. comm.).
1.6. CONCLUSIONS FROM LITERATURE REVIEW

‘Winter scours’ is a commonly reported syndrome of young Merino sheep grazing improved perennial pastures during the winter in south-eastern Australia. Affected animals have persistent diarrhoea, and become systemically ill and lethargic. A significant proportion of sheep can be affected (up to 90%) and mortalities can approach 20% in affected mobs. During an outbreak, animals often have low burdens of gastrointestinal nematodes and so the diarrhoea does not respond to an anthelmintic treatment, but does tend to improve following treatment with an antibiotic. Consequently, it is considered most likely that a bacterial agent is the primary cause of this syndrome.

The review presents strong evidence that pathogenic *Yersinia* species are the primary agents of the winter scours syndrome in sheep. Outbreaks of diarrhoea due to *Campylobacter* occur more frequently during the summer, are more sporadic and have far fewer deaths than with outbreaks of yersiniosis. Salmonellosis is uncommon and affected animals exhibit even more severe disease than those with yersiniosis.

It is unlikely that infectious protozoal organisms, such as *Cryptosporidium*, *Giardia* or *Eimeria*, play a major role in the winter scours syndrome due the age of animals affected (>3 months of age). In addition, these agents are typically associated with intensive rearing systems, such as those seen in the UK, Europe and America, and the clinical signs are comparatively mild.

Similarly, a major role for ‘nutritional scouring’, or exposure to toxic plants, can be largely excluded on the basis of the timing of the syndrome, the number of animals affected and the lack of any consistent history of a change in diet.

Until now, farmers and veterinary advisors have treated this syndrome empirically and have often not sought an accurate diagnosis. Scouring during the winter was often assumed to be associated with heavy burdens of gastrointestinal nematodes, and so the first response was to administer an anthelmintic treatment. If this failed, antibiotics were typically the second line of treatment. Consequently, the epidemiology and risk factors
associated with the winter weaner scours syndrome, as well as the importance of pathogenic *Yersinia* species, remains unclear. However, a number of risk factors have been proposed. These include inclement weather (low temperatures, rain and wind, producing a high ‘chill index’), the presence of gastrointestinal nematodes, below optimum feed availability and any recent management activities that interrupt grazing, such as shearing or weaning. In the face of an outbreak, the clinical experience of many sheep veterinarians is that antimicrobial therapy and the provision of alternative pastures reduces the severity of disease, but no other management strategies have been systematically investigated.

Consequently, the aims of this study were to identify the primary agent responsible for the winter scours syndrome in south-eastern Australia, to describe some aspects of the epidemiology and risk factors associated with the disease, to evaluate the efficacy of current treatment protocols and propose integrated strategies for the prevention, treatment and control of the disease.
CHAPTER 2 MATERIALS AND METHODS

2.1. DESCRIPTION OF FARMS

Selection of cooperating farms for the epidemiology (Chapter 4) and vaccine trial (Chapter 5) was based on annual rainfall (those with >500mm/year), flock size (those with >3000 breeding ewes), the history and prevalence of winter scouring and both their willingness and ability to participate in each study (Table 2.1). All selected farms were pasture based Merino enterprises that lambed in spring (August to October) and had reported multiple outbreaks of the winter scours syndrome within the last five years.

Table 2.1. Location, farm characteristics and regional weather conditions for the four Victorian farms involved in the epidemiology and autogenous vaccine studies.

<table>
<thead>
<tr>
<th>Farm characteristics</th>
<th>Flock description</th>
<th>Climate averages(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rainfall(^c) (mm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Winter</td>
</tr>
<tr>
<td>Farm ID</td>
<td>Location</td>
<td>Land (ha)</td>
</tr>
<tr>
<td>A(^d)</td>
<td>Woolsthorpe</td>
<td>480</td>
</tr>
<tr>
<td>B</td>
<td>Maindample</td>
<td>3000</td>
</tr>
<tr>
<td>C(^d)</td>
<td>Mansfield</td>
<td>1200</td>
</tr>
<tr>
<td>D</td>
<td>Winchelsea</td>
<td>3500</td>
</tr>
</tbody>
</table>

\(^a\)Australian bureau of statistics; \(^b\)Dry sheep per hectare; \(^c\)Means for the months of June, July and August; \(^d\)Vaccine trial conducted on these farms only; \(^e\)One third of ewes are mated to a terminal sire

2.1.1. FARM A

Farm A is 30 km north of Warrnambool and has improved and native pastures which are predominantly comprised of perennial ryegrass (Lolium perrenne) and subterranean clover (Trifolium subterraneum). At the start of this study, the self-replacing Merino flock consisted of 3300 ewes, 1500 wethers and 190 rams, and there were also 200 dairy heifers on the farm. Ewes were joined for seven weeks in late April, and so started lambing in mid-September. Lambs were weaned in November, 10-12 weeks after the start of lambing, and had an average weaning weight of 17kg (range 12 – 25kg). Weaner mobs typically comprised 500-600 animals.
The winter scours syndrome had occurred in four of the previous five years, and more than one outbreak per season was common. During an outbreak, all animals in affected mobs were usually treated with oral sulphonamide.

2.1.2. FARM B
This farm is 15km west of Mansfield. It has a mix of undulating and steep terrain, and phalaris (*Phalaris aquatica*), subterranean clover (*Trifolium subterraneum*). Cocksfoot (*Dactylis glomerata*), annual ryegrass (*Lolium rigidum*) and silver grass (*Vulpia* spp.) are the predominant pasture species. At the beginning of this study, 5000 Merino ewes, 3000 wethers, 150 rams and 450 angus steers were grazing at an average farm stocking rate of 8 DSE/ha.

The Merino ewes were joined for five weeks in February and lambing occurred in July. Lambs were marked one week after the end of lambing and then weaned into mobs of 500, 10-12 weeks after the start of lambing. The average weaning weight was 21kg (range 14-28 kg).

The winter scours syndrome had occurred sporadically on this farm, but outbreaks had occurred in three of the past five years. The severity of outbreaks varied between years, with mortalities ranging between 1-15%. During an outbreak, if less than 10% of animals within a mob were affected, only the affected animals were treated with oral sulphonamide. When more than 10% of a mob was affected, all animals within the mob would receive the antibiotic.

2.1.3. FARM C
This farm is 15km south-east of Mansfield and lies 15 km from farm B. A majority of pastures are comprised of perennial ryegrass (*Lolium perrenne*), sub-clover species (*subterraneum trifolium*) and silver grass (*Vulpia* spp.), but summer fodder crops, including Brassica species, are grown opportunistically. At the start of this trial, 7600 Merino ewes, 5200 wethers, 340 rams and 1200 Angus cattle were grazing at a stocking rate of 19
DSE/ha, although the weaner mobs were run at a higher stocking rate of 30 DSE/ha in mobs of 500-1000 lambs per mob.

Ewes were joined for five weeks in April and lambing started in September. Lambs were marked two weeks after the end of lambing (mid-October) and weaned 12 weeks after the start of lambing (late November). The average weight of weaned lambs was 18 kg (range 14-26kg).

The winter scours syndrome had occurred annually for the last five years with more than 30% morbidity and 1-5% mortality. Historically, sulphonamide was used to treat all affected animals during an outbreak. However, the apparent efficacy of sulphonamide had decreased over the five years preceding this trial, so in 2010 this producer started using oxytetracycline to treat affected animals. When less than 10% of animals were affected, only the tail of the mob would receive antibiotics, but when morbidity exceeded 10% all animals within affected mobs were treated.

2.1.4. FARM D
This farm is 20 km north-west of Winchelsea and has phalaris (Phalaris aquatica) and subterranean clover (subterraneum trifolium) dominant pastures. At the beginning of this trial, this self-replacing Merino flock included 9000 ewes, 3000 wethers and 200 rams. The stocking rate was approximately 12 DSE/ha and weaner mobs had between 600 and 2000 lambs. Ewes were joined for five weeks in March and lambing started in August. Lambs were weaned in October at an average weaning weight of 19kg (range 14-26kg).

Outbreaks of winter scours had occurred in most of the past 10 years, with outbreaks in three of the five years preceding this study. During an outbreak of winter scours, all animals in affected mobs received oral sulphonamide.

2.2. OBSERVATIONS

2.2.1. LIVE-WEIGHT
Individual animals were weighed using a manual sheep handling crate fitted with weigh bars accurate to 0.5kg (Prattley, New Zealand). The scales were calibrated at each visit
and weights recorded on a monitor (X3000, Tru-test, Australia) corresponding to an electronic ear-tag (EID), scanned with a portable reader (XRS, Tru-test, Australia).

2.2.2. FAECAL SAMPLE COLLECTION

2.2.2.1. Collection of individual faecal samples for worm egg count (WEC) and bacterial culture

Faecal samples were collected for the estimation of WEC and bacterial culture. Animals were restrained in a permanent raceway and individual faecal samples extracted digitally from the rectum. Samples for microbiological assessment were collected first (1 gram) and transferred into a sterile container, then the remaining faeces was transferred into a 10-well plastic tray for WEC assessment (3 grams). A new latex glove was used for each animal to reduce the likelihood of cross contamination of microbiological samples. If no sample was retrieved, this was recorded. Containers were labelled with the animals’ unique ear tag number, mob and date of collection, and stored in a chilled, portable cooler (‘esky’) for the duration of sampling (2-8 hours) and for transport to the laboratory. They were refrigerated at 4°C at the laboratory and processed within 24 hours of collection.

2.2.2.2. Collection of bulk faecal samples for WEC and bacterial culture

Faecal samples for bulk WECs were collected by the producers. The sheep were held in a corner of the paddock for 5-10 minutes. A proportion of animals would defecate within this time, and then fresh faecal samples were collected at random from the ground after the sheep had moved away from the area. Samples were collected into a plastic tray with 10 individual wells that were labelled with their mob identification and the collection date. Only faecal deposits more than one meter apart were selected, to maximize the chance that each sample was from a different animal, and care was taken to exclude samples contaminated with soil or plant material. These samples were sent by express post the day of collection. They were refrigerated at 4°C and processed within 24 hours of receipt. Faecal samples that were collected for bulk WEC assessment were also processed for microbiology using methods outlined in section 2.5.3.1.
2.3. BLOOD COLLECTION

Blood was collected to estimate the antibody response to vaccination and natural exposure to *Yersinia* species (Chapter 5). Eighty animals from a single mob were systematically selected for inclusion in this part of the trial, and were fitted with a different coloured ear tag for easy visual identification (Section 5.3.2). Blood was collected from these same animals at five to six visits during the study.

For blood collection, each animal was caught and positioned on its hind quarters in a seated position. It was restrained with its head gently flexed laterally. The jugular vein was occluded and palpated before blood was collected into a single, 10 ml plain tube using a 21 gauge needle (BD vacutainer™, Becton, Dickinson and Company).

This was labelled with the animals' identification number and collection date. Following collection, each animal was marked with a temporary spray raddle before being returned to the mob. When all animals from the sampling mob had been bled, the number of blood samples was checked with the number raddled animals. These samples were stored in a polystyrene tray on wet ice for transport to the laboratory.

In the laboratory, blood samples were left to stand for approximately 3 hours to allow clot formation before centrifugation (3000 rpm for 5 minutes). Serum was aspirated and transferred into duplicate 2 mL Eppendorf tubes, each labelled with a unique laboratory code including animal identification, farm and date of collection. These samples were stored at -20°C for further processing.

2.4. NECROPSIES

During the initial investigations (Chapter 3), a post mortem was conducted on a selection of animals that had either died within 2 hours of a farm visit, or were euthanased on welfare grounds. The animal was placed in right lateral recumbency and its identification tags were recorded. The left fore and hind limbs were retracted to provide access to the thorax and abdomen, and then a standard field post mortem technique was employed to
access the internal organs. All organs were grossly examined and any abnormalities recorded.

The gastrointestinal tract, between the diaphragm and the rectum, was excised and placed in a plastic container. The rumen, reticulum and abomasum were palpated and then incised to examine the contents. The intestines were thoroughly palpated and examined for gross abnormalities. Tissue sections and faecal samples from the proximal duodenum, jejunum, ileum, caecum and colon were collected for microbiology and histology. Samples of the mesenteric lymph nodes were also collected. Samples for histology were placed in a screw top, plastic container that had a 1:10 ratio of tissue to 10% phosphate buffered formal saline. Samples for microbiology were placed in a sterile, plastic container and stored in a portable cooler for transportation. All containers were labelled with farm and animal identification, sample description and date.

2.5. LABORATORY METHODS

2.5.1. PARASITOLOGY

2.5.1.1. Individual WEC

Samples were retrieved from refrigeration prior to processing and WECs were performed in the Mackinnon parasitology laboratories using a modified McMaster technique (Anderson et al., 1991). Three grams of faeces was weighed into a clean plastic container using digital scales accurate to 0.1g and labelled appropriately. Forty two millilitres of tap water was added to the faecal sample and vortexed at low speed for 30 seconds. The homogenised sample was filtered through a 100 µm mesh sieve and the fluid component collected. It was then gently mixed, transferred into a 10ml test tube and refrigerated at 4°C for at least two hours, to allow nematode eggs to sink to the bottom of the tube.

The supernatant was then gently poured off the sample, leaving 1-1.5 cm of solids in the base of the test tube. The tube was three quarter filled with saturated salt solution and mixed by inversion. The tube was then completely filled with the salt solution and contents mixed with a pipette.
One millilitre of solution was transferred into each of the two chambers of a clean McMaster slide. Nematodirus eggs were recorded separately, but all other nematode species were counted together (Strongyles). The counts from each of the two chambers were recorded and raw averages calculated. This was then multiplied by a factor of 15, to account for dilution, and reported as worm eggs per gram of faeces (epg). The minimum detectable WEC was 15 epg. If Coccidia oocysts or tapeworm eggs were present, these were estimated using a scale 0 (absent) to 3+ (high).

**2.5.1.2. Bulk WEC**

For bulk WECs, faecal samples were pooled in groups of five by mob or treatment group. One gram of faeces was weighed from each of the five samples into a plastic container. Forty five mL of tap water was added to this bulked sample, and then processed using the individual WEC protocol outlined above. Bulk WECs were multiplied by a factor of 10 to account for dilution, with the minimum detectable WEC being 10 epg.

**2.5.2. FAECAL CONSISTENCY SCORE**

Faecal consistency was subjectively scored at the time of processing for bacterial culture as an indicator of faecal water content (diarrhoea), using a scale of 1 to 5 as follows; 1 (hard pellets), 2 (soft pellets), 3 (soft faeces), 4 (soft paste) and 5 (watery)(Jacobson, 2006).

**2.5.3. MICROBIOLOGY**

**2.5.3.1. Faecal culture for Yersinia species**

Immediately prior to the culture of faeces, each individual faecal sample was adjusted to be of similar visual consistency by the addition of sterile water. This helped to minimise any variation associated with different faecal moisture content. The tip of a single-use, sterile cotton swab was introduced to an individual faecal sample and then wiped onto a portion of one half of an agar plate with selective media for Yersinia (Cefsulodin, Irgasan, Novobiocin agar plate (CIN), Edwards group Pty Ltd, Australia). Faecal matter was streaked using a modified dilution streaking technique as illustrated in figure 2.1.
Inoculated agar plates were incubated aerobically at 30°C for 48 hours (Refrigerated Incubator, Thermoline Scientific). Bacterial colonies were then examined grossly for phenotypic characteristics of *Yersinia* species after 24 hours. All dark purple colonies of 0.5 to 5 mm in diameter were sub-cultured onto sheep blood and MacConkey agar (Edwards Pty Ltd, Australia) for further investigation. A culture growth score (CGS) was assigned to phenotypically positive samples. If suspect colonies were present in either the faecal sample inoculation region, or the first, second or third streak, it was scored as a 1, 2, 3 or 4, respectively (Figure 2.1). For analysis, these scores were further categorized into negative (CGS 0), light growth (CGS 1 or 2) or heavy growth (CGS 3 or 4).

![Figure 2.1](image.png)

**Figure 2.1. Method of inoculation of agar plates with faecal samples for phenotypic identification and culture growth scoring.**

From all positive cultures, a sample from a representative, suspect colony was sub-cultured onto Sheep blood agar (SBA) and incubated for an additional 48 hours at 30°C for deoxyribonucleic acid (DNA) extraction and long term storage at -80°C.

### 2.5.3.2. Extraction and storage of DNA from faecal samples

From the SBA sub-cultures, an amount of bacteria approximately the size of a matchstick head was collected from each positive sample. The sample then was transferred into an individual 2 ml Eppendorf tube containing 150 µl of sterile diethylpyrocarbonate (DEPC) water (Bioline, Australia). Samples were heated to 100°C for 10 minutes and then
centrifuged at 10,000 g for two minutes (Miller et al., 2006). A 20 µL aliquot of supernatant containing lysed DNA was transferred into a 0.5 ml micro-Eppendorf tube containing 180 µL of DEPC water. Tubes were labelled with a unique laboratory accession number and then stored at -20°C.

A cross-section of positive bacterial isolates were also stored whole for further analysis. The remaining bacterial matter was harvested from the SBA using a sterile 10 µL inoculation loop, transferred onto cryobeads and stored at -80°C. (Protect microorganism preservation system, Thermo Fisher, Australia).

2.5.4. SEROTYPING OF Y. PSEUDOTUBERCULOSIS

_Yersinia pseudotuberculosis_ isolates were serotyped using a modified slide agglutination technique using commercial antisera I-VI (Denka Seiken Co. Ltd, Tokyo) (Mair, 1965; Hodges et al., 1984). Test samples were revived from cryobeads using nutrient agar and incubated for 48 hours at low temperature to avoid auto-agglutination (15-20°C).

An amount of bacteria, approximately the size of a matchstick head, was collected from the agar plate and transferred into a sterile Eppendorf tube containing 100 µL of 0.9% physiological saline. The solution was homogenized and seven, 10 µL aliquots of bacterial suspension were transferred onto a clean glass plate. An equal volume of antisera (I to VI) was added to one of the seven aliquots of bacterial suspension. Ten microliters of 0.9% saline was added to the last aliquot as a negative control to check for auto-agglutination. Samples were mixed thoroughly using a fresh sterile inoculation loop for each test suspension, and observed for one minute. A positive result was recorded if the solution took on a marked granular or sandy appearance within one minute (typically 10-20 seconds), indicating agglutination.

Where agglutination was absent for all serotypes, or auto-agglutination was observed in saline, samples were checked for contamination, re-cultured if required, and the process repeated. If the result remained ambiguous, the samples were deemed ‘untypeable’.
2.5.5. MOLECULAR TESTING

2.5.5.1. Optimisation of Polymerase Chain Reactions (PCR) assays

Test optimisation and determination of specificity was undertaken using five negative and two positive bacterial control strains (*Citrobacter freundii* NCTC 9750; *Enterobacter cloacae* NCTC 11936; *Escherichia Coli* 0157 NCTC 12900; *Salmonella typhimurium* NCTC 12023; *Serratia marcescens* NCTC 11935; *Yersinia enterocolitica* NCTC 10460; *Yersinia pseudotuberculosis* IFM 2802, IFM quality services, Ingleburn, NSW).

A commercial GoTaq® DNA polymerase kit was used for all PCR assays (Promega Corp, USA). A hot start, 25 μL reaction protocol was developed for each PCR protocol using synthetic, custom made primers (Table 2.2)(GeneWorks Pty Ltd, Australia). Amplification of DNA products was achieved using a Bio-Rad TM100™ Thermal cycler. Test samples were visualized using a 1.5% agarose gel containing gel red (ChemiDoc XR S+, Bio Rad Laboratories Inc.). The optimised conditions for all PCRs are detailed in Table 2.2.

2.5.5.2. Identification of pathogenic Yersinia species by PCR

All culture positive samples were screened using a modified multiplex PCR that identified the two major pathogenic species of *Yersinia* of sheep – *Y. enterocolitica* and *Y. pseudotuberculosis* (Stenkova et al., 2008). This PCR was developed to detect a protein coding sequence within the *ompF* gene specific to *Y. enterocolitica* (270 base pair product) and *Y. pseudotuberculosis* (756 base pair product).

2.5.5.3. Identification of members of the Yersinia genus by PCR

Samples lacking the *Yersinia* species specific *ompF* region were additionally tested for a genus level coding sequence within the same region (Stenkova et al., 2008). Isolates that produced the genus product (428-465 base pair), but lacked the species specific product were categorized as an ‘other *Yersinia* species’.

2.5.5.4. Identification of virulent *Yersinia enterocolitica* isolates by PCR

The virulence of all *Y. enterocolitica* isolates was assessed using a two-step simplex PCR that detected the presence of plasmid or chromosomal virulence gene sequences
Samples were initially assessed for the presence of a virulence coding region within the plasmid (YadA).

Table 2.2. Optimised conditions of four polymerase chain reaction assays used to identify pathogenic Yersinia species isolated from faecal samples.

<table>
<thead>
<tr>
<th></th>
<th>Yersinia genus PCR</th>
<th>Yersinia species PCR</th>
<th>Yersinia virulence PCR (plasmid)</th>
<th>Yersinia virulence PCR (chromosome)</th>
</tr>
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<tbody>
<tr>
<td><strong>Master mix:</strong></td>
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<tr>
<td>MgCl₂</td>
<td>2.5 mM</td>
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<tr>
<td>dNTPs</td>
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<td>Primers</td>
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<td>YadA F: 1 µM</td>
<td>YadA F: 0.5 µM</td>
</tr>
<tr>
<td></td>
<td>669R: 0.3 µM</td>
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<td>YadA R: 1 µM</td>
<td>YstA R: 0.5 µM</td>
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<td></td>
<td>YPS2R: 0.2 µM</td>
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<td></td>
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<tr>
<td></td>
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<tr>
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<tr>
<td><strong>PCR product</strong></td>
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<td>YE: 270 bp</td>
<td>681-849 bp</td>
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<td>(base pair, bp)</td>
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<td>YE: 756 bp</td>
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<tr>
<td>(x30)</td>
<td></td>
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<td>(x30)</td>
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<tr>
<td>Cycle 3:</td>
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<td>72°C for 5 mins</td>
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<td>YPS2R:</td>
<td>ACGTCGTCTGTATGATTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>YadA F:</td>
<td>CTTCAGACTGATGGCTGGCTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>YadA R:</td>
<td>ATGCCTGACTAGAGCGATATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>YstA F:</td>
<td>ATCGACACCAATAACCGCTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>YstA R:</td>
<td>CCAATCACTACTGACTTTCGGCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The presence of the plasmid virulence gene was sufficient to classify an organism as ‘virulent’. All samples lacking the plasmid gene were screened for the chromosomal virulence gene (YstA) (Thoerner et al., 2003). Samples that were plasmid negative and chromosome negative were classified as ‘avirulent’. Isolates that were plasmid negative and chromosome positive were classified as ‘plasmid-less virulent’, but no such isolate was detected.

2.5.6. ANTIBIOTIC SUSCEPTIBILITY TESTING

2.5.6.1. Disc diffusion method
A sub-set of stored isolates were revived from -80°C, cultured onto SBA and incubated at 30°C for 48 hours. Commercial Sensitest agar plates were used for disc diffusion testing (Edwards group Pty Ltd, Australia). Plates were prepared and interpreted according to the CDS model (Bell et al., 2014). In brief, a single colony (1-2 mm diameter) was inoculated into 2.5 ml of sterile, isotonic saline using a flame sterilised wire loop. The homogenized solution had an approximate turbidity of 0.5 McFarland standard solution. The bacterial suspension was transferred onto a Sensitest agar plate and distributed evenly across it with gentle rocking. Excess fluid was removed with a sterile pasture pipette and discarded. Agar plates were air dried at room temperature for a maximum of 20 minutes. Amoxicillin (25 µg), tetracycline (10 µg), trimethoprim (5 µg) and sulfafurazole (300 µg) antibiotic discs (Oxoid Ltd, UK) were dispensed onto test plates and incubated at 30°C for 18 hours. The shortest distance from the disc edge to the edge of confluent bacterial growth (annular radii) were measured and recorded. Isolates with an annular radius of ≤6 mm were classified as resistant to the specific antibiotic, and those >6 mm were classified as susceptible.

2.5.6.2. Sulphafurazole agar dilution method
Sulphafurazole disc diffusion results were ambiguous, and so these results were verified using a two-fold dilution technique using Muller Hinton plates in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2013). Plates contained a sulphafurazole concentration of 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 or 1024 µg/ml (Media preparation unit, University of Melbourne, Australia).
For each isolate, three to five colonies were transferred from SBA into 5 mL of warmed typtcise soy broth (TSB) and incubated at 30ºC overnight. In addition to the field Yersinia strains, one susceptible and one resistant isolate of E. coli were used as controls. The following day, 100 µL of individual bacterial solutions was transferred into 5 mL of warmed TSB and incubated with shaking at 30ºC for 4 hours. When required, the suspension was adjusted with TSB to a 0.5 McFarland standard (approximately 1.5 x 10^8 colony forming units/mL, cfu/ml).

A large, 52 finger rubber replicator with 3 mm pins, was used to transfer inoculum onto the Muller Hinton plates. For this method of sensitivity testing, the final inoculum required was 10^4 cfu per spot of 5 to 8 mm in diameter. The 3 mm applicator delivers approximately 2 µL of inoculum onto plates, and to account for, this each inoculum was further diluted 1:10 with TSB (final concentration of 1 x 10^7 cfu/ml). All suspension adjustments were completed within 15 minutes of preparation.

Tubes containing the adjusted bacterial suspensions were arranged in order in a rack. Each suspension was well mixed and an aliquot was then transferred into their corresponding wells of replicator block. Agar plates and the rubber replicator were marked to ensure correct orientation of the inoculum spots. The bacterial suspensions were transferred from the replicator block onto the Muller Hinton plates using the 52 finger rubber replicator.

Samples were first inoculated onto a control plate (no sulphafurazole) and then inoculum was transferred onto Muller Hinton plates in order of increasing antibiotic concentration, starting with the lowest concentration of antibiotic. A second control plate was inoculated last to ensure that there was no contamination or antibiotic carry-over during the inoculation process. A sample of each bacterial suspension was also streaked onto SBA and incubated at 30ºC overnight to detect mixed cultures.

Inoculated plates were allowed to dry for up to 15 minutes at room temperature and they were then incubated at 30ºC for 16 to 20 hours. Following this, plates were placed on a dark, non-reflective surface to determine end points (Figure 2.2). Sulphonamides may
contain antagonists in the medium that can enable slight growth, and so the end point was determined to be the concentration where there was an >80% reduction in growth compared with the initial control plate. The endpoint for sulphafurazole was ≥64 µg/ml and the growth of susceptible organisms was inhibited at higher concentrations (CLSI, 2016).

Figure 2.2. Muller Hinton agar plates used for agar dilution antibiotic sensitivity testing of Yersinia species to sulphafurazole. From left to right, top to bottom; Muller hinton plate with; no sulphafurazole, 64µg/ml, 128 µg/ml, 256 µg/ml, 512 µg/ml and 1024 µg/ml or sulphafurazole. Twenty-six samples were tested.

2.6. ETHICS APPROVAL

The research described in chapter four and five was approved the University of Melbourne Animal Ethics Committee. (Chapter 4, 1212405.1; Chapter 5, 1413067.1).
CHAPTER 3  AN INVESTIGATION OF OUTBREAKS OF THE ‘WINTER WEANER SCOURS’ SYNDROME IN YOUNG MERINO WEANERS

3.1. ABSTRACT

The objective of this study was to identify the cause of outbreaks of winter scouring in weaned Merino sheep in the high rainfall regions of south-eastern Australia and to determine the efficacy of antimicrobials used to treat this syndrome.

Forty-five outbreaks were investigated in 24 flocks. Faecal samples from affected animals were cultured for *Yersinia*, *Campylobacter* and *Salmonella* species. Risk factors, including rainfall, temperature and WEC, were assessed. The susceptibility of *Yersinia* species isolates to four common antimicrobials was also determined.

*Yersinia pseudotuberculosis* serotype III and virulent *Y. enterocolitica* were most frequently isolated. No single risk factor precipitated an outbreak, with the frequency and severity of disease varying between region, farm and year. *Yersinia pseudotuberculosis* was detected only during winter, but *Y. enterocolitica* was present in all seasons. Pathogenic *Yersinia* species were more often isolated when WECs exceeded 500 eggs per gram.

A high proportion of *Y. enterocolitica* and *Y. pseudotuberculosis* were resistant to sulphafurazole (64% and 86.9%, respectively). Most *Y. enterocolitica* were also resistant to ampicillin (89%) and some to tetracycline (2%).

Pathogenic *Yersinia* cause bacterial enteritis that is responsible for the winter scours syndrome. No clear association between weather, WEC and disease was established, so complex interactions between risk factors are probably more important than any single factor. Resistance by *Yersinia* to antibiotics used to treat winter scours was common, so
during an outbreak the use of antibiotics should be integrated with grazing management, including moving affected mobs onto lower risk pastures and decreasing stocking rate.

3.2. INTRODUCTION

Diarrhoea during winter (‘winter scours’) is a common problem of sheep in the high rainfall regions of south-eastern Australia, and a winter scouring syndrome has been described in young Merino sheep (<16 months old) in this area. Outbreaks typically occur between July and October, when temperatures are low, rainfall is high (>500 mm/year) and there is often limited pasture availability (1-2 cm or 400-600 kg dry matter/hectare, DM/ha) (Glastonbury, 1990; Philbey et al., 1991). It has been hypothesised that a complex interaction between environmental, management and animal risk factors precipitate an outbreak, but studies examining these interactions are lacking. Recent observations from sheep veterinary consultants in this region are that the frequency and severity of outbreaks are increasing, especially on farms with high stocking rates (Lane et al., 2015).

During an outbreak, up to 60% of sheep are affected and there can be significant mortalities of up to 10%. Affected animals present with acute or subacute enteritis characterised by profuse, watery diarrhoea, teeth grinding and signs of abdominal discomfort. A chronic scouring syndrome associated with weight loss and ill thrift has also been reported, but is less common (Slee and Skilbeck, 1992).

Anecdotal evidence suggests that there is some interaction between high WECs and disease, but the acute syndrome can often occur when WECs are low and persists despite treatment with an effective anthelmintic. Affected animals frequently respond to treatment with antibiotics, including sulphonamides or tetracycline. Consequently, although interactions between risk factors are necessary to precipitate an outbreak, bacterial enteritis is believed to be an important component of this syndrome.

To date, no studies have specifically investigated the epidemiology of risk factors for the winter scours syndrome in this region, and current understanding is limited to clinical reports and case series investigations. The collective costs of the winter scours syndrome have not been estimated, but costs associated with bacterial enteritis, including mortalities,
lost productivity and treatment, have been estimated at $10-29 million annually (Sackett and Holmes, 2006; Lane et al., 2015). However, these estimates may be conservative because there is limited epidemiological data to support the assumptions used in these reports. In addition, gastrointestinal parasitism is often a presumptive diagnosis for scouring weaners, and so winter scouring associated with bacterial enteritis may be more common than realised.

*Campylobacter, Salmonella* and *Yersinia* species are the bacterial agents that could contribute to this syndrome, with virulent *Y. enterocolitica* and *Y. pseudotuberculosis* most commonly isolated from sheep with acute gastroenteritis. Of these, *Y. pseudotuberculosis* is the most frequently isolated, whereas *Y. enterocolitica* is found only sporadically (Slee and Button, 1990a; Slee and Button, 1990b; Philbey et al., 1991). The clinical signs associated with each *Yersinia* species are similar, although outbreaks associated with *Y. pseudotuberculosis* are typically confined to winter, whereas those associated with *Y. enterocolitica* have been reported throughout the year.

In addition to outbreaks of disease, these organisms can be isolated from the faeces of healthy animals, and so it is thought that a complex interaction between environmental, management and animal factors, such as weather and weaning or shearing, are necessary for the development of disease (Mackintosh and Henderson, 1984a; Slee and Skilbeck, 1992; Gill, 1996). Results from case series investigations have identified several risk factors, including inclement weather and an increased burden of gastrointestinal nematodes, but these are not always associated with disease (McSporran et al., 1984; Philbey et al., 1991).

Although yersiniosis is thought to play the major role in the winter scours syndrome, sporadic outbreaks associated with *Campylobacter or Salmonella* species have also been reported. However, outbreaks of salmonellosis are typically associated with periods of nutritional stress, confinement feeding, transportation or overcrowding and there are usually more mortalities (Glastonbury, 1990; Radostits et al., 2007). Outbreaks associated with *C. jejuni* or *C. coli* occur most frequently during summer, and typically have fewer mortalities than outbreaks of yersiniosis (Glastonbury, 1990).
The aims of this study were to identify the primary agents responsible for outbreaks of the winter scours syndrome in south-eastern Australia, to identify potential risk factors for this disease and evaluate the efficacy of antibiotics used for treatment.

3.3. MATERIALS AND METHODS

3.3.1. STUDY DESIGN

A case series investigation was conducted over a 16 month period, using targeted sampling during outbreaks of winter scours. Outbreaks were identified using a network of veterinary consultants, Government veterinarians and existing Mackinnon Project clients. Only outbreaks in 3-16 month old Merino weaners in the high rainfall regions of south-eastern Australia (those with >500 mm annual rainfall) were investigated.

The definition of an outbreak was the acute onset of diarrhoea of less than one week duration, with a minimum morbidity of 25% and evidence of systemic disease, including inappetence, bruxism, lethargy and dehydration. Primary gastrointestinal parasitism, principally associated with *Teladorsagia* and *Trichostrongylus* species, was excluded by sheep having a low WEC or being recently treated with an effective anthelmintic. Faecal samples were collected before the administration of antibiotics, from a representative sample of affected sheep for parasitology and microbiology. Where possible, a full post mortem was conducted on representative animals from a subset of cases that had either died within two hours of a farm visit, or were showing signs of severe disease.

3.3.2. MICROBIOLOGY

Faecal and post mortem samples were screened for the presence of *Yersinia*, *Campylobacter* and *Salmonella* species by inoculation onto selective media. All positive samples were given a semi-quantitative culture growth score and characterised further to light or heavy bacterial growth (Section 2.5.2). *Yersinia* species were isolated using Cefsulodin-Irgasan-Novobiocin agar (CIN; Edwards Pty Ltd, Australia) incubated aerobically for 24 to 48 hours at 30°C (Section 2.5.3.1). Suspect isolates were tested for an oxidase reaction and all oxidase negative samples were stored on PROTECT™ cryobeads at -80°C (Technical Service Consultants, Heywood, Lancs., UK).
Campylobacter species were detected using Skirrows agar (Media preparation unit, University of Melbourne, Aust.). Samples were incubated in micro-aerophilic conditions (5% O₂, 10% CO₂, and 85% N₂, CampyGen™, Oxoid Ltd., UK.), at both 37°C and 42°C for 48 hours. Suspect colonies were stained with dilute carbol fuchsin and morphologically positive samples were sub-cultured for further analysis. Isolates that showed enhanced growth at 42°C and were positive for sodium hippurate hydrolysis were identified as Campylobacter jejuni. Those showing enhanced growth at 42°C but were negative for hippurate hydrolysis was identified as Campylobacter coli.

Salmonella was detected using selenite mannitol enrichment broth incubated at 37°C for 24 hours. A subculture of the broth was made onto Xylose lysine deoxycholate agar (Media preparation unit, University of Melbourne, Aust.) and incubated at 37°C for 24 hours. No Salmonella isolates were detected during this study.

### 3.3.2.1. Identification and serotyping of Yersinia species

Approximately 10% of suspect Yersinia species were biochemically typed using commercial kits (API 20E®, Biomerieux, USA). However, due to problems with repeatability and interpretation of those biochemical reactions, a multiplex PCR to identify Y. enterocolitica and Y. pseudotuberculosis, and a simplex PCR to identify members of the Yersinia genus, were developed (Section 2.5.5). Briefly, genomic DNA was extracted from pure culture by heat lysis. (Miller et al., 2006) All suspect samples were screened using a multiplex PCR to identify if they were either Y. enterocolitica (270 base pair product) or Y. pseudotuberculosis (756 base pair product). (Stenkova et al., 2008) A hot start, 25 µL reaction containing 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of each primer (227F, YER, YPS2R, YE2R), 1 U of GoTaq® DNA polymersase and 2 µL of genomic DNA was performed. The PCR mixture was amplified in a Bio-Rad TM100™ Thermal cycler using the following cycle: 5 mins at 95°C, 30 cycles of 94°C for 20 sec, 66°C for 20 sec and 72°C for 45 sec, then a final 5 min cycle at 72°C (Table 2.1).

All negative samples were further analysed using a Yersinia genus, simplex PCR. (Stenkova et al., 2008) Positive samples were classified as ‘other Yersinia species’
which were non-pathogenic for sheep. Reaction conditions were; 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.3 µM of each primer (227F, 669R), 0.5 U GoTaq® DNA polymerase and 2 µL of genomic DNA. Amplification conditions were: 95°C for 5 mins, 30 cycles of 94°C for 20 sec, 68°C for 20 sec and 72°C for 15 sec, followed by 72°C for 5 mins.

Virulent *Y. enterocolitica* were identified using a two-step simplex PCR that identified plasmid (YadA) and chromosomal (YstA) virulence genes. (Thoerner et al., 2003) Optimised PCR conditions for YadA detection were: 2 mM MgCl₂, 0.1 mM dNTPs, 1 µM of each primer (YadA F, YadA R), 0.5 U GoTaq® DNA polymerase and 2 µL of genomic DNA. Optimised PCR conditions for YstA detection were: 2 mM MgCl₂, 0.1 mM dNTPs, 0.5 µM of each primer (YstA F, YstA R), 0.5 U GoTaq® DNA polymerase and 2 µL of genomic DNA. Amplification conditions for both virulence PCRs were: 95°C for 5 mins, 30 cycles of 95°C for 15 sec, 61°C for 30 sec and 72°C for 1 min, followed by 72°C for 5 mins. *Yersinia enterocolitica* isolates lacking both virulence genes were classified as ‘avirulent’.

*Yersinia pseudotuberculosis* isolates were serotyped by slide agglutination using commercial antisera against ‘O’ antigens I-VI (Denka Seiken Co. Ltd, Tokyo).

### 3.3.3. Parasitology

Faecal samples were collected from the ground by cooperating producers (section 2.5.1.2). Bulk WECs were performed using methods a modified McMaster technique described in section 2.5.1. Burdens of coccidia and *Moniezia* species were each estimated using a scale of 0 (none) to 3 (heavy).

If WECs were not conducted in house, the producer verbally reported the results from the most recent WEC test, but these values were not included in this study.

### 3.3.4. Post Mortem Examinations

A total of 14 post mortems, which included one to three animals from 7 farms (4 from south-west Victoria (Farm A, E, I, U), 1 from north-central Victoria (Farm F), 2 from Gippsland (Farm H, X)) were conducted. Faecal samples and mucosal scrapings were submitted for bacterial culture. Faecal samples were also examined for WECs and, fresh
and formalin-fixed tissues from the duodenum, ileum, jejunum, large intestine and caecum were cultured and examined by histopathology. Samples were collected within 4 hours of death or immediately following euthanasia of the animal.

3.3.5. ANTIMICROBIAL SUSCEPTIBILITY TESTING

The antimicrobial susceptibility of at least half of the *Yersinia* isolates from each farm was determined. Susceptibility to amoxicillin (25 µg), tetracycline (10 µg), trimethoprim (5 µg) and sulphafurazole (300 µg) (Oxoid Ltd, UK) was assessed using a disc diffusion technique (Section 2.5.6.1)(CLSI, 2013).

The disc diffusion results for sulphafurazole were ambiguous, and so these were verified using a two-fold agar dilution technique with Muller Hinton agar plates (Media preparation unit, University of Melbourne, Aust.) containing a sulphafurazole concentration of either 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 or 1024 µg/ml (CLSI, 2013) The resistance breakpoint was 64 µg/mL (Section 2.5.6.2).

3.4. RESULTS

3.4.1. FIELD INVESTIGATIONS

Forty five outbreaks were investigated on 24 farms between June 2012 and October 2013 (2 from north-central Victoria, 2 from north-east Victoria, 10 from south-west Victoria, 4 from Gippsland, 6 from New South Wales (NSW)). The average farm was 1700 ha (range 240-3500 ha), with an average annual rainfall of 675 mm (range 540-960 mm), an average stocking rate of 14 dry sheep equivalents per hectare (DSE/ha, range 7-22 DSE/ha) and had 1000-9000 breeding Merino ewes.

Farm visits were conducted by a veterinarian for seven of the 45 outbreaks. At these visits, faecal samples were collected from 10-40 scouring sheep from each affected mob, and post mortem examination of one to three animals, were conducted. On all other occasions, faecal samples were collected by producers.
3.4.2. IDENTIFICATION OF BACTERIAL SPECIES
A total of 1020 faecal samples were collected from the 24 farms (Table 3.1), *Yersinia pseudotuberculosis* was the most frequently isolated pathogen (18.5% of samples), followed by virulent *Y. enterocolitica* (13.6% of samples). One outbreak of *Campylobacter jejuni* was recorded on a farm in north-central Victoria in October 2012, but *Campylobacter* was not detected at any other time. No *Yersinia* species were detected on this farm during the outbreak of Campylobacter. No *Salmonella* species were detected during this study.

One hundred and thirty five of the 189 isolates of *Y. pseudotuberculosis* were serotyped; 98% (n=132) were serotype III, 1% serotype II (n=1) and the remaining two isolates were inconclusive. One hundred and seven of the isolates of 139 *Y. enterocolitica* were screened for the presence of virulence genes by PCR. Of these, 92% were virulent, having either the plasmid (85%) or chromosomal (7%) gene sequence. All avirulent samples (8%) originated from an outbreak investigation on a single farm in October 2013, but no alternate cause of disease was identified in this case.

3.4.3. DETECTION OF YERSINIA SPECIES
In both years, and across all farms and regions, the isolation of pathogenic *Yersinia* species was highest during winter, with the exception of *Y. enterocolitica* in spring 2012 (Table 3.1). Of the samples collected in winter, *Y. enterocolitica* was isolated from 9% of samples from 2012, and 30% of samples from 2013. In comparison, *Y. pseudotuberculosis* was isolated from 27% of samples 2012 and 29% of samples from 2013. The proportion of animals shedding each species varied markedly between region and farm, ranging from 0-75% and 0-55%, for *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively.

Ten of the 45 outbreak investigations occurred during summer or autumn, with seven of these recorded in NSW. Of these 10 investigations, *Y. enterocolitica* and *Y. pseudotuberculosis* were either absent or detected in only a very low proportion of samples (<8%).
In 2013, when the number of submissions from Victoria (16 outbreaks, 300 samples) and NSW (10 outbreaks, 226 samples) were similar, isolation of \( Y. \) pseudotuberculosis was significantly higher in Victoria (26% vs 0.5%, \( \chi^2 = 69.7, P < 0.001 \)). No difference for isolation of \( Y. \) enterocolitica was detected in 2013 (20% vs. 14%; \( \chi^2 = 3.1, P = 0.08 \)).

To more accurately describe the winter scours syndrome, data collected during the winter and spring was collated and is presented by region, farm and year in Table 2. The proportion of animals shedding pathogenic \( Yersinia \) species, and the primary species detected, varied between farms within the same region. For example, Farms B and C were located within a 15km radius, but the proportion of each \( Yersinia \) species detected differed markedly.

In both years, farms B and C each recorded outbreaks of scouring during the winter. On farm B in 2012, the diarrhoea was not associated with either \( Yersinia \) species, but in 2013, \( Y. \) pseudotuberculosis was detected in 55% of samples. In contrast, on farm C, \( Y. \) pseudotuberculosis was the primary species detected (61%) in 2012, whereas \( Y. \) enterocolitica (48%) was the primary species detected in 2013.

### 3.4.4. WEATHER CONDITIONS

The north-central, north-east and south-west regions of Victoria have a winter-dominant rainfall pattern, typically recording more than a third of their annual rainfall between June and August each year. Average winter rainfall and winter temperatures recorded at Bureau of Meteorology sites closest to each of the farms with outbreaks within these regions, was comparable between study years, with the exception of Lake Eildon (Farms B and C) which recorded a higher than average rainfall in 2013.

In contrast, Gippsland and NSW have a more uniform rainfall distribution, with significant rainfall occurring in both summer and winter. In these regions, average winter temperatures as well as average winter rainfall were higher in 2013 than in 2012.

When comparing averages across all regions, winter rainfall (+44 mm) and minimum (+0.7°C) and maximum (+0.7°C) winter temperature ranges were higher in 2013, indicating
that it was a wetter but warmer year than in 2012. The largest differences between rainfall (+12 to 188mm) and temperatures (Min: +0.3°C to +1.9°C; Max: -0.1°C to +1.8°C) in 2013 were recorded in Gippsland and NSW.

3.4.5. PARASITOLOGY
Worm egg counts were available from 28 outbreaks in 17 flocks during this study (Table 3.2). Of these, 8 flocks had counts that exceeded the accepted threshold for anthelmintic treatment in Merino weaners (>300 epg) (Niven et al., 2002a). On all occasions where WECs were markedly higher than this threshold (>550 epg), the combined proportion of pathogenic *Yersinia* species detected was greater than 50%. When WECs ranged from 400 and 550 epg, the detection of pathogenic *Yersinia* species was more variable, ranging from 3-61%. It was not possible to assess the association between the presence of pathogenic *Yersinia* species and WECs below 400 epg, because in these cases, low WECs may have been associated with either a low parasite burden or a recent anthelmintic treatment. For all farms, burdens of Coccidia and *Moniezia* species were 0 or +1 and there was no evidence of disease on post mortem examination.

3.4.6. POST MORTEM INVESTIGATIONS
Fourteen post-mortems, which included one to three affected weaners from 7 farms were undertaken. Due to autolysis, the samples from the large intestines of two animals were not examined by histology.

On all occasions, moderate to severe lymphocytic-histiocytic enteritis and colitis were the primary abnormalities reported. Inflammatory cell infiltrate typically contained lymphocytes, macrophages, plasma cells, neutrophils and in some cases, eosinophils. Ulceration or abscessation was observed in 5 of 14 small intestinal samples and 6 of 12 large intestinal sections. Microabscessation with bacterial aggregates was reported on 2 of 14 occasions. Heavy growths of either *Y. enterocolitica* or *Y. pseudotuberculosis* on *Yersinia* selective agar were reported on all occasions.
3.4.7. ANTIMICROBIAL SENSITIVITY TESTING OF YERSINIA SPECIES

Most *Y. enterocolitica* isolates were resistant to ampicillin (89%), with resistant isolates detected on all farms (Table 3.3). The disc diffusion test indicated that only 8% of *Y. enterocolitica* isolates were resistant to sulphafurazole, but the proportion assessed as resistant was much higher when using the agar dilution test (64%). A small proportion (2%) of *Y. enterocolitica* isolates were resistant to tetracycline, however all of these originated from one farm. No resistance by *Y. enterocolitica* to trimethoprim was detected.

Resistance of *Y. pseudotuberculosis* to sulphafurazole was detected on all farms and the agar dilution test detected more resistance than the disc diffusion test (87 vs. 30%). Resistance to ampicillin (10%) was less than that recorded for *Y. enterocolitica*, a small proportion of isolates were resistant to trimethoprim (3%) and no resistance to tetracycline was detected.
Table 3.1. A summary of *Yersinia* species isolated from investigations of outbreaks of diarrhoea in weaned Merino lambs, on 24 farms in South-eastern Australia in 2012 and 2013.

<table>
<thead>
<tr>
<th>Region</th>
<th>Season</th>
<th>2012 No. of invest.</th>
<th>Number (%) of samples</th>
<th>Total samples</th>
<th>2013 No. of invest.</th>
<th>Number (%) of samples</th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Y. <em>entero</em></td>
<td>Y. <em>pseudo</em></td>
<td>Other <em>Yersinia</em></td>
<td>Not <em>Yersinia</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South-west Victoria*</td>
<td>Autumn</td>
<td>0</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>6</td>
<td>21 (15%)</td>
<td>41 (28%)</td>
<td>2 (1%)</td>
<td>80 (55%)</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>2</td>
<td>7 (16%)</td>
<td>6 (13%)</td>
<td>0 (0%)</td>
<td>32 (71%)</td>
<td>45</td>
</tr>
<tr>
<td>Gippsland, Victoria*</td>
<td>Winter</td>
<td>2</td>
<td>4 (4%)</td>
<td>18 (16%)</td>
<td>4 (4%)</td>
<td>86 (76%)</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>1</td>
<td>6 (75%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (25%)</td>
<td>8</td>
</tr>
<tr>
<td>North-east Victoria*</td>
<td>Autumn</td>
<td>0</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>2</td>
<td>3 (5%)</td>
<td>30 (51%)</td>
<td>0 (0%)</td>
<td>20 (34%)</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>2</td>
<td>1 (3%)</td>
<td>0 (0%)</td>
<td>1 (3%)</td>
<td>29 (94%)</td>
<td>31</td>
</tr>
<tr>
<td>North-central Victoria*</td>
<td>Winter</td>
<td>1</td>
<td>3 (9%)</td>
<td>14 (42%)</td>
<td>1 (3%)</td>
<td>15 (46%)</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>2</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>30 (100%)</td>
<td>30</td>
</tr>
<tr>
<td>New South Wales</td>
<td>Summer</td>
<td>0</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autumn</td>
<td>0</td>
<td></td>
<td></td>
<td>7</td>
<td>12 (8%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>1</td>
<td>1 (3%)</td>
<td>1 (3%)</td>
<td>0 (0%)</td>
<td>28 (93%)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>0</td>
<td></td>
<td></td>
<td>1</td>
<td>8 (40%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>OVERALL</td>
<td>Summer</td>
<td>0</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autumn</td>
<td>0</td>
<td></td>
<td></td>
<td>10</td>
<td>16 (7%)</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>12</td>
<td>32 (9%)</td>
<td>104 (27%)</td>
<td>7 (2%)</td>
<td>229 (60%)</td>
<td>380</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>7</td>
<td>14 (12%)</td>
<td>6 (5%)</td>
<td>1 (1%)</td>
<td>93 (82%)</td>
<td>114</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>19</td>
<td>46 (9%)</td>
<td>110 (23%)</td>
<td>8 (2%)</td>
<td>322 (65%)</td>
<td>494</td>
</tr>
</tbody>
</table>

*Non-pathogenic *Yersinia* isolates were identified by PCR to genus level only; *No outbreak was recorded in this region during summer; *Two mixed cultures of *Y. enterocolitica* and *Y. pseudotuberculosis* were detected in 2012; *No outbreak was recorded in this region during autumn; *Six mixed cultures of *Y. enterocolitica* and *Y. pseudotuberculosis* were detected in 2013 and one mixed culture detected in 2013.
Table 3.2. A summary of the number of investigations and faecal samples, worm egg counts (WEC) and number (%) of samples positive for each *Yersinia* species during outbreaks of winter scours during winter and spring in 2012 and 2013.

<table>
<thead>
<tr>
<th>Year</th>
<th>Region</th>
<th>Farm</th>
<th>No. of invest.</th>
<th>WEC (epg)</th>
<th>Number (%) of samples</th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Y. <em>enterocolitica</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Y. <em>pseudotuberculosis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Other <em>Yersinia</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Not <em>Yersinia</em></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>South-west Victoria</td>
<td>A</td>
<td>5</td>
<td>267</td>
<td>17 (12%) 38 (27%) 0 (0%) 86 (60%)</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>2</td>
<td>211</td>
<td>7 (21%) 7 (18%) 0 (0%) 20 (61%)</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>1</td>
<td></td>
<td>0 (0%) 1 (14%) 2 (29%) 4 (57%)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>1</td>
<td></td>
<td>4 (50%) 2 (25%) 0 (0%) 2 (25%)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>2</td>
<td>10</td>
<td>3 (7%) 12 (27%) 3 (7%) 26 (59%)</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>1</td>
<td></td>
<td>6 (75%) 0 (0%) 0 (0%) 2 (25%)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>X</td>
<td>2</td>
<td>0</td>
<td>1 (1%) 6 (9%) 1 (1%) 60 (88%)</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Gippsland, Victoria</td>
<td>B</td>
<td>2</td>
<td>401</td>
<td>1 (3%) 0 (0%) 1 (3%) 39 (94%)</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>1</td>
<td>790</td>
<td>3 (6%) 30 (61%) 0 (0%) 10 (20%)</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>2</td>
<td>597</td>
<td>3 (9%) 14 (42%) 1 (3%) 15 (46%)</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K</td>
<td>1</td>
<td>0</td>
<td>0 (0%) 0 (0%) 0 (0%) 30 (100%)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>J</td>
<td>1</td>
<td>445</td>
<td>1 (3%) 1 (3%) 0 (0%) 28 (94%)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TOTAL</td>
<td>21</td>
<td></td>
<td>46 (9%) 111 (23%) 8 (2%) 322 (66%)</td>
<td>487</td>
</tr>
<tr>
<td>2013</td>
<td>South-west Victoria</td>
<td>A</td>
<td>1</td>
<td>413</td>
<td>1 (2%) 25 (56%) 0 (0%) 19 (42%)</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>1</td>
<td></td>
<td>0 (0%) 12 (92%) 0 (0%) 1 (8%)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>1</td>
<td></td>
<td>0 (0%) 1 (10%) 0 (0%) 9 (90%)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>1</td>
<td></td>
<td>0 (0%) 0 (0%) 0 (0%) 10 (100%)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q</td>
<td>1</td>
<td></td>
<td>9 (90%) 0 (0%) 0 (0%) 1 (10%)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>1</td>
<td>360</td>
<td>2 (7%) 8 (29%) 0 (0%) 18 (64%)</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U</td>
<td>2</td>
<td>205</td>
<td>10 (38%) 11 (42%) 0 (0%) 5 (19%)</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W</td>
<td>1</td>
<td>0</td>
<td>0 (0%) 0 (0%) 0 (0%) 20 (100%)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Gippsland, Victoria</td>
<td>L</td>
<td>1</td>
<td></td>
<td>0 (0%) 0 (0%) 0 (0%) 4 (100%)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>1</td>
<td>155</td>
<td>10 (50%) 0 (0%) 0 (0%) 10 (50%)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>North-east Victoria</td>
<td>B</td>
<td>1</td>
<td>2015</td>
<td>0 (0%) 11 (55%) 0 (0%) 9 (45%)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>2</td>
<td>470</td>
<td>26 (48%) 10 (19%) 0 (0%) 18 (33%)</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>New South Wales</td>
<td>M</td>
<td>1</td>
<td>413</td>
<td>11 (37%) 0 (0%) 0 (0%) 19 (63%)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>1</td>
<td></td>
<td>0 (0%) 0 (0%) 1 (10%) 9 (90%)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>O</td>
<td>1</td>
<td></td>
<td>9 (47%) 0 (0%) 0 (0%) 10 (52%)</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V</td>
<td>1</td>
<td></td>
<td>8 (40%) 0 (0%) 0 (0%) 12 (60%)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>18</td>
<td>86 (25.4%)</td>
<td>78 (23%) 1 (0.3%) 174 (51.3%)</td>
<td>339</td>
<td></td>
</tr>
</tbody>
</table>

*a Only farms with outbreak investigations during winter or spring are reported in this table; *Arithmetic means reported; *Mixed infections with *Y. enterocolitica* and *Y. pseudotuberculosis* are not reported in this table.*
Table 3.3. The proportion (%) of *Y. enterocolitica* and *Y. pseudotuberculosis* isolates, collected during outbreak investigations of winter scouring, that are resistant to ampicillin, tetracycline, trimethoprim and sulphafurazole.

<table>
<thead>
<tr>
<th>Region</th>
<th>Farm</th>
<th>No. tested</th>
<th>AMP(^a)</th>
<th>TETRA(^b)</th>
<th>TRIM(^c)</th>
<th>SULPH DISC(^d)</th>
<th>SULPHA AGAR(^e)</th>
<th>No. tested</th>
<th>AMP(^a)</th>
<th>TETRA(^b)</th>
<th>TRIM(^c)</th>
<th>SULPH DISC(^d)</th>
<th>SULPHA AGAR(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>South-west Victoria</td>
<td>A</td>
<td>43</td>
<td>30%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>20%</td>
<td>16</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>50%</td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>20</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>100%</td>
<td>6</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>33%</td>
<td>83%</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>20%</td>
<td>40%</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>4</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>50%</td>
<td>100%</td>
<td>3</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>33%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Q</td>
<td>7</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>100%</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>2</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>50%</td>
<td>0</td>
<td>4</td>
<td>25%</td>
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<td>0%</td>
<td>50%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>6</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>100%</td>
<td>5</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>100%</td>
</tr>
<tr>
<td>Gippsland, Victoria</td>
<td>H</td>
<td>3</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>33%</td>
<td>8</td>
<td>0%</td>
<td>0%</td>
<td>13%</td>
<td>38%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>5</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>100%</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>7</td>
<td>71%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>71%</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>33%</td>
<td>67%</td>
</tr>
<tr>
<td>North-east Victoria</td>
<td>C</td>
<td>43</td>
<td>38%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>25%</td>
<td>4</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>North-central Victoria</td>
<td>R</td>
<td>3</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>100%</td>
<td>9</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>22%</td>
<td>78%</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td>0%</td>
<td>0%</td>
<td>27%</td>
<td>18%</td>
<td>100%</td>
</tr>
<tr>
<td>New South Wales</td>
<td>J</td>
<td>1</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>100%</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>5</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>20%</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>5</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>20%</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>6</td>
<td>100%</td>
<td>33%</td>
<td>0%</td>
<td>0% (0%)</td>
<td>0</td>
<td>0(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td>160</td>
<td>89%</td>
<td>2%</td>
<td>0%</td>
<td>8% (0%)</td>
<td>64%</td>
<td>89</td>
<td>10%</td>
<td>0%</td>
<td>3%</td>
<td>30%</td>
<td>87%</td>
</tr>
</tbody>
</table>

\(^a\) Ampicillin 25 µg disc; \(^b\) Tetracycline 10 µg disc; \(^c\) Trimethoprim 5 µg disc; \(^d\) Sulphafurazole 300 µg disc; \(^e\) Sulphafurazole serial agar dilution technique: 1-1024 µg/ml, breakpoint 64 µg/ml. \(^f\) No isolates were available for testing.
3.5. DISCUSSION

This cross-sectional study investigated 45 outbreaks of diarrhoea (‘scouring’) in Merino weaners, in 24 flocks in south-eastern Australia. The aim was to determine the primary cause of diarrhoea in weaner sheep during the winter that was not caused by gastrointestinal parasites (‘winter scours syndrome’). The most frequently isolated pathogens were Y. pseudotuberculosis serotype III and virulent Y. enterocolitica. No Salmonella species were isolated and only one outbreak was associated with Campylobacter species. From this, we conclude that bacterial enteritis caused by Yersinia species is a major contributor to the winter scour syndrome. These outbreaks were not a random sample, but sourced from a network of producers with a known history of scouring in post-weaned lambs. However, these results support previous case series investigations in this region, which identified pathogenic Yersinia species as an important cause of diarrhoea of young sheep during the winter (Slee and Button, 1990a; Slee and Button, 1990b; Philbey et al., 1991), but this is the first cross-sectional study to provide a firm link between the winter scour syndrome and Yersinia species.

In this study, a similar proportion of outbreaks of diarrhoea in Merino weaners were associated with Y. pseudotuberculosis and Y. enterocolitica. This was in contrast to previous studies, which found that enteritis associated with Y. enterocolitica was either uncommon or sporadic, and that Y. pseudotuberculosis was the primary organism associated with the winter scour syndrome (McSporran et al., 1984; Slee and Button, 1990a; Philbey et al., 1991). In the current study, the molecular techniques used to identify pathogenic Yersinia species were far more specific than the biochemical methods used previously and the results from this study indicate that Y. enterocolitica may play a far more important role in outbreaks of diarrhoea in young sheep than previously recognised.

Inclement weather, recent stress from shearing, poor nutrition, high challenge with gastrointestinal nematode larvae and higher stocking rates have been hypothesised as risk factors for outbreaks of this syndrome (Slee and Skilbeck, 1992; Gill, 1996). Although identification of specific risk factors was beyond the scope of the present study, the timing
of outbreaks, as well as their association with season, region, climate and burdens of gastrointestinal nematodes, was assessed.

Previous research has found that the faecal shedding of *Y. pseudotuberculosis* is highest during winter and spring, whereas the shedding of *Y. enterocolitica* is less seasonal, with peaks in both spring and summer (Slee and Skilbeck, 1992). The high prevalence of shedding of *Yersinia* species during winter has previously been associated with periods of severe cold, wet weather, but no such clear relationship was established in this study. However, it is likely that the cumulative effects of weather, such as wind chill, low temperatures and increased rainfall, together with management or nutritional factors, form complex interactions that are more significant than any single risk factor.

It was not possible to determine peak shedding periods for either organism during the current study, since the investigation concentrated primarily on outbreaks of diarrhoea occurring during the winter and spring. However, information from the present study provides evidence that *Y. enterocolitica*, as well as *Y. pseudotuberculosis*, can cause outbreaks of bacterial enteritis and diarrhoea during the winter and spring. In the summer and autumn, however, outbreaks of scouring were predominantly associated with *Y. enterocolitica*. This indicates that *Y. pseudotuberculosis* is unlikely to cause diarrhoea in summer or autumn, whereas, under suitable conditions, *Y. enterocolitica* can cause disease throughout the year.

These observations suggest a different epidemiology for the two *Yersinia* species, including how they are maintained within a population of sheep and circulate to initiate an annual recurrence of disease. The ability of pathogenic *Yersinia* species to survive in the environment, or establish a latent infection, may contribute to such differences.

For example, a recent laboratory study investigating the effect of high and low temperatures and a lack of moisture on the survival of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* in soil, found the latter species was significantly more resilient (Thomson, 2014). Both species survived fixed and fluctuating temperatures ranging from -20°C to 20°C for more than 40 days, but when soil temperature was fixed at 20°C, *Y.
*Enterocolitica* survived only 14 days. When soil temperature was fixed at 40°C, a temperature frequently exceeded in faecal masses in this region during the summer, neither species survived for more than 48 hours.

From these observations, and the typical pattern of a progressively increasing rate of cases during an outbreak of winter scours, it is likely that pasture contamination and environmental survival during cooler months might play an important role in oral-faecal cycling of *Yersinia* within affected populations of sheep. Consequently, moving affected mobs onto low risk pastures (those with reduced risk of gross contamination with *Yersinia* species) may be a suitable adjunct to antibiotic treatment to control a disease outbreak during the winter.

During the summer and autumn, outbreaks were only associated with *Y. enterocolitica*. Since this organism was detected in faecal samples during this period, oral-faecal infection may contribute to circulation of disease. However, given that *Y. enterocolitica* survives in the environment for only a short time in temperatures above 20°C, it is expected that outbreaks would most likely occur when a high proportion of animals were shedding the bacteria and when stocking rates are high. Since the detection of *Y. pseudotuberculosis* in faeces was negligible during summer and autumn, and that environmental survival was also poor at high temperatures, circulation of this organism may rely on ‘carrier’ animals or a maintenance host.

In addition to differences in the seasonal shedding of *Yersinia* species, this study demonstrated that the nature of outbreaks varied between year, region and farm. However, due to the relatively small number of outbreaks investigated during this study and that only 5 farms reported outbreaks in consecutive years, no firm conclusions can be made about such variability.

This syndrome is most often reported in Victoria, but is endemic throughout the high rainfall regions of south-eastern Australia, including NSW. The prevalence of shedding of both *Yersinia* species was markedly higher in Victoria than in NSW, although sampling bias associated with distance and access to farms during an outbreak, may have
influenced results. However, these results may indicate that the winter scours syndrome in NSW is different to that which occurs in Victoria or, alternatively, that too few ‘typical’ outbreaks were investigated in NSW. A prospective epidemiological study to assess and compare patterns of excretion and specific risk factors associated with Yersinia species in each region would be needed to establish this relationship.

This study also provided some evidence in support of a previous association between total worm burden as indicated by high WECs and bacterial enteritis caused by Yersinia species (McSporran et al., 1984; Philbey et al., 1991). The isolation of pathogenic Yersinia species was greatest when WECs exceeded 500 epg, but there were too few observations to clearly define this relationship. In young sheep (<9mo), the quantification of WEC is a reliable, indirect indicator of total worm burden, however this relationship does weaken following anthelmintic treated and in sheep greater than 12 months of age (Niven et al., 2002b). Consequently, further studies that examine WEC as a risk factor for bacterial enteritis caused by Yersinia species, are needed.

Results from this study indicate that reaching a definitive diagnosis for clinical cases of weaner scours is difficult. In most cases, the microscopic changes observed for animals euthanased for necropsy were indicative of lymphocytic-histiocytic enteritis, and typical abscessation lesions associated with Yersinia species infection, including mucosal abscessation in distal small intestine and colon (Philbey et al., 1991; Jubb and Grant, 2016), were present in only 2 of 14 cases. The reasons for this are unclear, although only 1 to 3 animals were sacrificed for a detailed post mortem examination and this was usually done early in an outbreak. This highlights the importance of quantifying bacterial growth when investigating outbreaks of diarrhoea, as this can help to determine the biological significance of isolating pathogenic organisms. Investigations of winter scours should therefore include a culture growth score for Salmonella, Campylobacter, as well as for Yersinia species. Taken together, or by themselves, neither histology, nor culture results without growth scores provide enough information to accurately interpret results.

Historically, the treatment of winter scours was not often based on a definitive diagnosis. Typically, affected sheep are treated first with an anthelmintic because the diarrhoea is
assumed to be associated with gastrointestinal parasites. When animals do not respond to this treatment, they are often treated with sulphonamides and, on some farms, the severity and recurrence of outbreaks results in multiple antibiotic treatments each year.

To date, very few studies have investigated the antimicrobial sensitivity of *Yersinia* species isolated from sheep. Our results are similar to those reported by Philbey et al (1991), although a higher proportion of *Y. enterocolitica* were resistant to ampicillin and sulphonamide and fewer resistant to tetracycline. Similarly, a large proportion of *Y. pseudotuberculosis* isolates were resistant to sulphonamide, but fewer were resistant to ampicillin and none resistant to tetracycline. Our results are probably a more accurate estimate of antimicrobial resistance, because a larger number of *Yersinia* species isolates, from a wide geographical area were tested, compared with the study conducted om 1991 by Philbey *et al* (n= 202 vs n=44, respectively).

In this study, the compound to which *Yersinia* species had developed resistance, as well as the proportion of resistant isolates, varied markedly between farms. This was not investigated in detail, but may be associated with the frequency and severity of previous outbreaks, the strategy for antibiotic use (e.g. all animals or only those affected were treated) and the compound most often used on each farm. Given the extremely high proportion of *Yersinia* species resistant to sulphafurazole, an innate rather than acquired resistance to this antibiotic may have contributed to this result. Irrespective of the mechanism, these results indicate that sulphonamides are unlikely to be effective in many cases, and should therefore not be recommended for the routine treatment of yersiniosis. Given that resistance to tetracycline was not common, this may be a more appropriate choice for the treatment of bacterial enteritis associated with *Yersinia* species. However, to minimise the development of resistance, it might be better to use this broad spectrum drug strategically, treating only clinically affected animals rather than the whole mob, unless a very high proportion have diarrhoea and are ill.

Whenever possible, and in all cases that do not respond to treatment, bacterial culture and sensitivity testing should be undertaken to determine the most suitable treatment. Given the difficulties interpreting the disc diffusion techniques for assessing resistance to the
sulphonamides, and large discrepancy between the disc and agar diffusion plating results, sensitivity testing for these compounds should be based solely on agar dilution methods.

3.6. CONCLUSIONS

Yersinia pseudotuberculosis and Y. enterocolitica were most likely the primary organisms responsible for outbreaks of the winter weaner scours syndrome reported by producers and a network of veterinary advisors in the high rainfall regions of Victoria, but their role in NSW was less clear. Outbreaks associated with Y. pseudotuberculosis occurred only in winter. However, those associated with Y. enterocolitica occurred throughout the year, and so this organism should be included as a differential diagnosis for outbreaks of scouring in Merino weaners at all times. The frequency and severity of outbreaks varied between year, region and farm, with no specific risk factors identified during this study. However, it is likely that contamination of pasture with Yersinia species during the winter plays an important role in establishing new infections through oral-faecal cycling. Consequently, decreasing the stocking rate and moving affected mobs onto pastures with a lower risk of bacterial contamination are simple management strategies that could be important adjuncts to antibiotic treatment.

Given the results of the antimicrobial resistance testing, the continued use of sulphonamides for the treatment of bacterial enteritis associated with the winter scours syndrome is not recommended. Rather, the targeted use of tetracycline, together with the management interventions discussed above, are likely to be a more effective and sustainable approach. High burdens of adult worms and challenge with infective larvae of gastrointestinal nematodes may contribute to development of winter scours, but this relationship was not clear in the 28 outbreaks we investigated that had WEC data. Further studies on the environmental, management and animal factors that increase the prevalence or severity of disease are needed. These will provide a more complete understanding of the epidemiology of this syndrome and enable the development of more effective control strategies.
CHAPTER 4  A LONGITUDINAL STUDY OF FAECAL SHEDDING OF Y. ENTEROCOLITICA AND Y. PSEUDOTUBERCULOSIS BY MERINO WEANERS IN SOUTH-EAST AUSTRALIA

4.1. ABSTRACT

A longitudinal study was conducted to investigate potential risk factors for faecal shedding of *Yersinia enterocolitica* and *Y. pseudotuberculosis* by Merino lambs in four flocks in south-eastern Australia. The aims of the study were determine the seasonal patterns of shedding of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*, and to evaluate putative risk factors for faecal shedding of these organisms, including WEC, live-weight and growth rate.

The risk of shedding each *Yersinia* species varied between farm, season and year. Shedding of *Y. pseudotuberculosis* occurred predominately in winter, whereas *Y. enterocolitica* was commonly isolated from faeces throughout the year. Moderate to high prevalences of shedding of each organism occurred in the absence of clinical outbreaks of bacterial enteritis. In general, animals with moderate or high WECs were at an increased risk of shedding both organisms compared with animals with low WECs. Sheep with higher average daily weight gains were at decreased risk of shedding *Y. enterocolitica* but increased risk of shedding *Y. pseudotuberculosis*. Live-weight was not significantly associated with risk of shedding either species.

This study highlighted that exposure to determinants of shedding *Y. enterocolitica* and *Y. pseudotuberculosis* differed between farms and over time within farm. Shedding was likely influenced by environmental, animal and management factors. We propose that risk the factors for clinical disease are different to those that cause faecal shedding of *Yersinia*, because moderate to high prevalences of shedding were not always associated with outbreaks of clinical disease.

4.2. INTRODUCTION

Fine wool production in south-eastern Australia typically involves self-replacing Merino flocks grazing pasture and lambing in the winter and spring (July to September). Spring
lambing systems minimise the requirement for supplementary feeding, but can expose young lambs to inclement weather in winter and periods of limited feed availability and undernutrition during the following summer and autumn (Allworth, 1994). On some farms, lamb mortality between birth and weaning is as high as 30%, with most deaths associated with starvation and mis-mothering within the first 48 hours of life (Hinch and Brien, 2014). In the 12 months following weaning, the average mortality of Merino lambs was can be as high as 15% (range 4.5 - 26.8%), with poor nutrition, gastrointestinal parasitism and infectious diseases, including bacterial enteritis, accounting for the majority of these deaths (Campbell et al., 2009). In addition to mortalities, these conditions can also have a significant, long term impact on animal welfare and productivity.

In the high rainfall regions of south-eastern Australia, persistent diarrhoea of Merino weaners that is not responsive to anthelmintic treatment has been described (‘winter scours syndrome’) (Chapter 1). Sheep less than 16 months of age are most commonly affected and, during an outbreak, clinical disease and mortality can exceed 30% and 10%, respectively. Affected animals are lethargic and rapidly dehydrate.

Results from the outbreak investigation trial suggested that yersiniosis associated with Y. enterocolitica and Y. pseudotuberculosis could be the primary cause of the winter scours syndrome in Victorian Merino flocks (Chapter 3). This causal inference was made on the basis of histological lesions showing lymphocytic-histiocytic enteritis and some microabscessation, isolation of the organisms, a positive response to treatment with antibiotics and the absence of other infectious agents, including Salmonella and Campylobacter (Chapter 1Chapter 3). The precise costs and losses associated with yersiniosis are not known, but annual costs to the Australian sheep industry associated with bacterial enteritis, including mortalities, production losses and treatments, have been estimated at between $10-29 million (Sackett and Holmes, 2006; Lane et al., 2015).

On some farms, the syndrome recurs annually and multiple outbreaks can occur each winter. High stocking rate, inclement weather, increased total worm burden as indicated by high WECs and management activities including weaning or shearing are thought to increase the risk of an outbreak (Slee and Skilbeck, 1992; Gill, 1996). No clear association between any risk factor and the winter scours syndrome has been established, but it is believed to occur following complex interactions between multiple risk factors in the presence of the causative organism.
To date, few studies have investigated the epidemiology of yersiniosis in sheep. Consequently, current preventative and management strategies are based mainly on results from case investigations, but also extrapolated from a well-established knowledge of the epidemiology of yersiniosis in deer (Mackintosh and Henderson, 1984a; Mackintosh, 1993; Gill, 1996). In deer, yersiniosis most frequently occurs when there is limited pasture available, or when animals have been transported, yarded or handled, managed at high stocking rates or subjected to extreme climatic conditions. In these situations, prophylactic vaccination has an important role in the prevention and control of this disease (Mackintosh and Henderson, 1984a; Mackintosh, 1993). There is currently no vaccine for sheep, but in addition to the treatment of affected animals with antibiotics, strategies to manage yersiniosis during outbreaks have included decreasing the stocking rate and providing affected animals with pastures of a lower risk of contamination with *Yersinia*. However, the outbreak investigation study Chapter 3 demonstrated that over 80% of pathogenic *Yersinia* species tested were resistant to sulphonamides, the class of antibiotic most commonly used to treat yersiniosis in sheep (Table 3.3). This emphasized the need for a more detailed study to investigate patterns of and risk factors for the shedding of pathogenic *Yersinia* species by sheep, to enable the development of more effective management and prevention strategies.

In sheep, studies investigating the epidemiology of yersiniosis have assessed the prevalence of this organism in faeces as an indicator of infection within flocks, rather than simply the proportion of clinical cases (Slee and Button, 1990b; Slee and Skilbeck, 1992). The early clinical signs of yersiniosis are often subtle and indistinct, and so detection of faecal shedding may be an effective way to monitor large mobs of sheep and potentially pre-empt outbreaks of clinical disease. Modification of this approach, to include sampling of individual sheep within a flock, enables the detection of both passive shedding of *Yersinia* species as well as shedding associated with clinical disease. The use of culture growth scores as a semi-quantitative measure of shedding also provides information about the biological importance of the *Yersinia* species detected.

In addition to the detection and quantification of faecal shedding, scoring of faecal consistency has recently been used to evaluate the risk of heavy shedding of *Escherichia coli* in dairy heifers (Williams et al., 2015). This study found that the risk of shedding this organism in faeces was significantly higher in animals with high (more
liquid) faecal scores, and so this approach may be useful when evaluating the risk of heavy shedding of *Yersinia* in faeces.

The aims of this study were to determine seasonal patterns for the faecal shedding of pathogenic *Yersinia* species by sheep, and to evaluate some potential risk factors for this shedding. The diagnostic sensitivity of faecal consistency score as an indicator of heavy shedding was also assessed.

### 4.3. MATERIALS AND METHODS

#### 4.3.1. STUDY DESIGN

A prospective longitudinal panel study was conducted in farms that had reported outbreaks of yersiniosis in weaned lambs in the previous three years. Two farms in Victoria, in south-eastern Australia, were enrolled in 2012 (Farm B and C). They were visited three times at approximately 9-12 weekly intervals for six months between May and October. For farm B and C, lambing commenced in July or September, respectively, and lambs were weaned approximately 12 weeks after the start of weaning (2-3 months of age).

For visits in 2012, lambs initially aged 8 to 9 months, were weighed and faeces collected for microbiological culture and evaluation of WEC (Section 2.2). Two additional farms were enrolled in 2013 (Farm A and D). For farm A and D, lambing commenced in September and August, respectively, and lambs were weaned approximately 12 weeks after the start of weaning (2-3 months of age). In 2013, the four farms were visited six times at approximately 4-9 weekly intervals for ten months, from January to October, with the lambs initially aged 4 to 5 months. Lambs were balanced for gender in both years. This study was approved by the University of Melbourne Animal Ethics Committee (Approval no. 1212405.1).

The selection of farms was based on their average annual rainfall (>500 mm/year), flock size (>3000 breeding ewes), time of lambing (July and August) and history of a high prevalence of clinical yersiniosis in lambs. All farms had reported multiple outbreaks of yersiniosis in the past 3 years, with up to 30% of sheep affected and 10% mortalities.

In 2012, on a separate day on each farm, approximately at least fifty 6-7 month-old lambs were systematically selected from a larger mob as each sheep moved through a race. Selected lambs were identified using a radio frequency identification (RFID) ear
tag. At each visit, individual faecal samples were collected from each enrolled animal. The same procedures were used in 2013 where, on each farm, approximately 100 lambs were systematically selected and enrolled. To replicate the typical weaner management that occurs on large-scale farms, enrolled animals were grazed together with the larger mob of 400-500 lambs from which they were selected. At each visit, each enrolled animal was weighed using electronic scales and individual faecal samples collected for WECs and bacterial culture. An assessment of a sheep (weighing and faecal sampling) at a particular visit constituted a ‘sheep-sampling’. To eliminate inaccurate or unreliable weights, outlier live-weights (those more than 2 standard deviations from the mean) were excluded from analyses. Average daily rates of live-weight change (‘average daily gains’) were calculated for each sheep as the change in live-weight from one sampling to the next (live-weight at most recent sampling minus live-weight at the immediately preceding sampling for that flock) divided by the number of days between those samplings; average daily gains were calculated only for sheep with non-excluded live-weights at both samplings.

4.3.2. BACTERIOLOGY

Bacterial culture and molecular testing was undertaken using methods previously described (Section 2.5). The culture of Yersinia species was performed at 30°C with 5% CO₂ (without cold enrichment), using Cefsulodin Irgasan Novobiocin (CIN) selective agar plates (Edwards Pty Ltd, Australia) inoculated with a sterile cotton swab after it was immersed in faecal material. A less sensitive culture technique (46%, (Hussein et al., 2001) was selected to minimise the detection of very low numbers of Yersinia species that were unlikely to be biologically important, although as few as one colony of Yersinia species was detected using CIN agar. Suspect colonies were classified as Y. enterocolitica, Y. pseudotuberculosis, ‘other Yersinia’ (another member of the Yersinia genus), or negative (not Yersinia) using polymerase chain reaction (PCR) assays (Section 2.5.5). The specificity of the multiplex PCR was determined using purified DNA from two Yersinia strains and five strains of closely related bacteria (Section 2.5.5.1). Specific amplification products were detected for both Yersinia strains and no products were detected from amplification of Enterobacteriaceae strains.

In 2013, each sample was assigned a semi-quantitative culture growth score based on growth on CIN agar: (0) no growth or no colonies suspected to be Yersinia species, (1) growth of suspected Yersinia species in the primary streak, (2) growth of suspected Yersinia species extending into the first dilution streak, (3) growth of suspected Yersinia species extending into the second dilution streak, (4) growth of suspected Yersinia species into the third dilution streak (Section 2.5.2).
4.3.3. PARASITOLOGY

Individual WECs were performed based on the technique described by Anderson et al. (1991), with a minimum detectable limit of 15 eggs per gram (epg)(Section 2.5.1). Worm egg counts were categorised as either low (0-99 epg), moderate (100-299 epg) or high (>300 epg)(Larsen et al., 1994). These categories are consistent with those used by diagnostic parasitology laboratories in south-eastern Australia for areas in which *Haemonchus* is not an endemic parasite.

Faecal consistency of each sheep's sample was subjectively scored at the time of culture using a scale from 1 to 5: (1) firm pellet, (2) soft pellet, (3) soft faeces, (4) soft paste, (5) watery (Jacobson, 2006).

4.3.4. STATISTICAL ANALYSES

All analyses were conducted using Stata (version 13; StataCorp, Texas, USA). Four outcome variables were analysed. For each of *Y. enterocolitica* and *Y. pseudotuberculosis*, sheep samplings were classified for shedding (any growth and PCR positive, compared to no growth) and for heavy shedding (culture growth score of 3 or 4 and PCR positive, compared to a growth score of 0 to 2). Heavy shedding was analysed only for 2013 because culture growth scores were not recorded in 2012. Exact binomial confidence intervals for prevalences were calculated for each sampling within each farm using Stata's `-cii-` command.

The primary interest of this study was in both total and direct effects of each putative risk factor. Based on biological interactions and prior knowledge, a directed acyclic graph was developed to describe postulated causal interrelationships between exposure variables that could directly and/or indirectly affect the risk of shedding or the risk of heavy shedding of *Y. enterocolitica* or *Y. pseudotuberculosis* (Figure 4.1).

Only unidirectional arrows were used, and only exposure variables that were measured in the study were included. With directed acyclic graphs, the direct effect of a particular exposure variable is indicated by a single arrow directly linking the exposure and outcome variables while an indirect effect of that exposure variable is indicated by a pathway through one or more intervening variables to the outcome variable (Greenland et al., 1999). There can be more than one indirect pathway from a particular exposure variable to the outcome variable.

Thus, direct effects are direct only in the context of the exposure variables included in the directed acyclic graph; these effects may, in fact, be mediated by other
unmeasured variables. The total effect of an exposure variable is the sum of the direct and all indirect effects of that exposure on the outcome variable (Dohoo et al., 2009). Directed acyclic graphs are also used to identify confounders (Greenland et al., 1999; Shrier and Platt, 2008; Dohoo et al., 2009; Textor and Liskiewicz, 2011) including variables that become confounders through conditional associations (Shrier and Platt, 2008). Minimal sufficient adjustment sets for both total and direct effects of each exposure variable were identified using the DAGitty® software (Textor et al., 2011). A sufficient adjustment set is a set of variables that appropriately controls confounding of the association between the particular exposure variable of interest and the outcome variable, assuming the directed acyclic graph is valid and comprehensive. For direct effects, the sufficient adjustment set also includes variables from each indirect pathway between the exposure and outcome variables. This approach was used by (Hay et al., 2014) to assess the risk factors for bovine respiratory disease in Australian feedlot cattle. Minimal sufficient adjustment sets are shown in Table 4.1. Farm, day of year of sampling, live-weight at sampling, average daily gain since previous sampling, WEC and gender were incorporated into the directed acyclic graph and the effects of each subsequently assessed in separate multivariable generalised estimating equations (GEE) models.

Separate GEE models were fitted for each of the four outcome variables for each of 2012 and 2013, using the -xtgee- command in Stata. GEE models were selected because they can be validly used for analysing repeated measures data with a binary outcome variable using uncorrelated correlation structures. Additionally, GEE models were deemed more appropriate than generalised mixed models because GEE models provide population-average effect estimates rather than subject-specific estimates. Thus, they estimated the strength of association between each factor and the outcomes averaged across all sheep (ie across all clusters), rather than the strength of association for changing the exposure status within a particular sheep (Twisk, 2003; Dohoo et al., 2009). Population-averaged effect estimates were generally more appropriate given the context of the study, as they estimated how much the odds of shedding change with exposure across many sheep, rather than with exposure within any particular sheep.

The logit link and binomial error term were used. The individual sheep-sampling was the unit of analyses and the panel variable was the individual sheep. The within-sheep correlation structure was unstructured and the robust (i.e. sandwich or Huber-White) estimator of variance was used. Within-sheep correlation structures were obtained using the -estat wcorrelation- command in Stata.
Figure 4.1. Directed acyclic graph indicating postulated causal pathways between putative risk factors (farm, day of year, worm egg count (WEC), average daily gain (ADG), live-weight and gender) and from these to the occurrence of faecal shedding *Yersinia* species in Merino lambs.

Farm was included in all models as a fixed effect to account for clustering of sheep within farm. Interactions between day of year and farm were hypothesised *a priori* for all outcome variables, so main effects and interaction terms (farm by each of linear and quadratic terms for day of year) were fitted for all models. For live-weight, average daily weight gain and day of year, linearity in the logit was assessed using visual assessment of lowess (locally weighted regression) plots and after simultaneously fitting grand mean centred linear and quadratic terms. The relationships for average daily weight gain and day of year were further explored with fractional polynomial models and plots. Day of year and average daily weight gain were curvilinear in the logit, and low p-values for quadratic terms were low in at least one year, so both linear and quadratic terms were fitted. For live-weight, p-values for quadratic terms were high and lowess plots and fractional polynomial models did not indicate marked non-linearity in the logit so only the linear term was fitted. Worm egg count was highly right-skewed and was categorised with the same cut points as used for diagnostic testing by our laboratory. Odds ratios were calculated to give an estimate of risk for total and direct effects for each variable.
Table 4.1. Minimal sufficient adjustment sets for estimation of total and direct effects of each exposure variable on the shedding of *Yersinia* species (these sets were based on the directed acyclic graph shown in Figure 4.1).

<table>
<thead>
<tr>
<th>Exposure variable</th>
<th>Description/categories</th>
<th>Total effect</th>
<th>Direct effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worm egg count (WEC)</td>
<td>Low: 0-99 epg&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Farm, day of year (both linear and quadratic terms)</td>
<td>Farm, day of year (both linear and quadratic terms), gender, ADG (both linear and quadratic terms)</td>
</tr>
<tr>
<td></td>
<td>Mod: 100-300 epg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High: &gt;300 epg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Farm, day of year (both linear and quadratic terms)</td>
<td>Farm, day of year (both linear and quadratic terms), WEC, ADG (both linear and quadratic terms), live-weight at sampling</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average daily weight gain (ADG)</td>
<td>Average grams/day change in live-weight since last sampling</td>
<td>Farm, day of year (both linear and quadratic terms), WEC, gender</td>
<td>Farm, day of year (both linear and quadratic terms), WEC, gender, live-weight at sampling</td>
</tr>
<tr>
<td>Live-weight at sampling</td>
<td>Live-weight (kg)</td>
<td>Farm, day of year (both linear and quadratic terms), gender, ADG (both linear and quadratic terms)</td>
<td>Same as for total effect.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Clustering of sheep-sampling within sheep was accounted for by fitting sheep as the panel variable in each GEE model; <sup>b</sup>Eggs per gram

Faecal consistency was postulated to be an effect, rather than a cause, of shedding, and so its usefulness as a marker for *Yersinia* shedding was also assessed using GEE models as described above. In young sheep (>12 months old), WEC is positively associated with faecal consistency and was associated with some of the *Yersinia* shedding outcome variables. Accordingly, WEC was fitted as a covariate to remove any confounding due to this variable. Farm and day of year (both linear and quadratic terms) were also fitted, to remove any confounding due to these variables; farm was fitted to also account for clustering of sheep within farm.

Diagnostic sensitivity of faecal consistency scores for detecting heavy shedding, using cut points of 3 and 5, was assessed as the proportion of heavy shedding sheep-samplings where the faecal consistency score was at or above the cut point. Confidence intervals accounting for clustering of sheep-sampling within farm were
calculated using Stata's -svy: proportion- command; farm was the primary sampling unit and Taylor-linearized variance estimation was used.

4.4. RESULTS

4.4.1. NUMBER OF SHEEP-SAMPLINGS, DEATHS AND OVERALL BACTERIOLOGY RESULTS

A total of 520 sheep were enrolled in the study; 147 in 2012 (53 and 94 on farms B and C, respectively) and 373 in 2013 (95, 93, 91 and 94 on farms A to D, respectively). These contributed 357 and 1896 sheep-samplings, and 39 and 72 sheep were absent at the final visit, in 2012 and 2013, respectively. An additional 53 and 155 sheep were missing at one sampling but then present at a subsequent sampling, in 2012 and 2013, respectively.

In 2012, both farms had outbreaks of scouring and mortalities between the second sampling in July and the third sampling, 81 to 90 days later in October, with 8% and 9% of sheep in the study mobs dying on farms B and C, respectively. In 2013, a high prevalence of faecal shedding of *Y. enterocolitica* in July (22 of 40 faecal samples cultured) was associated with clinical disease in the mob containing the study sheep on farm C. In addition, outbreaks of scouring associated with faecal shedding of *Y. pseudotuberculosis* occurred in mobs not containing the study sheep on farms A, B and C in Jul, Sep and Aug, respectively.

Of the 2253 sheep-samplings, results from CIN culture were available for all samples. Corresponding PCR results when isolates suspected to be *Yersinia* species were detected were available for 577 (88%) of the 648 *Yersinia*-positive samples. Of these, *Y. enterocolitica* was confirmed by PCR in 37% (41 of 110) and 45% (210 of 467) of samples, and *Y. pseudotuberculosis* in 50% (55 of 110) and 41% (191 of 467) of samples, in 2012 and 2013, respectively. Both species were detected at the same time on 8 (1.4%) of the 567 sheep-sampling occasions.

4.4.2. LIVE-WEIGHT

The average live-weight and daily weight gain at each sampling for both years of the study are summarised in Table 4.2. In 2013, the live-weight measurements could not be matched to all individual animals on farm D, and so these were not included in the statistical analysis.
In 2012, animals on farm B were always heavier than those on farm C. The sheep on farm C lost weight between May and July (-21 g/head/day), but had a higher average daily weight gain between July and October than those on farm B (125 vs. 75 g/head/day). Within each farm the average live-weights were similar in each year, except on farm C in May when animals were 4 kg heavier in 2012 (29.0 kg versus 25.0 kg in 2013). In 2013, the sheep on farm B were always the heaviest, whereas those on farm A were always the lightest. However, the study mob on farm A was the only one to have a positive average daily weight gain at every visit (from 10 g/head/d in Mar to 73 g/head/d in July). On all other farms the average daily weight gain varied considerably between January and July, and was negative on 4 of 9 occasions over the four farms. However for all farms, all mobs had positive average daily gains from July to Sept (range 58 to 174 g/head/d, Table 4.2).

Table 4.2. Live weight and average daily weight gain (ADG) since last sampling for Merino lambs on Victorian farms in 2012 (2 farms) and 2013 (4 farms).

<table>
<thead>
<tr>
<th>Year</th>
<th>Farm</th>
<th>Live weight (kg; mean (sd))</th>
<th>ADG since previous sampling (g/day; mean (sd))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Jan</td>
<td>Feb</td>
</tr>
<tr>
<td>2012</td>
<td>B</td>
<td>33.0(5.1)</td>
<td>34.0(5.5)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>21.9(3.0)</td>
<td>27.6(3.7)</td>
</tr>
<tr>
<td>2013</td>
<td>A</td>
<td>18.7(2.6)</td>
<td>21.2(2.7)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>27.6(4.8)</td>
<td>29.9(4.4)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>22.5(2.7)</td>
<td>24.7(3.0)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>22.2(2.0)</td>
<td>24.5(2.8)</td>
</tr>
</tbody>
</table>

- *Animals were aged 3 to 5 months on 1st January; These data were not included in statistical analyses because they could not be matched to individual sheep*

**4.4.3. BACTERIOLOGY**

In 2012, *Y. enterocolitica* was detected on more occasions, and a higher proportion of sheep were shedding this organism on farm B compared with farm C (47% versus 23% of sheep-samplings, Figure 4.2). The sampling prevalence of *Y. pseudotuberculosis* was usually less than for *Y. enterocolitica* on both farms, except when 89% of sheep were shedding *Y. pseudotuberculosis* on farm B in July.
In 2013, the number of sampling occasions that *Y. enterocolitica* was detected, and the proportion of animals shedding, varied considerably between the four farms (Figure 4.3). The prevalence was highest on farm A, with 5 to 32% of animals shedding this organism at each visit. *Yersinia enterocolitica* was also detected at most visits on farms B and D, although the prevalence of shedding was more variable and generally less than on farm A. On farm C, *Y. enterocolitica* was detected at only 2 of 6 visits, with the highest prevalence of shedding in June (65%). The highest proportion of heavy shedders of *Y. enterocolitica* coincided with the highest proportion of total shedders on all farms except farm B.

In 2013, *Y. pseudotuberculosis* was detected on fewer occasions than *Y. enterocolitica* on all farms. The isolation of *Y. pseudotuberculosis* occurred exclusively in winter and early spring (June to Sept, Figure 4.4), except for a low proportion of animals that were shedding the organism on farm B in May (1%).

The highest prevalence of faecal shedding of *Y. pseudotuberculosis* occurred on farm A (July, 85%), with 73% of animals classified as 'heavy' shedders at this time. The prevalence of *Y. pseudotuberculosis* shedding was also high on farm C in August, (57%), although only 8% of these were classified as heavy shedders.

On farm C, the highest prevalence of faecal shedding of both *Y. enterocolitica* and *Y. pseudotuberculosis* occurred in winter. On the other three farms, the prevalence of *Y. enterocolitica* shedding was similar throughout the year, whereas the peak for shedding of *Y. pseudotuberculosis* occurred between July and August.
Figure 4.2. Prevalence of faecal shedding (proportion of mob shedding) of *Yersinia enterocolitica* (orange bars) and *Yersinia pseudotuberculosis* (blue bars) by Merino sheep from two flocks in south-eastern Australia in 2012. The same sheep were sampled at each sampling other than those that were culled or died during the study, with from 40 to 94 sheep sampled on each farm at each sampling (bars indicate exact binomial 95% confidence intervals and a red arrow indicates an outbreak of yersiniosis in the trial mob).
Figure 4.3. Prevalences of light (CGS 1-2; orange sections) and heavy (CGS ≥3; blue sections) faecal shedding of *Yersinia enterocolitica* by Merino lambs from four flocks in Victoria, Australia, in 2013. The same sheep were sampled at each sampling other than those that were culled or died during the study with 74 to 94 sheep were sampled on each farm at each sampling (bars indicate exact binomial 95% confidence intervals for total shedding of *Yersinia* species and a red arrow indicates an outbreak of yersiniosis in the trial mob).
Figure 4.4. Prevalences of light (CGS 1-2; orange sections) and heavy (CGS ≥3; blue sections) faecal shedding of *Yersinia pseudotuberculosis* by Merino lambs from four flocks in Victoria, Australia, in 2013. The same sheep were sampled at each sampling other than those that were culled or died during the study with 74 to 94 sheep were sampled on each farm at each sampling (bars indicate exact binomial 95% confidence intervals for total shedding of *Yersinia* species and a hatched arrow indicates an outbreak of yersiniosis in a non-trial mob).
4.4.4. MULTIVARIABLE GEE MODELS

The effects of each exposure variable on shedding and heavy shedding of *Y. enterocolitica* and *Y. pseudotuberculosis* are shown in Tables 4.3 and Table 4.4, and Table 4.5 and Table 4.6, respectively. Unless otherwise stated, the estimated total and direct effects of variables on each exposure outcome were similar or identical. The minimal adjustment sets for the total and direct effects of live-weight on each shedding outcome were identical.

Interactions between day of year and farm were hypothesised *a priori* for all outcome variables because differences in farm microclimate, animal management and husbandry were thought to impact the risk of shedding *Yersinia* species. For each year, estimation of these interactions was attempted for both organisms, and for each shedding outcome. In 2013, there was a strong interaction between farm and day of year on total shedding of *Y. enterocolitica*, but no other model converged. Consequently, only main effects of farm and day of year were fitted in all models and interpretations of these exposure variables were based on the descriptive data.

4.4.4.1. *Yersinia enterocolitica*

In each year, for both shedding and heavy shedding of *Y. enterocolitica*, sheep gaining weight had a moderately lower risk of shedding *Y. enterocolitica* than those not gaining weight (Table 4.3 and Table 4.4). This protective effect was larger in 2013. However, there was no significant effect of live-weight for any shedding outcome in either year.

In 2013, animals with high WECs unexpectedly had a decreased risk of shedding, with the odds ratios for total and direct effects being 0.7 and 0.5, respectively. When all sheep-samplings were pooled, the prevalence of faecal shedding for the low (0-100 epg) and high (> 300 epg) WEC categories on each farm were 22% and 13% (farm A), 3% and 0% (farm B), 12% and 3% (farm C) and 11% and 7% (farm D). Over all farms, the number of sheep in each WEC category varied from 216 to 366 sheep-samplings for the low, and from 58 to 114 sheep-samplings for the high WEC category. Assuming the directed acyclic graph shown in Figure 4.1 is valid; the similar odds ratios for total and direct effects indicate that the apparent protective effect of factors associated with a higher WEC was not mediated through average daily weight gain.

Males had a higher direct risk of shedding than females in 2012, but not in 2013. The point estimate for the total effect was smaller than for the direct effect, indicating that this effect was not mediated through average daily weight gain or live-weight.
4.4.4.2. Yersinia pseudotuberculosis

In 2012, sheep gaining an additional 50 g/head/day and those with 1 kg additional live-weight had a lower risk of shedding Y. pseudotuberculosis (Table 4.5). In contrast, in 2013 sheep gaining an additional 50 g/head/day had an increased risk for both shedding and of being a heavy shedder, although there was no significant effect of weight on either shedding outcome (Table 4.5 and Table 4.6). The risk of both shedding outcomes for Y. pseudotuberculosis was higher in sheep with high WECs in both years, and those with moderate or high WECs were more likely to be heavy shedders of Y. pseudotuberculosis compared with animals with low WECs.

Gender had no effect on the risk of either shedding outcome for Y. pseudotuberculosis.

4.4.4.3. Correlations

Within-sheep correlations between samplings were obtained after fitting farm, day of year (linear and quadratic terms) average daily weight gain (linear and quadratic terms), live-weight, gender and WEC. For each shedding outcome in both years, the within-sheep correlations were generally small (mostly between -0.5 and +0.23). These results indicate that, after accounting for these covariates, neither outcome for the shedding of Yersinia within individual sheep was consistent over time.

4.4.5. FAECAL CONSISTENCY

The estimated sensitivities of faecal consistency score with a cut point of greater than 3, to detect heavy shedding of Yersinia enterocolitica and Y. pseudotuberculosis, were 0.8 and 0.7, respectively (Table 4.7). The estimated sensitivities were close to zero for both shedding outcomes when using a faecal consistency score of 5 as the cut point, but all sensitivity estimates were imprecise.
Table 4.3. Estimated odds ratios for the total effects of exposure variables on the risk of shedding *Y. enterocolitica* by Merino sheep from flocks in Victoria, Australia in 2012 and 2013, adjusted for minimal sufficient adjustment sets detailed in Figure 4.1. Results for direct effects are also shown in brackets if these were largely different from the total effects.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Year and term or category</th>
<th>No. sheep-samplings&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean (SD)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total effect</th>
<th>95% confidence interval</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADG (Odds ratio for each additional 50 g/day)</td>
<td>2012</td>
<td>211</td>
<td>38</td>
<td>37 (72)</td>
<td>65 (69)</td>
<td>0.002</td>
</tr>
<tr>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadratic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>1428</td>
<td>147</td>
<td>28 (56)</td>
<td>62 (79)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadratic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live-weight (Odds ratio for each additional kilogram)</td>
<td>2012</td>
<td>293</td>
<td>40</td>
<td>36 (8)</td>
<td>34 (7)</td>
<td>1.0</td>
</tr>
<tr>
<td>2013</td>
<td>1823</td>
<td>208</td>
<td>23 (5)</td>
<td>28 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WEC (eggs per gram (epg))</td>
<td>2012</td>
<td>105</td>
<td>8</td>
<td>7.6%</td>
<td>Reference group</td>
<td>0.248</td>
</tr>
<tr>
<td>0-99 epg (low)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.4</td>
<td>0.5 to 3.8</td>
</tr>
<tr>
<td>100-300 epg (mod)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.3)</td>
<td>(0.1 to 1.3)</td>
</tr>
<tr>
<td>&gt;300 epg (high)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.2</td>
<td>0.8 to 5.9</td>
</tr>
<tr>
<td>2013</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.3)</td>
<td>(0.1 to 1.5)</td>
</tr>
<tr>
<td>Gender</td>
<td>2012</td>
<td>129</td>
<td>13</td>
<td>10.1%</td>
<td>Reference group</td>
<td>0.017</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>0.8 to 2.9</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.3)</td>
<td>(0.5 to 1.4)</td>
</tr>
<tr>
<td>2013</td>
<td>976</td>
<td>121</td>
<td>12.4%</td>
<td>Reference group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.7</td>
<td>0.5 to 1.0</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.7)</td>
<td>(0.5 to 1.0)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers and statistics are for sheep-samplings included in the total effects model for that exposure variable; <sup>b</sup>Bolded p-values are joint overall Wald p-values for the variable.
Table 4.4. Estimated odds ratios for the total effects of exposure variables on the risk of heavy shedding of *Y. enterocolitica* by Merino sheep from flocks in Victoria, Australia in 2013, adjusted for minimal sufficient adjustment sets detailed in Figure 4.1.

<table>
<thead>
<tr>
<th>Variable and category</th>
<th>No. sheep-samplings&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean (SD)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total effect</th>
<th>Adjusted odds ratio</th>
<th>95% confidence interval</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Heavy shedders</td>
<td>Crude %</td>
<td>Heavy shedders</td>
<td>Not heavy shedders</td>
<td></td>
</tr>
<tr>
<td>ADG (Odds ratio for each additional 50 g/day)</td>
<td>1882</td>
<td>90</td>
<td></td>
<td>133 (74)</td>
<td>145 (84)</td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadratic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live-weight (Odds ratio for each additional kilogram)</td>
<td>1822</td>
<td>89</td>
<td></td>
<td>23 (5)</td>
<td>27 (7)</td>
<td></td>
</tr>
<tr>
<td>WEC (eggs per gram (epg))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-99 epg (low)</td>
<td>1229</td>
<td>56</td>
<td>4.6%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100-300 epg (mod)</td>
<td>229</td>
<td>11</td>
<td>4.8%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;300 epg (high)</td>
<td>373</td>
<td>10</td>
<td>2.7%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>976</td>
<td>53</td>
<td>5.4%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>896</td>
<td>36</td>
<td>4.0%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers and statistics are for sheep-samplings included in the total effects model for that exposure variable; <sup>b</sup>Bolded p-values are joint overall Wald p-values for the variable.
Table 4.5. Estimated odds ratios for the total effects of exposure variables on the risk of shedding *Y. pseudotuberculosis* by Merino sheep from flocks in Victoria, Australia in 2012 and 2013, adjusted for minimal sufficient adjustment sets detailed in Figure 4.1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Year and term or category</th>
<th>No. sheep-samplings*</th>
<th>Mean (SD)*</th>
<th>Total effect</th>
<th>95% confidence interval</th>
<th>P**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Shedders</td>
<td>Crude %</td>
<td>Shedders</td>
<td>Non-shedders</td>
</tr>
<tr>
<td>ADG (Odds ratio for each additional 50 g/day)</td>
<td>2012&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>211</td>
<td>51</td>
<td>-13 (60)</td>
<td>84 (56)</td>
</tr>
<tr>
<td></td>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quadratic</td>
<td></td>
<td>1428</td>
<td>186</td>
<td>83 (67)</td>
<td>55 (78)</td>
</tr>
<tr>
<td>Live-weight (Odds ratio for each additional kilogram)</td>
<td>2012&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>293</td>
<td>51</td>
<td>28 (4)</td>
<td>36 (6)</td>
</tr>
<tr>
<td></td>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quadratic</td>
<td></td>
<td>1823</td>
<td>188</td>
<td>29 (6)</td>
<td>27 (7)</td>
</tr>
<tr>
<td>WEC (eggs per gram (epg))</td>
<td>2012&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>0-99 epg (low)</td>
<td>105</td>
<td>2</td>
<td>1.9%</td>
</tr>
<tr>
<td></td>
<td>100-300 epg (mod)</td>
<td></td>
<td></td>
<td>58</td>
<td>11</td>
<td>19.0%</td>
</tr>
<tr>
<td></td>
<td>&gt;300 epg (high)</td>
<td></td>
<td></td>
<td>119</td>
<td>40</td>
<td>33.6%</td>
</tr>
<tr>
<td></td>
<td>2013&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>0-99 epg (low)</td>
<td>1230</td>
<td>52</td>
<td>4.2%</td>
</tr>
<tr>
<td></td>
<td>100-300 epg (mod)</td>
<td></td>
<td></td>
<td>229</td>
<td>32</td>
<td>14.0%</td>
</tr>
<tr>
<td></td>
<td>&gt;300 epg (high)</td>
<td></td>
<td></td>
<td>373</td>
<td>87</td>
<td>23.3%</td>
</tr>
<tr>
<td>Gender</td>
<td>2012&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>Female</td>
<td>129</td>
<td>28</td>
<td>21.7%</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td></td>
<td></td>
<td>166</td>
<td>27</td>
<td>16.3%</td>
</tr>
<tr>
<td></td>
<td>2013&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>Female</td>
<td>976</td>
<td>96</td>
<td>9.8%</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td></td>
<td></td>
<td>897</td>
<td>94</td>
<td>10.5%</td>
</tr>
</tbody>
</table>

*Numbers and statistics are for sheep-samplings included in the total effects model for that exposure variable; Bolded p-values are joint overall Wald p-values for the variable; Convergence was not achieved for direct effect models.
Table 4.6. Estimated odds ratios for the total effects of exposure variables on the risk of heavy shedding of *Y. pseudotuberculosis* by Merino sheep from flocks in Victoria in 2013, Australia, adjusted for minimal sufficient adjustment sets detailed in Figure 4.1.

<table>
<thead>
<tr>
<th>Variable and category</th>
<th>No. sheep-samplings&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean (SD)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Heavy shedders</td>
<td>Crude %</td>
</tr>
<tr>
<td><strong>ADG (Odds ratio for each addition 50 g/day)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td>1428</td>
<td>94</td>
<td>81 (61)</td>
</tr>
<tr>
<td>Quadratic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Live-weight (Odds ratio for each additional kilogram)</strong></td>
<td>1823</td>
<td>95</td>
<td>27 (6)</td>
</tr>
<tr>
<td><strong>WEC (eggs per gram (epg))</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-99 epg (low)</td>
<td>1230</td>
<td>14</td>
<td>1.1%</td>
</tr>
<tr>
<td>100-300 epg (mod)</td>
<td>229</td>
<td>27</td>
<td>11.8%</td>
</tr>
<tr>
<td>&gt;300 epg (high)</td>
<td>373</td>
<td>47</td>
<td>12.6%</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>976</td>
<td>54</td>
<td>5.5%</td>
</tr>
<tr>
<td>Male</td>
<td>897</td>
<td>42</td>
<td>4.7%</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers and statistics are for sheep-samplings included in the total effects model for that exposure variable; <sup>b</sup>Bolded p-values are joint overall Wald p-values for the variable.
Table 4.7. Numbers of sheep-samplings with heavy shedding of *Yersinia enterocolitica* and *Y. pseudotuberculosis* and proportions of these that had faecal consistency scores greater than 3 out of 5 or equal to 5 out of 5 (i.e. diagnostic sensitivity of these faecal consistency categories for detection of heavy shedding of *Yersinia* species)

<table>
<thead>
<tr>
<th>Yersinia species and farm</th>
<th>No. sheep-samplings</th>
<th>Faecal consistency ≥3</th>
<th>Faecal consistency = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (No.)</td>
<td>Sensitivity</td>
<td>95% confidence</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>37</td>
<td>54.1% (17)</td>
<td>0.8</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>100% (1)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>32</td>
<td>96.9% (31)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>100% (5)</td>
<td></td>
</tr>
<tr>
<td>Y. pseudotuberculosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>68</td>
<td>57.4% (39)</td>
<td>0.7</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>100% (4)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>8</td>
<td>87.5% (7)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>8</td>
<td>100% (8)</td>
<td></td>
</tr>
</tbody>
</table>
4.5. DISCUSSION

A longitudinal trial was conducted on four farms to establish the seasonal variation in shedding of pathogenic *Yersinia* species, and to examine potential risk factors for faecal shedding of these organisms. It was found that a high proportion of animals could be shedding pathogenic *Yersinia* species in the absence of clinical disease, and not all mobs within a farm experienced clinical outbreaks of bacterial enteritis simultaneously. A number of factors that increased the risk of shedding were identified, but no factor was a single or predominant determinant of an outbreak.

Shedding of *Y. pseudotuberculosis* occurred predominately during the winter, whereas shedding of *Y. enterocolitica* was detected throughout the year. There was a significant effect of farm, indicating that the prevalence of shedding varied within farms between years. A positive average daily weight gain was associated with a reduced risk of shedding *Y. enterocolitica*, but paradoxically, an increased risk of shedding *Y. pseudotuberculosis*. However, the live-weight of an animal had no consistent influence on the risk of shedding of either organism.

Sheep with a moderate or high WEC generally had a greater risk of shedding each *Yersinia* species. However, in one year of the study (2013) the inverse of this relationship was observed, with the risk of shedding *Y. enterocolitica* being unexpectedly lower in sheep with a high WEC. Males had a higher risk of shedding *Y. enterocolitica* than females in 2012, but not in 2013.

The effect of farm on the shedding of *Yersinia* species was complex. Variation in seasonal patterns of shedding between farms was likely associated with differences in seasonal conditions and management, which were further compounded by WEC and average daily weight gain. This interaction between farm and day of year highlights the multifactorial nature of this syndrome, and strongly suggests that neither the prevalence of shedding nor the occurrence of an outbreak can be reliably predicted based on exposure to the risk factors identified in this study.

Historically, outbreaks of enteritis associated with *Y. enterocolitica* were thought to be sporadic and occur primarily in the warmer months (Slee and Button, 1990a; Slee and Button, 1990b; Philbey et al., 1991). However, results from the outbreak investigation study of the winter weaner scours syndrome associated with yersiniosis, found that the prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* in faeces was 13.6% and
18.5%, respectively (Chapter 3). Results from the present study further support this, demonstrating that, in flocks with a history of yersiniosis, *Y. enterocolitica* was commonly present in winter. Importantly, moderate prevalences of faecal shedding of *Y. enterocolitica* were also detected throughout the year. Thus, whilst *Y. enterocolitica* may cause outbreaks of disease during winter, it may also cause scouring at other times and so should be a differential diagnosis for scouring at any time of the year. Although outbreaks of yersiniosis appear to be more common in winter, scouring associated with the shedding of *Y. enterocolitica* in summer may also increase the risk of breech strike and contribute to production losses associated with ill-thrift (Morley et al., 1976).

The year round shedding of *Y. enterocolitica*, as opposed to the winter-dominant shedding of *Y. pseudotuberculosis*, suggests that the epidemiology of infection may differ between these organisms. For example, for *Y. enterocolitica*, sheep may be the maintenance host and continually contribute to environmental contamination and reinfection within affected mobs. In contrast, the winter-dominant shedding of *Y. pseudotuberculosis* suggests that some sheep, or another host, may be latent carriers of this organism during the summer. Alternatively, this species may survive better in the environment and reinfection occurs from this source.

In support of this, both *Yersinia* species were found to survive in faeces at temperatures below 20°C for over 40 days (Thomson, 2014). Therefore, the faecal contamination of pastures during winter, when average daily temperatures are consistently below 20°C, may be cumulative and contribute to oral-faecal infection, with the risk of an outbreak increasing over time. However, in south-eastern Australia, the environmental survival of either *Yersinia* species during a typical summer, when average daily temperatures are >20°C, is unlikely since neither species can withstand temperatures above 20°C for more than 48 hours (Thomson, 2014). Previous studies have indicated that feral cats, dogs, rats, mice, birds and rabbits may also contribute to pasture contamination with *Yersinia* species (Mackintosh and Henderson, 1984b; Fukushima and Gomyoda, 1991; Cork et al., 1995; Liang et al., 2015). However, the low numbers of these potential hosts cohabiting with sheep and the amount of faeces matter they produce, make their role in this syndrome less likely.

Live-weight at weaning and post weaning growth rate has previously been shown to have a significant impact on the survival of Merino lambs, with sheep less than 20 kg at weaning, and those failing to gain at least 1 kg per month, having a higher risk of death.
through any cause (Campbell et al., 2007; Hatcher et al., 2008; Campbell et al., 2009). Consequently, the absence of any significant association between live-weight and the risk of shedding *Yersinia* species in the current study, was unexpected. Weaning weights were not recorded, but the average live-weights at the first sampling visit, which was 2-3 months after weaning, were close to this critical target for most farms in 2013. Consequently, it was expected that the risk of faecal shedding of *Yersinia* species would be increased for lighter animals, but the results did not support this. For this study, live-weight rather than body condition score was recorded, because it is a more accurate and repeatable measure for immature animals (Foot et al., 1987; Allworth, 1994).

In the present study, average daily weight gain had a significant impact on the risk of shedding for both *Yersinia* species. A positive average daily weight gain was associated with a decreased risk of shedding *Y. enterocolitica*, but an increased the risk of shedding *Y. pseudotuberculosis*. The protective effect of average daily gain on shedding of *Y. enterocolitica* appears intuitive, but the negative association for *Y. pseudotuberculosis* is not. However, if this observation is correct, one explanation may be that the risk of shedding and infection with *Y. pseudotuberculosis* is related to the infective dose of bacteria ingested. Animals that are gaining weight will have a higher feed intake and therefore may ingest more bacteria. However, additional studies would be necessary to explore this hypothesis.

An association between moderate and high WEC and an increased risk of shedding *Yersinia* species was also identified in this study. In this region of Australia, *Teladorsagia circumcincta* and *Trichostrongylus* species, including *T. vitrinus*, *T. colubriformis* and *T. rugatus*, are the most important gastrointestinal parasites infecting sheep (Anderson et al., 1978). *Teladorsagia circumcincta* typically inhabit the gastric glands of the abomasum and cause destruction of parietal cells, leading to an increase in the pH of the abomasum and impaired protein digestion (Bown et al., 1989; Radostits et al., 2007; Williams and Palmer, 2012). *Trichostrongylus* species inhabit the proximal third of the small intestine causing villous atrophy and epithelial damage which impairs the absorptive capacity of the intestine (Beveridge et al., 1989; Bown et al., 1989). In contrast, *Yersinia* species typically cause erosive enteritis of the jejunum and ileum and congestion of the large intestine, and lesions are uncommon in the duodenum (Philbey et al., 1991; Jubb and Grant, 2016).
Given that *Yersinia* species and gastrointestinal parasites establish infection at anatomically separate sites, it is unlikely that the physical damage cause by the parasites facilitates infection with *Yersinia* species. However, elevated pH within the abomasum may favour the survival and passage of *Yersinia* species to the distal small intestine (Radostits et al., 2007). In addition, the risk factors associated with high burdens of gastrointestinal parasites and yersiniosis are similar. Each requires cool, moist conditions for environmental survival, and the risk of infection is increased by short pasture and increased stocking rate. However, the association between elevated WEC, indicating increased populations of nematodes, and decreased shedding of *Yersinia* species observed in this study remains unexplained and may simply be circumstantial. The correlation between WEC and total worm burden is reasonably strong in young sheep (<9mo), but does decrease following anthelmintic treatment and with age (Niven et al., 2002b).

Male sheep had an increased risk of shedding *Y. enterocolitica* in 2012. A similar effect has been reported in deer, with males 4-9 times more likely to develop yersiniosis than females, although no explanation was given for this observation (Mackintosh and Henderson, 1984a). In the present study, the direct effects of gender were larger than the total effects, suggesting that animal effects, such as genetic or hormonal differences, may have contributed to this finding. This gender effect was observed in one year for one shedding outcome and so, the importance of this finding is unclear.

In addition to the evaluation of risk factors, the diagnostic sensitivity of faecal consistency score was assessed as a predictor of heavy shedding of *Yersinia* species. This sensitivity was moderate when the cut point was 3, out of a score of 1 (firm pellets) to 5 (watery diarrhoea). It was extremely low when the cut point was 5, but there were only 9 animals with a FCS of 5. Thus, in the absence of clinical disease, faecal consistency will not be a sensitive indicator of yersiniosis because many other factors, including gastrointestinal parasite burden and nutrition, can affect this score (Larsen et al., 1999; Jacobson, 2006). Nevertheless, during an outbreak of scouring, there is evidence that bacterial culture is warranted when faecal consistency scores are 3 or more, but not when they are 1 or 2.

**4.6. CONCLUSIONS**

It was found that the prevalence of faecal shedding within a flock is not a reliable means to predict a clinical outbreak of winter scours. Shedding of *Y. pseudotuberculosis* occurred only during the winter, whereas *Y. enterocolitica* was
detected throughout the year. Consequently, *Y. enterocolitica* should be included as a differential diagnosis for diarrhoea in weaned Merino sheep at all times of the year.

There was a significant interaction between farm and day of year, highlighting the multifactorial nature of this syndrome and suggesting that no single risk factor can reliably predict the timing of an outbreak. Although environmental conditions, animal and management factors may partly explain this variation, differences between mobs within a farm also affected the risk of an outbreak. Not all mobs of weaners on the same farm experienced disease or, alternatively, different mobs experienced outbreaks at different times.

Of the risk factors that were evaluated in this study, WEC and average daily weight gain were found to affect the risk of shedding *Yersinia* species. For *Y. pseudotuberculosis*, the risk of shedding was increased when WECs were >300 eggs per gram. Therefore, management strategies that aim to decrease gastrointestinal parasite burden, such as grazing lower risk pastures and allocating animals to paddocks with more feed on offer, may have the added benefit of reducing the risk of yersiniosis in the winter. A positive average daily weight gain reduced the risk of shedding *Y. enterocolitica*. Paradoxically, this relationship was reversed for *Y. pseudotuberculosis*, with a negative average daily gain (weight loss) being associated with a decreased risk of shedding. Despite this, aiming for slow positive growth rates for Merino weaners until 16 months of age is still recommended (Hatcher et al., 2008; Campbell et al., 2009).
CHAPTER 5  THE EFFICACY OF AN AUTOGENOUS VACCINE AGAINST *Y. PSEUDOTUBERCULOSIS*

5.1. ABSTRACT

A study was conducted on two farms in Victoria to investigate the efficacy of an autogenous, bacterin vaccine against *Y. pseudotuberculosis* III in young Merino lambs. The aims of the study were to determine if vaccination affected the faecal shedding of *Y. pseudotuberculosis* and *Y. enterocolitica* or the growth rate of animals in the study. The humoral response to vaccination and natural exposure to *Yersinia* spp. was also evaluated.

In the absence of an outbreak of yersiniosis, there were no detectable effects of vaccination in this study. Vaccination did not affect the average live weight, average daily gain, the prevalence of faecal shedding of *Yersinia* species or the proportion of animals that were seropositive for *Yersinia* outer membrane proteins (*Yops*). Further studies are needed to determine the efficacy of this vaccine during an outbreak or following experimental challenge with pathogenic *Yersinia* spp.

5.2. INTRODUCTION

*Yersinia pseudotuberculosis* serotype III was found to be a significant contributor to outbreaks of the winter scours syndrome in weaned Merino sheep, isolated from 18.5% of 1020 faecal samples (Chapter 3). Similarly, a more detailed study of the epidemiology of winter scours on two farms found that outbreaks of yersiniosis were regularly associated with a high proportion (>10%) of sheep shedding this organism in their faeces (Chapter 3). Furthermore, a study of the resistance of *Yersinia* species to common antimicrobials found that there was significant resistance to sulphonamides (64-89%), the main compound used to treat this syndrome (Chapter 3). From this, it was concluded that there was an urgent
need to develop alternative treatment and prevention strategies for bacterial enteritis associated with yersiniosis.

In New Zealand, yersiniosis associated with *Y. pseudotuberculosis* is the most common infectious disease of farmed deer (Mackintosh and Henderson, 1984a; Mackintosh, 2001). This is a costly disease with morbidity, mortality and case fatality rates of up to 16%, 12% and 100%, respectively (Mackintosh and Henderson, 1984a; Mackintosh et al., 1992). Yersiniosis in deer is characterised by haemorrhagic enteritis and terminal septicaemia, and is typically more severe than the disease in sheep (Mackintosh and Henderson, 1984a). Consequently, ‘Yersiniavax’, a formalin-killed bacterin vaccine containing *Y. pseudotuberculosis* serotypes I, II and III, was developed and is widely used within the NZ deer industry (Mackintosh et al., 1992). During an outbreak of yersiniosis in weaned deer grazing pasture, vaccination decreased the average morbidity from 20% compared with 11% in the unvaccinated animals that were grazing with vaccinated deer, and reduced mortalities from 11% to 2% (Mackintosh et al., 1992).

Given that *Y. pseudotuberculosis* serotype III is involved in a large proportion of outbreaks in sheep (18.5%, Chapter 3), and that vaccination has been an effective management tool for the deer industry, an autogenous, formalin-killed bacterin vaccine was developed and evaluated as a potential control measure for yersiniosis in sheep. The aims of this study were to determine whether an autogenous vaccine consisting of killed *Y. pseudotuberculosis* serotype III reduced the faecal shedding of *Yersinia* species or positively affected live weight or the average daily weight gain of sheep, and to determine if animals developed antibodies following vaccination and after natural exposure to these organisms.

### 5.3. MATERIALS AND METHODS

The two farms which had the highest peak prevalence of shedding of *Yersinia pseudotuberculosis* during the study in Chapter 4 were enrolled in this study - one at Warrnambool in south-west Victoria (farm A, 86% shedding in Sept) and the other at Mansfield in north-east Victoria (farm C, 56% shedding in Aug). Lambs were weaned in
November and maintained as a single mob, balanced for gender for the duration of the trial. At the start of the trial, animals were between five and six months of age.

5.3.1. VACCINE DEVELOPMENT

A commercial vaccine containing strains of *Y. pseudotuberculosis* is available for use in deer in New Zealand, but this could not be imported for use in this trial. Additionally, this vaccine contained *Y. pseudotuberculosis* serotypes I, II and III and may not have been suitable for use in sheep. Consequently, two autogenous vaccines, one for each farm, were produced under an APVMA permit allowing small scale studies (Permit 7250). Each vaccine combined three strains of *Y. pseudotuberculosis* serotype III that were isolated from animals with yersiniosis on that farm the previous year. Two different vaccines were produced for each farm, one using standard laboratory techniques (‘STAND-vax’) and another produced under low calcium conditions (‘EDTA-vax’). The latter increases the secretion of *Yops*, potentially increasing the antigenicity of the vaccine (Heesemann et al., 1986).

5.3.1.1. Vaccine manufacture

Isolates of *Yersinia* were pooled to make a composite culture for each farm and these were incubated at 26°C overnight in trypticase soy broth (TSB). The culture for the STAND-vax was diluted 1:20 with TSB and incubated at 30°C until the bacterial suspension reached an optical density at 600 nm (OD$_{600}$) of 0.9, which was estimated to include $2 \times 10^8$ colony forming units per millilitre (cfu/ml). The culture for the EDTA-vax was diluted 1:20 with TSB, adjusted to an OD$_{600}$ of 0.07-0.1 or $1 \times 10^7$ cfu/ml, and incubated at 37°C for 90 minutes. When the OD$_{600}$ of the culture reached 0.4 ($6 \times 10^7$ cfu/ml), 5mM of EDTA was added and was incubated for a further 2½ hours at 37°C, to reach a final OD$_{600}$ of 0.9 or $2 \times 10^9$ cfu/ml.

The vaccine contained ≤0.2% volume to volume free formaldehyde according to the standard procedure at this laboratory (ACE laboratory services, Bendigo, Australia). Supernatant from each EDTA supplemented culture was included in the vaccine. Each vaccine was diluted with an aluminium hydroxide adjuvant to have a final concentration of $1 \times 10^9$ cfu/ml.


5.3.1.2. Vaccine safety

The vaccines were prepared by a commercial laboratory specialising in autogenous vaccine production for livestock (ACE laboratory services, Bendigo, Australia). Although there was a high level of confidence in the vaccines produced, because it was not a standard bacterin vaccine produced by that laboratory, the safety of the EDTA-vax was assessed on each farm, one week before the start of the vaccine trial. Twenty weaned lambs in the mob from which trial animals were to be drawn were chosen. The larger mob of sheep (400-600) was moved through the race and sheep were visually assessed to be either heavier, equal to or lighter than the average live-weight for that mob. For each of these live-weight categories, every 5th animal was enrolled in the pre-trial assessment group.

On each farm, all weaned sheep had received vaccines as part of the normal husbandry procedures, including 5-in-1 vaccine against clostridial infections (Virbac), before the trial commenced. To ensure that any reaction observed was associated with the EDTA-vax, any animal detected with a pre-existing vaccine reaction, such as a granulomatous swelling, was excluded from this pre-trial assessment.

Selected animals received a double dose (4ml) of the EDTA-Vax. The vaccine was administered subcutaneously, high up on the left side of the neck just below the ear, to minimise carcass damage and facilitate assessment of the local response to vaccination. Animals in the pre-trial assessment group were identified with a coloured ear tag and excluded from the main trial.

Animals were observed daily for one week following vaccination. This was done by the cooperating producer using an animal health checklist (Table 5.1) which provided guidelines for the assessment of illness, including lethargy, abnormal gait and altered mentation. The site of vaccination was also examined for signs of swelling, heat or pain.

For each animal, a score of 0 or 1 was assigned for mentation, the site of vaccination and gait (0=normal, 1=abnormal). Any abnormalities were photographed and assessed by the primary researchers. The animal health protocol for this vaccine trial required
administration of non-steroidal anti-inflammatories or antibiotics if animals were unwell, but no medical interventions were required.

5.3.2. STUDY DESIGN
For each farm, approximately 490 animals were systematically enrolled in the trial from the larger mob of approximately 2000 animals. To facilitate random allocation of animals to treatments, as sheep moved through the race, every fourth sheep was selected, assigned to one of the three trial mobs and fitted with a coloured ear tag.

Each trial mob included 75 unvaccinated (CONT) and 75 vaccinated animals (EDTA-vax). An additional 20 lambs were vaccinated with the STAND-vax and included in mob 1 (Figure 5.1). From mob 1, 20 animals from each of the three treatment groups were systematically selected for the evaluation of antibody response to vaccination and natural exposure to *Yersinia* species. These 60 animals were identified with a scourable brand in addition to an electronic (RFID) ear tag. To replicate the typical weaner management that occurs on large scale farms, trial animals were grazed on pasture with 300-400 non-trial sheep from the larger mob from which they were selected.
Table 5.1 Pre-trial animal health evaluation form that was used by producers to assess an animal's response to a double dose of an autogenous vaccine against *Y. pseudotuberculosis* serotype III.

<table>
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<tr>
<th>Farm location:</th>
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<tbody>
<tr>
<td>Observer’s name:</td>
<td>___________________________</td>
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<tr>
<th>General appearance</th>
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<td>0: Normal – bright, alert</td>
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<td>1: Abnormal – dull (d), slow to rise (s), look unwell (u)</td>
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<th>Site of injection</th>
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<tr>
<td>0: No reaction (N),</td>
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<td>1: Reaction – heat (h), pain (p), swelling (s)</td>
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<th>General movement</th>
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<th>17</th>
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<tr>
<td>0: Normal gait</td>
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<tr>
<td>1: Abnormal: stiff (s), lame (l), unable to rise (u)</td>
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On both farms, a higher proportion of females were included in the STAND-Vax group (farm A: 85%, farm C: 80%) because they were more likely to be retained on farm if further testing was required. Results from the epidemiology study in Chapter 4 indicated that gender did not affect the risk of faecal shedding of *Yersinia* species and so it was not anticipated that this would bias the outcome of this trial.

A 2ml dose of EDTA-vax or STAND-vax was administered subcutaneously, high up on the left side of the neck, behind the ear. CONT animals did not receive any treatment. For each group, the first vaccination was given in March (farm A: 10/03/14, farm C: 17/03/14) and the second dose administered 4 weeks later. Details of the date of sampling and type of samples collected are summarised in Table 5.2.

**Samples collected**
- Live-weight
- Faeces
- Blood (20 animals/group)

![Diagram of MOB 1]
- 75 X Unvaccinated (CONT)
- 75 X Vaccinated (EDTA-Vax)
- 20 X Standard vaccine (STAND-Vax)

![Diagram of MOB 2]
- 75 X Unvaccinated (CONT)
- 75 X Vaccinated (EDTA-Vax)

![Diagram of MOB 3]
- 75 X Unvaccinated (CONT)
- 75 X Vaccinated (EDTA-Vax)

Figure 5.1. The number of sheep in treatment groups within 3 mobs of Merino weaners on each of 2 farms. These were part of a study of the safety and efficacy of an autogenous vaccine against *Yersinia pseudotuberculosis*; ‘CONT’ denotes the control group (no treatment), ‘EDTA-Vax’ and ‘STAND-Vax’ were vaccinated with bacterins manufactured in low calcium and standard cultures, respectively.
5.3.3. SAMPLING PROCEDURE

There were five visits scheduled for each farm. For microbiological and parasitological assessment, 50 bulk faecal samples were collected from each trial mob at the first (farm A: day 7 (3-Mar), farm C: day 7 (17-Mar)) and second farm visit (farm A: day 28 (7-Apr), farm C: day 39 (16-Apr)), and individual faecal samples were collected at the final visit (farm A: 11-Sept, farm C, 8-Sept). Individual faecal samples were collected directly from the rectum of each animal with a gloved hand and bulk samples were collected from the ground as detailed in section 2.5.1. Individual animal live-weight was recorded at the first, second and final visits.

Every 2-5 weeks between farm visits, the cooperating producers collected 50 bulk faecal samples from each trial mob. Results from these samples were used to monitor the prevalence of shedding of *Yersinia* species in each trial mob.

The frequency of sampling increased during winter (20-June to 11-Sept), because the risk of an outbreak of yersiniosis was highest during this time. Producers were notified when the prevalence of faecal shedding of *Y. pseudotuberculosis* of a trial mob was greater than 10% because outbreaks of yersiniosis most often occurred when the prevalence of shedding exceeded this point (Chapter 3). When this occurred, the sheep were more closely monitored for signs of scouring, lethargy and abdominal discomfort so that clinical cases of yersiniosis could be detected promptly. A farm visit occurred if clinical cases were detected in approximately 2% of a mob. During the investigation of an outbreak (farm A: 17-Jun), live weight was recorded and individual faecal samples were collected from all trial animals on the farm affected by the outbreak, a total of 490 sheep.

All faecal samples were cultured for *Yersinia* using selective agar and a proportion (>30%) of CIN positive cultures were identified using a multiplex PCR with methods described in Chapter 2. All faecal samples were processed within 48 hours of collection.

Blood samples were collected from the sub-set of 20 of animals from each treatment group in mob 1 that were identified with a scourable brand (Section 5.3.2). Blood samples were collected to evaluate the antibody response to vaccination (farm A: day 28, farm C: day
Table 5.2. A summary of the timing of activities and samples collected from sheep enrolled in an autogenous vaccine trial against *Yersinia pseudotuberculosis* on two farms in Victoria in 2014.

<table>
<thead>
<tr>
<th>Sampling description</th>
<th>Date (day of trial)</th>
<th>Farm A</th>
<th>Farm C</th>
<th>Activities and samples collected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Farm visit</td>
</tr>
<tr>
<td>First vaccination</td>
<td>3-Mar (0)</td>
<td></td>
<td>17-Mar (7)</td>
<td>✓</td>
</tr>
<tr>
<td>Second vaccination</td>
<td>7-Apr (28)</td>
<td></td>
<td>16-Apr (39)</td>
<td>✓</td>
</tr>
<tr>
<td>Post vaccination</td>
<td>23-Apr (44)</td>
<td></td>
<td>30-Apr (51)</td>
<td>✓</td>
</tr>
<tr>
<td>Monitor sample</td>
<td>21-May (72)</td>
<td></td>
<td>21-May (72)</td>
<td>x</td>
</tr>
<tr>
<td>Blood collection</td>
<td>2-Jun (84)</td>
<td></td>
<td>5-Jun (87)</td>
<td>✓</td>
</tr>
<tr>
<td>Outbreak investigation</td>
<td>17-Jun&lt;sup&gt;a&lt;/sup&gt; (99)</td>
<td>-</td>
<td>17-Jun&lt;sup&gt;a&lt;/sup&gt; (99)</td>
<td>✓</td>
</tr>
<tr>
<td>Monitor sample</td>
<td>- 20-Jun&lt;sup&gt;b&lt;/sup&gt; (102)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Monitor sample</td>
<td>30-Jun (112)</td>
<td></td>
<td>30-Jun (112)</td>
<td>x</td>
</tr>
<tr>
<td>Monitor sample</td>
<td>14-Jul (126)</td>
<td></td>
<td>14-Jul (126)</td>
<td>x</td>
</tr>
<tr>
<td>Monitor sample</td>
<td>28-Jul (140)</td>
<td></td>
<td>28-Jul (140)</td>
<td>x</td>
</tr>
<tr>
<td>Monitor sample</td>
<td>11-Aug (154)</td>
<td></td>
<td>11-Aug (154)</td>
<td>x</td>
</tr>
<tr>
<td>Monitor sample</td>
<td>25-Aug (168)</td>
<td></td>
<td>25-Aug (168)</td>
<td>x</td>
</tr>
<tr>
<td>Final visit</td>
<td>11-Sep (185)</td>
<td></td>
<td>8-Sep (182)</td>
<td>✓</td>
</tr>
</tbody>
</table>

<sup>a</sup>An outbreak occurred on farm A only; <sup>b</sup>Monitor samples were collected for farm C only
30) and following a second vaccination (day 44), as well as following exposure to low (farm A: day 84, farm C: day 80) and high quantities of \textit{Yersinia} species on pasture (farm A: day 185, farm C: day 175). For farm A, the antibody response of these animals was assessed after the suspected outbreak of yersiniosis had occurred (day 99). Blood was collected from the jugular vein using methods described in section 2.3.

### 5.3.4. The Assessment of the Innate Immune Response to Vaccination and Natural Exposure to \textit{Y. pseudotuberculosis}

#### 5.3.4.1. Production of \textit{Yersinia} outer membrane proteins (‘Yops’)

Antibodies against \textit{Yersinia} outer membrane proteins (‘Yops’) were assessed by direct enzyme-linked immunosorbent assay (ELISA). Yops were produced in brain heart infusion broth rather than TSB, using the same conditions culture as the EDTA-Vax (Section 5.3.1.1), up to the point of formalin treatment. Bacterial cells were removed by centrifugation (7,000g, 20min at 4ºC) and the supernatant containing Yops was filter sterilised using a 0.45 µM polyvinylidene difluoride membrane syringe filter (Jet biofilm, China). Proteins were precipitated from the supernatant with solid ammonium sulphate (40 g/100ml) and pelletised by centrifugation (10,000g, 20 min, 20ºC) (Heesemann et al., 1986).

The pelletised proteins were resuspended in phosphate buffered solution (PBS) and quantified by spectrophotometry using the Biuret method (NanoDrop®, ND-1000, Thermo Scientific, Australia). Yops were stored at -20ºC and were used as the primary coating antigen for the ELISA assays described below. The presence of Yops was confirmed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Laemmli, 1970).

#### 5.3.4.2. Optimised ELISA conditions

The validity of Yops as a primary coating antigen was confirmed by western blot using serum from animals that had been diagnosed with \textit{Y. pseudotuberculosis} serotype III infection (Mahmood and Ping-Chang, 2012). These positive control serum samples were also used to generate a standard curve for all ELISA assays. Serum samples from recently weaned wethers that had been raised indoors, and had therefore been at extremely low
risk of previous exposure to *Yersinia* species, were used as negative control samples. The optimal concentration each reagent was determined by evaluating two-fold dilutions for each of *Yops* (0.15 – 10 µg/ml), antigen in serum (1:100 – 1:3200) and monoclonal anti-sheep/donkey IgG (1:2000 – 1:8000).

Briefly, 96-well microplates (Nunc, Denmark) were coated with 150 µL/well of 2.5 µg/mL *Yops* solution prepared in coating buffer (0.1 M carbonate-bicarbonate, pH 9.6) and incubated at 4°C overnight. Plates were then washed twice with phosphate buffer saline containing 0.05% Tween (PBS-T), and 150 µL of PBS with 5% newborn calf serum was added to all wells. Plates were then incubated at 37°C for 2 hours and then washed twice with PBS-T. Test serum was diluted 1:400 with 2.5% new born calf serum in PBS-T, and added to duplicate wells. A standard control curve was generated using a positive serum sample at concentrations between 1:100 and 1:12,800, and added to duplicate wells. A blank (PBS-T) and standard negative (1:400) control sample were added to duplicate wells.

Plates containing serum were incubated at room temperature for 2 hours with shaking. Plates were washed 4 times with PBS-T and 100 µL of 1:2000 peroxidase conjugate of monoclonal anti-sheep/donkey IgG (Sigma, USA) was added to each well. Plates were incubated for a further 45 mins, then washed 4 times with PBS-T. Following this, ABST substrate was added and plates were incubated for 20 minutes at room temperature. The reaction was stopped with 1% sodium dodecyl sulphate solution and the optical densities were recorded at a wave length of 405 nm. Cut points for negative results were set at 2 standard deviations above the average optical density for the negative control.

**5.3.5. STATISTICAL ANALYSIS**

Faecal samples were categorised as positive or negative based on the morphological appearance of the bacterial colonies on CIN plates (Section 2.5.3.1). A proportion (range 33-100%) of these positive samples were identified to the species level using the multiplex PCR described in section 2.5.5.2. An estimate of the mob prevalence of faecal shedding of *Yersinia* species was derived from this sub-set of PCR results and extrapolated to the whole population.
All analyses were conducted using Stata version 13 (StataCorp, Texas, USA). To eliminate inaccurate or unreliable weights, outlier live-weights (those more than two standard deviations from the population mean) were excluded from analysis. The distribution of the live-weight and average daily weight gains were visualised using histograms. Exact binomial confidence intervals for prevalences were calculated for each sampling within each farm using Stata's `-cii-` command.

Average daily rates of live-weight change ('average daily weight gains') were calculated for each sheep as the change in live-weight from one sampling to the next (live-weight at most recent sampling minus live-weight at the immediately preceding sampling for that flock) divided by the number of days between those samplings. An average daily gain was calculated only for sheep with non-excluded live-weights at sequential samplings.

The effect of treatment was determined independently for each farm. The effect of EDTA-Vax was compared with the effect of no treatment (CONT) and then the effect of STAND-Vax. Live-weight was assessed after adjusting for farm, gender and the initial live weight. The effect of treatment on average daily gain was assessed after adjusting for farm and gender. The effect of treatment on the shedding (any growth and PCR positive, rather than no growth) and heavy shedding (culture growth score of 3 or 4 and PCR positive rather, than growth score of 0 to 2) of *Y. enterocolitica* and *Y. pseudotuberculosis* was assessed after adjusting for farm and gender.

For the analysis of the effects of EDTA-vax compared with no vaccination (CONT), treatment group was the unit of analysis and the panel variable was the mob on farm. Population-average linear models were fitted for live-weight and for average daily weight gain using the `-xtreg-` command in Stata. The `-xtlogit-` command was used for analysis of shedding and heavy shedding of *Yersinia* species.

On each farm, all animals within the STAND-vax group were grazed in a single mob. Only animals within that mob were included in the analysis of the effects of EDTA-vax compared with STAND-vax. Therefore, linear models were fitted for using `-regress-` and –
exlogistic- commands for live-weight and average daily weight gain, and for shedding and heavy shedding of *Yersinia*, respectively.

The cut point for a negative titre was set at OD$_{405}$ of less than 100 and results were categorised accordingly (0 = OD$_{405}$ less than 100; 1 = OD$_{405}$ greater than 100). Any animal that had a positive antibody titre at the first sampling (d0) was excluded from further analysis. For each sampling on both farms, the proportion of seropositive control and vaccinated animals was independently compared by a Fisher’s exact test using the –row exact- command in Stata.

**5.4. RESULTS**

Three of the 40 animals vaccinated with EDTA-Vax, 2 on farm A and 1 on farm C, developed small, localised swellings at the site of vaccination, but no systemic signs of disease were observed.

### 5.4.1. NUMBER OF SHEEP SAMPLINGS, DEATHS AND OVERALL BACTERIOLOGY RESULTS

In total, 973 sheep were enrolled in the study (farm A 487; farm C 486) and they contributed 3,263 sheep-samplings. Sixty and 42 sheep died, or were not mustered for the sampling, between the first and final samplings, for farm A and C, respectively. Six animals from farm A and five sheep from farm C missed at least one sampling, but had a subsequent sampling.

Of the 3,263 sheep samplings, 1,330 samples were cultured with CIN agar (41%). An associated PCR result was available for 375 of the 467 positive samples (80%). Of these, *Y. enterocolitica* was detected by PCR in 57% and 9% (farm A: 166/292; farm C: 15/175), and *Y. pseudotuberculosis* was detected in 6% and 22% (farm A: 17/292; 38/175) of samples from farms A and C, respectively.

For farm A, *Y. enterocolitica* was detected in faecal samples on 9 of 10 occasions (3 to 26% of samples), and *Y. pseudotuberculosis* detected on 4 of 10 occasions (1 to 8% of samples)(Figure 5.2). At all sampling occasions, less than 10% of the mob were shedding
Y. pseudotuberculosis, with the total combined shedding of both Yersinia spp. being less than 31%.

For farm C, Y. enterocolitica was detected in faecal samples on 7 of 9 occasions (2 to 77% of samples), and Y. pseudotuberculosis detected on 6 of 9 occasions (12 to 30% of samples). In June, July and August, more than 10% of the mobs were shedding Y. pseudotuberculosis in faeces, but no clinical outbreak was detected.

On farm A, a higher proportion of animals were shedding Y. enterocolitica compared with Y. pseudotuberculosis on all occasions, with the total proportion of the mob shedding pathogenic Yersinia species being less than 31%. On farm C, the proportion of animals shedding Y. enterocolitica and Y. pseudotuberculosis was more variable. The proportion of animals shedding Y. pseudotuberculosis was highest between June and September. The proportion of sheep shedding of pathogenic Yersinia species was greater than 30% on three occasions in June and July, and peaked at 93% in June (77% Y. enterocolitica, 16% Y. pseudotuberculosis).

5.4.2. OUTBREAK INVESTIGATION

On farm A in June (Figure 5.2), the producer reported that approximately 40% of a trial mob had developed diarrhoea, 5% of that mob (8 animals) had died over a 3 day period and individual sheep were exhibiting signs of lethargy and abdominal discomfort. At the previous sampling, 16% of all trial sheep were shedding Y. enterocolitica in faeces and so an outbreak investigation was conducted (Section 5.3.3). Individual faecal samples were collected from all trial animals and 14% were shedding Y. enterocolitica and 1% shedding Y. pseudotuberculosis in their faeces (Figure 5.2). Fourteen percent of these isolates had a culture growth score ≥3.

5.4.3. LIVE-WEIGHT AND AVERAGE DAILY WEIGHT GAINS

Average live-weight at sampling and average daily weight gains (±SD) for each group on both farms are shown in Table 5.3 and Table 5.4, respectively. Across all treatments, animals on Farm A were 0.3kg heavier than those on farm C (22.2±3.8 kg vs 21.9±3.2 kg) at the first vaccination, but were 6.2 kg lighter at the conclusion of the study (28.1±3.8 kg
vs 34.3±4.5 kg). On all occasions, the average live-weights were comparable between the treatment groups within each farm. Animals on farm A gained an average of 1 kg/month (±0.5 kg) whereas those on farm C gained an average of 2 kg/month (±0.6 kg).

Average daily weight gains were positive on all but one occasion for both farms (25.7-54.9 g/head/day and 3.3-77.3 g/head/day on farms A and C, respectively). The exception was between the 1st and 2nd vaccination on farm A, when animals in both vaccinated groups lost weight (-3.3 g/head/day and -11.6 g/head/day in STAND-Vax and EDTA-Vax, respectively). On farm A, where there were four visits, the average daily gains were highest between the 2nd vaccination in April and the outbreak investigation in June.

### 5.4.4. WORM EGG COUNTS

On farm A, the average WEC for each trial mob was less than 300 eggs per gram (epg) at every sampling occasion, except at the suspected outbreak investigation in June. At that sampling, the average WEC was 2932, 1667 and 489 epg for mobs 1, 2 and 3, respectively.

On farm C, the average WEC was more variable between the mobs and sampling occasions. For all mobs, the average WEC was less than 300 epg on 5 of 9 sampling occasions. Two of the trial mobs had WECs greater than 300 epg on one occasion in June and twice in July. The maximum average WEC of 811 epg was recorded in June.
Figure 5.2. a) Prevalence of faecal shedding of *Yersinia enterocolitica* (orange sections) or *Y. pseudotuberculosis* (blue sections) by Merino lambs grazing in mobs enrolled in an autogenous vaccine trial for *Y. pseudotuberculosis* serotype III (error bars indicate exact binomial 95% confidence intervals). b) The proportion of non-vaccinated (CONT) and vaccinated (EDTA-Vax) animals with antibodies to *Y. pseudotuberculosis* from two flocks in Victoria, Australia in 2014 (Solid dot represents CONT group, Cross indicates EDTA-Vax group). A black arrow indicates when vaccine was administered and a red arrow indicates a suspected outbreak of yersiniosis in the trial mob.
On farm A, there were no significant differences between the average live-weight or average daily weight gain of CONT animals and those vaccinated with the EDTA-vax on any sampling occasions (Table 5.3 and 5.4).

At the second vaccination for farm C, animals vaccinated with EDTA-vax were lighter (Coefficient: -0.7, 95% confidence interval -1.0 to -0.4, p<0.001) and had lower average daily weight gains (Coefficient: -2.6, 95% confidence interval -3.5 to -1.6, p<0.001) than CONT animals. There was no difference between the average live-weight or average daily weight gains between these treatment groups at the final sampling (Sept).

The risk of shedding and of heavy shedding of *Y. enterocolitica* were comparable for animals in the EDTA-vax and CONT groups, at all sampling occasions for both farms (Table 5.5). At the final sampling for farm A, there were a higher proportion of CONT animals that were shedding and were heavy shedders of *Y. enterocolitica*, but these estimates were imprecise. At the final sampling for farm C, more CONT animals were shedding *Y. enterocolitica* compared with EDTA-vax animals, and a slightly higher proportion of EDTA-vax group were heavy shedders of this organism, but these estimates were not significant (P>0.387).

There was no significant difference between the proportion of CONT and EDTA-vax animals that were shedding or were heavy shedders of *Y. pseudotuberculosis*, on any sampling occasions for either farm (Table 5.6).
Table 5.3. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III (EDTA-vax) compared with no treatment (CONT) on the live-weight of weaned Merino lambs from two Victorian flocks in 2014.

<table>
<thead>
<tr>
<th>Farm and treatment</th>
<th>No. of samples</th>
<th>% Males</th>
<th>1st vaccination (March)</th>
<th>2nd vaccination (April)</th>
<th>Outbreak investigation (June)</th>
<th>Trial conclusion (Sept)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Live weight&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Live weight&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Coeff. 95% confidence interval</td>
<td>P</td>
</tr>
<tr>
<td><strong>Farm A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>225</td>
<td>46</td>
<td>22.3 (3.8)</td>
<td>22.4 (4.0) Reference category</td>
<td>25.7 (4.1) Reference category</td>
<td>28.3 (3.8) Reference category</td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>223</td>
<td>55</td>
<td>22.2 (3.9)</td>
<td>22.1 (4.0) -0.2 -0.5 to 0.1</td>
<td>0.178</td>
<td>25.8 (4.1) 0.1 -0.3 to 0.5</td>
</tr>
<tr>
<td><strong>Farm C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>225</td>
<td>52</td>
<td>21.7 (3.4)</td>
<td>23.6 (3.2) Reference category</td>
<td>34.3 (4.8) Reference category</td>
<td></td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>222</td>
<td>54</td>
<td>22.3 (3.2)</td>
<td>23.3 (3.1) -0.7 -1.0 to -0.4</td>
<td>&lt;0.001</td>
<td>34.5 (4.4) -0.4 -1.1 to 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Farm A only; <sup>b</sup>kilograms (sd)

Table 5.4. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III (EDTA-vax) compared with no treatment (CONT) on the average daily weight gain of weaned Merino lambs from two Victorian flocks in 2014.

<table>
<thead>
<tr>
<th>Farm and treatment</th>
<th>No. of samples</th>
<th>ADG between 1st and 2nd vaccine</th>
<th>ADG between the 2nd vaccine and outbreak investigation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ADG between the outbreak investigation&lt;sup&gt;a&lt;/sup&gt; or 2nd vaccine&lt;sup&gt;b&lt;/sup&gt; to trial conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ADG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Coeff. 95% confidence interval</td>
<td>P</td>
</tr>
<tr>
<td><strong>Farm A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>223</td>
<td>3.6 (61.8)</td>
<td>Reference category</td>
<td>20.6 (25.2) Reference category</td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>216</td>
<td>-3.0 (63.9)</td>
<td>-7.5 -18.8 to 3.9 0.199</td>
<td>48.3 (28.4) 4.2 -1.8 to 10.1 0.168</td>
</tr>
<tr>
<td><strong>Farm C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>214</td>
<td>5.9 (5.0)</td>
<td>Reference category</td>
<td>73.8 (23.3) Reference category</td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>218</td>
<td>3.3 (5.0)</td>
<td>-2.6 -3.5 to -1.6 &lt;0.001</td>
<td>76.9 (23.1) 2.2 -2.0 to 6.4 0.305</td>
</tr>
</tbody>
</table>

<sup>a</sup>Farm A; <sup>b</sup>Farm C; <sup>c</sup>Average daily weight gain grams/day (sd)
Table 5.5. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III (EDTA-vax) compared with no treatment (CONT) on the faecal shedding and heavy shedding of *Yersinia enterocolitica* by weaned Merino lambs from two Victorian flocks in 2014.

<table>
<thead>
<tr>
<th>Farm and treatment</th>
<th>No. of samples</th>
<th>Total faecal shedding</th>
<th>Heavy faecal shedding</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Outbreak investigation(a)</td>
<td>Trial conclusion</td>
<td>Outbreak investigation(a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(June, day 99)</td>
<td>(Sept, day 185)</td>
<td>(June, day 99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crude %</td>
<td>Adjusted odds ratio</td>
<td>95% confidence interval</td>
</tr>
<tr>
<td>Farm A</td>
<td></td>
<td>Reference category</td>
<td>Reference category</td>
<td>Reference category</td>
</tr>
<tr>
<td>CONT</td>
<td>198</td>
<td>13.6</td>
<td>0.6 to 1.9</td>
<td>0.898</td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>189</td>
<td>15.3</td>
<td>1</td>
<td>0.6 to 1.9</td>
</tr>
<tr>
<td>Farm C</td>
<td></td>
<td>Reference category</td>
<td>Reference category</td>
<td>Reference category</td>
</tr>
<tr>
<td>CONT</td>
<td>197</td>
<td>0.0</td>
<td>0.6 to 1.9</td>
<td>0.898</td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>201</td>
<td>0.0</td>
<td>0.6 to 1.9</td>
<td>0.898</td>
</tr>
</tbody>
</table>

\(a\)Farm A
Table 5.6. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III (EDTA-vax) compared with no treatment (CONT) on the faecal shedding and heavy shedding of *Yersinia pseudotuberculosis* by weaned Merino lambs from two Victorian flocks in 2014.

| Farm and treatment | No. of samples | Shedding | |  |  | Heavy shedding | |  |  |
|--------------------|----------------|----------|---|---|---|----------------|---|---|---|---|
|                    |                | Crude %  | Adjusted odds ratio | 95% confidence interval | P  | Crude %  | Adjusted odds ratio | 95% confidence interval | P  |
| Farm A             |                |          |                      |                          |    |          |                      |                          |    |
| CONT               | 198            | 5.1      | Reference category   |                            |    | 2.0      | Reference category   |                            |    |
| EDTA-vax           | 189            | 3.7      | 0.7                  | 0.2 to 1.9                | 0.473 | 1.6      | 0.9                  | 0.2 to 4.0                | 0.850 |
| Farm C             |                |          |                      |                          |    |          |                      |                          |    |
| CONT               | 197            | 9.1      | Reference category   |                            |    | 3.0      | Reference category   |                            |    |
| EDTA-vax           | 201            | 10.4     | 1.2                  | 0.6 to 2.3                | 0.676 | 5.5      | 1.9                  | 0.7 to 5.2                | 0.243 |

5.4.6. A COMPARISON OF THE EFFECTS OF EDTA-VAX AND STAND-VAX ON ANIMAL GROWTH AND FAECAL SHEDDING OF YERSINIA SPECIES

On both farms the average live-weight and daily weight gains of animals vaccinated with EDTA-vax or STAND-vax were comparable for all sampling occasions (Table 5.7 and Table 5.8).

At the outbreak investigation for farm A, animals treated with EDTA-vax had a lower risk of shedding *Y. enterocolitica* compared with animals that had been treated with the STAND-vax, but this was not significant (Table 5.9: OR: 0.8, 95% confidence interval 0.0 to 5.6, P = 0.78). A higher proportion of the EDTA-vax group were heavy shedders of *Y. enterocolitica*, but the risk of heavy shedding was increased for the STAND-vax group although these estimates were extremely imprecise (OR: 3.6, 95% confidence interval 0.0 to 28.5, P = 0.6). At the final sampling for farm A, the risk of shedding and heavy shedding of this organism was similar between treatment groups.

For farm C, a higher proportion of EDTA-vax animals were shedders and heavy shedders of *Y. enterocolitica*. The risk of each shedding outcome was higher for animals in the STAND-vax group, although estimates were again imprecise.

At the final sampling for farm C, there were more EDTA-vax animals shedding *Y. pseudotuberculosis* compared with the STAND-Vax group (EDTA-vax 4.5%, STAND-vax
0%), but the risk of shedding was greater for the animals in the STAND-vax group. These estimates were very imprecise because there were small numbers of animals in each comparison group (OR 3.5, 95% confidence interval 0 to 28, P=0.602)(Table 5.9). No shedding or heavy shedding was detected at any other sampling for either farm.

5.4.7. THE SEROLOGICAL RESPONSE TO VACCINATION AND NATURAL EXPOSURE TO YERSINIA SPECIES

Four animals from the CONT, six from the EDTA-vax and three from the STAND-vax groups were seropositive at the first vaccination for farm A, and so were excluded from all further analysis. No seropositive animals were detected at the first vaccination on farm C.

There was no significant effect of vaccination on the proportion of seropositive animals on either farm (Table 5.10). The proportion of seropositive animals for each treatment group was low or zero for all sampling occasions, except at the trial conclusion for farm A (Figure 5.2). Similarly, on farm C the proportion of seropositive animals for all treatment groups remained low until the visit in June. There was no marked difference between the proportions of seropositive animals in any treatment group following the 1st or 2nd vaccination.

The highest proportion of seropositive animals in each of the treatment groups was detected in September, approximately 5 months after vaccination (farm A, CONT: 86%, EDTA-Vax: 75%, STAND 57%; farm C, CONT: 76%, EDTA-Vax: 78%, STAND 79%). The proportion of seropositive animals was comparable between treatment groups within and between farms at all times.

A post hoc power analysis was conducted to determine the likelihood that seroconversion following vaccination would be detected in this study. Three scenarios were modelled, using a sample size of 15 animals per treatment and an alpha level of 0.05. Where the true proportion of unvaccinated compared with vaccinated animals was 5% versus 90%, 10% versus 80% or 20% versus 70%, the power was 1.00, 0.996 and 0.82, respectively.
Table 5.7. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III manufactured in a low-calcium culture (EDTA-vax) compared with one manufactured using standard culture methods (STAND-vax) on the live-weight of weaned Merino lambs from two Victorian flocks in 2014.

<table>
<thead>
<tr>
<th>Farm and treatment</th>
<th>No. of samples</th>
<th>% of Males</th>
<th>1st vaccination (March)</th>
<th>2nd vaccination (April)</th>
<th>Outbreak investigation* (June)</th>
<th>Trial conclusion (Sept)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Live-weight*</td>
<td>Coeff.</td>
<td>95% confidence interval</td>
<td>P</td>
</tr>
<tr>
<td>Farm A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>72</td>
<td>68</td>
<td>22.6 (3.8)</td>
<td></td>
<td>Reference category</td>
<td></td>
</tr>
<tr>
<td>STAND-vax</td>
<td>20</td>
<td>15</td>
<td>20.7 (3.0)</td>
<td>-0.2</td>
<td>-1.2 to 0.8</td>
<td>0.692</td>
</tr>
<tr>
<td>Farm C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>74</td>
<td>58</td>
<td>22.3 (3.3)</td>
<td>0.3</td>
<td>-0.5 to 1.1</td>
<td>0.403</td>
</tr>
<tr>
<td>STAND-vax</td>
<td>19</td>
<td>15</td>
<td>20.7 (3.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Farm A; **Farm C; *kilograms (sd)

Table 5.8. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III manufactured in a low-calcium culture (EDTA-vax) compared with one manufactured using standard culture methods (STAND-vax) on the average daily weight gain of weaned Merino lambs from two Victorian flocks in 2014.

<table>
<thead>
<tr>
<th>Farm and treatment</th>
<th>No. of samples</th>
<th>ADG between 1st and 2nd vaccine</th>
<th>ADG between the 2nd vaccine and outbreak investigation*</th>
<th>ADG between the outbreak investigation* or 2nd vaccine* to trial conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ADG*</td>
<td>Coeff.</td>
<td>95% confidence interval</td>
</tr>
<tr>
<td>Farm A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>72</td>
<td>5.9 (5.0)</td>
<td>Reference category</td>
<td>50.9 (27.0)</td>
</tr>
<tr>
<td>STAND-vax</td>
<td>20</td>
<td>-11.6 (68.6)</td>
<td>-6.0</td>
<td>-42.5 to 30.5</td>
</tr>
<tr>
<td>Farm C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>73</td>
<td>2.9 (4.8)</td>
<td>Reference category</td>
<td>76.7 (24.7)</td>
</tr>
<tr>
<td>STAND-vax</td>
<td>19</td>
<td>5.0 (5.9)</td>
<td>1.9</td>
<td>-0.8 to 4.7</td>
</tr>
</tbody>
</table>

*Farm A; **Farm C; *Average daily weight gain grams/day (sd)
Table 5.9. The effect of an autogenous vaccine for *Yersinia pseudotuberculosis* serotype III manufactured in a low-calcium culture (EDTA-vax) compared with one manufactured using standard culture methods (STAND-vax) on the faecal shedding and heavy shedding of *Y. enterocolitica* by weaned Merino lambs from two Victorian flocks in 2014.

<table>
<thead>
<tr>
<th>Farm and treatment</th>
<th>No. of samples</th>
<th>Outbreak investigation* (June)</th>
<th>Trial conclusion (Sept)</th>
<th>Outbreak investigation* (June)</th>
<th>Trial conclusion (Sept)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total faecal shedding</td>
<td></td>
<td>Heavy faecal shedding</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crude</td>
<td>Adjusted odds ratio</td>
<td>95% confidence interval</td>
<td>P</td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>54</td>
<td>10</td>
<td>Reference category</td>
<td>18.8</td>
<td>Reference category</td>
</tr>
<tr>
<td>STAND-vax</td>
<td>14</td>
<td>0.0</td>
<td>0.0 to 5.6</td>
<td>0.780</td>
<td>22.2</td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>64</td>
<td>4.5</td>
<td>Reference category</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAND-vax</td>
<td>18</td>
<td>0.0</td>
<td>0.0 to 27.8</td>
<td>0.602</td>
<td></td>
</tr>
</tbody>
</table>

*Farm A only;

Table 5.10. The proportion of seropositive weaned Merino lambs following vaccination for *Yersinia pseudotuberculosis* serotype III (EDTA-vax) and natural exposure to *Yersinia* species compared with untreated lambs animals (CONT)

<table>
<thead>
<tr>
<th>Farm and treatment</th>
<th>1st vaccination (Mar, day 0)</th>
<th>2nd vaccination (Apr, day 28)</th>
<th>Post vaccination (Apr, day 44)</th>
<th>Pre-exposure (June, day 84)</th>
<th>Outbreak investigation* (June, day 99)</th>
<th>Trial conclusion (Sept, day 185)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of samples</td>
<td>% Pos*</td>
<td>No. of samples</td>
<td>% Pos</td>
<td>P</td>
<td>% Pos</td>
</tr>
<tr>
<td>Farm A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>21</td>
<td>19</td>
<td>14</td>
<td>0.0</td>
<td>Ref. category</td>
<td>0.0</td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>19</td>
<td>31.6</td>
<td>8</td>
<td>12.5</td>
<td>0.364</td>
<td>12.5</td>
</tr>
<tr>
<td>STAND-vax</td>
<td>20</td>
<td>15</td>
<td>14</td>
<td>7.1</td>
<td>1.000</td>
<td>0.0</td>
</tr>
<tr>
<td>Farm C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>21</td>
<td>0</td>
<td>21</td>
<td>4.8</td>
<td>Ref. category</td>
<td>0.0</td>
</tr>
<tr>
<td>EDTA-vax</td>
<td>20</td>
<td>0</td>
<td>19</td>
<td>0.0</td>
<td>1.000</td>
<td>5.3</td>
</tr>
<tr>
<td>STAND-vax</td>
<td>19</td>
<td>0</td>
<td>19</td>
<td>0.0</td>
<td>1.000</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Farm A only; Seropositive animals at the 1st vaccination were excluded from analysis
5.5. DISCUSSION

Virulent *Yersinia* species, including *Y. pseudotuberculosis*, produce *Yersinia* outer membrane proteins (‘Yops’) that play an integral part in the cellular invasion of host cells and the establishment of infection (Pujol and Bliska, 2005). Laboratory studies have demonstrated that the production of Yops can be increased if virulent *Yersinia* species are cultured in low-calcium conditions during their exponential growth phase (Heesemann et al., 1986). Under these conditions, Yops are secreted into the culture broth and can be extracted by centrifugation.

Consequently, to increase the antigenicity of the vaccine used in this study, a novel method for vaccine manufacture was used whereby *Yersinia* isolates were cultured in a low-calcium environment (‘EDTA’). Thus, the EDTA-Vax contained formalin-killed *Yersinia* cells and concentrated Yops from the culture supernatant. Despite this modification, there were very few vaccination reactions in animals given a double dose of the EDTA-Vax and so it was judged safe to use in the field trial.

Animals also develop antibodies to Yops following natural exposure to virulent *Yersinia* species (Robins-Browne et al., 1993; Iwata et al., 2010). Consequently, the detection of antibodies to Yops was used to measure the humoral immune response to both vaccination and natural exposure to *Yersinia* species.

During the current study, there were no outbreaks of diarrhoea associated with bacterial enteritis due to *Yersinia* species. This occurred despite the proportion of sheep shedding virulent *Yersinia* species in faeces being greater than 10% on 13 of 19 (68%) sampling occasions. Only one outbreak of diarrhoea was detected in this study, on farm A in July. This outbreak of diarrhoea was due to primary gastrointestinal parasitism, not yersiniosis. This conclusion was supported by very high WECs (1696 epg), only moderate faecal shedding of *Y. enterocolitica* (14%) and low shedding of *Y. pseudotuberculosis* (1%), with 86% of positive cultures having only a light growth of *Yersinia* species (CGS ≤2).
The absence of an outbreak of yersiniosis was unexpected, because results from the preliminary outbreak study (Chapter 3) indicated that most outbreaks occurred when the prevalence of faecal shedding of pathogenic *Yersinia* species was greater than 10%. However, results from the outbreak investigation study also indicated, that during an outbreak, affected animals were typically heavy shedders of *Yersinia* species (a CGS ≥3). In the current vaccine study, a large proportion of animals were shedding *Yersinia* species, but very few were heavy shedders. These results further highlight that a complex and multifactorial interaction between risk factors are necessary to precipitate an outbreak.

In the absence of an outbreak, there was no evidence to indicate that vaccination with EDTA-Vax had any beneficial effect on live-weight (Table 5.3), average daily weight gain (Table 5.4) or the risk of shedding or heavy shedding of *Yersinia* species (Table 5.5 and Table 5.6). In fact, on farm C there was a significant decrease in the average live-weight and average daily weight gain of animals vaccinated with EDTA-vax between the 1st and 2nd vaccination. This reduced growth might have been due to a short period of anorexia following vaccination or mustering. However, this effect was temporary and there was no significant difference between the growth of vaccinated and unvaccinated animals at any other sampling time.

Similarly, the method of vaccine manufacture had no effect on the average live-weight, average daily weight gain or the prevalence of faecal shedding of *Yersinia* species. These results suggest that, despite altering the culture methods used to produce the EDTA-vax, there was no measurable difference in the antigenicity of this vaccine compared with the STAND-Vax.

Results from this study are consistent with those from trials that assessed the efficacy of a formalin-killed bacterin vaccine containing *Y. pseudotuberculosis* serotypes I, II and III, in deer. The efficacy of that vaccine was assessed in a field trial that included 4,958 deer calves grazing pasture, on 17 farms across New Zealand (Mackintosh et al., 1992). In the absence of an outbreak, there was no difference between the number of clinical cases or mortalities between vaccinated and unvaccinated deer. The effect of vaccination on live weight and faecal shedding of *Yersinia* was not reported in this paper.
During the study of deer in New Zealand, an outbreak of yersiniosis was detected on three farms, and the prevalence of clinical disease and mortality was significantly lower for vaccinated compared with unvaccinated animals. The average incidence of clinical disease and mortalities for vaccinated deer were 6% and 2%, compared with 25% and 16% for unvaccinated deer, respectively. A similar result was reported in another study that challenged vaccinated and unvaccinated deer with a high oral dose of *Y. pseudotuberculosis* (8 x 10^9 cfu) (Mackintosh, 1993). In that study, clinical disease was reported in 31% of vaccinated and 54% of unvaccinated animals. Consequently, it is still possible that the autogenous vaccine developed for sheep in the current study may have reduced mortalities during an outbreak of yersiniosis, but further investigation of this is needed.

In the present study, the proportion of EDTA-Vax and CONT animals that were seropositive one month after the first vaccination, and two weeks after the second vaccination, were low and comparable. Following immunisation or exposure to a pathogenic organism, antibodies are usually produced and can be detected in blood within 2 days (Siegrist, 2008). Antibody concentration typically peaks four weeks following exposure, and a second vaccine is often administered at this time to reactivate immune memory and increase the concentration of circulating immunoglobulin G (Siegrist, 2008). Results from the present study indicate that the EDTA-vax did not stimulate a measurable humoral immune response, as determined by the production of antibodies to *Yops*. The studies investigating the efficacy of vaccination for deer have not reported the serological response of animals to vaccination. Therefore, although there was no measureable change in antibody titre following vaccination in the present study, it is possible that vaccination induced a cell-mediated immune response which was not assessed in this study.

A high proportion of both vaccinated and control sheep were seropositive at the final sampling in September. This indicated that although animals did not seroconvert following vaccination, they did develop antibodies to *Yops* following natural exposure to the virulent *Yersinia* species that were known to be present in faeces. This supports earlier studies
which found that sheep and deer produced antibodies to *Yersinia* species following natural exposure and further supports the use of vaccination as a means to control the disease (Mackintosh et al., 1986; Robins-Browne et al., 1993).

A number of other factors can affect the efficacy of a vaccine, including the site of vaccination, the adjuvant used and whether the bacterial cells contained in the vaccine are live or have been killed. In the present study, an aluminium hydroxide adjuvant was used and bacterial cells were killed with formalin.

Killed bacterin vaccines with an aluminium adjuvant are commonly used for sheep and can significantly reduce the risk of systemic disease, such as the most frequently used 5-in-1 vaccines against clostridial pathogens. However, the efficacy of bacterin vaccines against enteric pathogens is variable. For example, studies that have assessed a killed vaccine containing *Salmonella typhimurium* for dairy calves have shown inconsistent results, ranging from ‘good’ to ineffective. These differences may be related to the concentration of antigen, the adjuvant used, the age of animals at immunisation or the amount bacterial challenge calves were exposed to (House et al., 2001). These factors may have also influenced the efficacy of the vaccine against *Y. pseudotuberculosis* that was evaluated in the present study.

Many studies have also demonstrated that live-attenuated vaccines can stimulate the innate and cell mediated immune responses more effectively than killed or inactivated vaccines (Siegrist, 2008). For example, a study that compared a killed bacterin vaccine containing virulent *Y. pseudotuberculosis* with an oral, live-attenuated vaccine that contained a virulent, but less pathogenic strain of *Y. pseudotuberculosis* (it had the plasmid but not the chromosomal high pathogenicity genes), found that the live vaccine was significantly more effective (Quintard et al., 2010). Guinea pigs that were vaccinated with the killed vaccine mounted a significant antibody response (IgG), but the mortalities were comparable with the non-vaccinated animals following a high oral challenge with *Y. pseudotuberculosis*. In contrast, none of the guinea pigs that were vaccinated with the live, less pathogenic vaccine, developed disease. Oral vaccines are not commonly used for
sheep and cattle, but this delivery method certainly warrants further investigation for enteric diseases for sheep.

5.6. CONCLUSION

In the absence of an outbreak of yersiniosis, vaccination did not affect the average live weight, average daily gain, the prevalence of faecal shedding of *Yersinia* species or the proportion of animals that were seropositive for *Yersinia* outer membrane proteins (*Yops*). Similarly, the method used to manufacture the vaccine did not affect these factors, which indicates that the antigenicity of the EDTA-Vax and STAND-vax were comparable.

Vaccination did not stimulate a measurable antibody response, but a large proportion of animals in each treatment group were seropositive after natural exposure to *Yersinia* species. This indicates that sheep are capable of mounting a humoral immune response to *Yersinia*, but did not mount a response to the vaccine assessed during this study. Alternative vaccine formulations, such as a live-attenuated vaccine or one using a different adjuvant for the bacterin vaccine, require further investigation.

Although there were no detectable effects of vaccination in this study, the efficacy of this vaccine during an outbreak or following experimental challenge with differing numbers of organisms needs to be assessed.
CHAPTER 6  CONCLUSIONS

Forty five outbreaks of the winter scours syndrome were investigated, with *Y. pseudotuberculosis* and virulent *Y. enterocolitica* isolated from 18.5% and 13.6% of 1020 faecal samples, respectively (Chapter 3). Consequently, these organisms were identified as the primary agents most likely to be responsible for this syndrome in young Merino sheep in south-eastern Australia.

Initially, pathogens were isolated from faeces by selective bacterial culture (CIN agar) and identified with commercial biochemical test kits that were specific for members of the Enterobacteriaceae family. However, the sensitivity and repeatability of these commercial test kits was quite poor for *Y. pseudotuberculosis* and *Y. enterocolitica*. Due to these inaccuracies, it is likely that yersiniosis may have been underdiagnosed in laboratories using these kits and that yersiniosis is far more prevalent than previously recognised. Consequently, molecular techniques including PCR assays were developed for use in the current studies.

The PCR assays developed for these studies provided a rapid, specific and sensitive tool to identify the two pathogenic *Yersinia* species that were found to be the primary cause of the winter scours syndrome in sheep. The use of molecular tests in conjunction with traditional bacterial culture methods was efficient and cost effective, and it would be a useful approach for commercial veterinary diagnostic laboratories. An increasing proportion of these laboratories have the equipment required for PCR testing, and so the adoption of molecular techniques for routine veterinary investigations is feasible. The development of a direct, quantitative multiplex PCR to identify the major causes of bacterial enteritis in ruminants, such as *Yersinia, Campylobacter* and *Salmonella*, would further improve the utility of this method.

In addition to increasing the sensitivity of testing, an estimate of the biological importance of the pathogens isolated was also needed. Many enteric organisms including virulent *Yersinia* species can be detected in the faeces of healthy animals. Consequently, a semi
quantitative culture growth score (CGS) was used to estimate the amount of *Yersinia* growth on selective agar. There were no additional costs associated with the assessment of the growth score for *Yersinia* species, and so this method could be easily applied to other bacterial agents. Therefore, it may be beneficial for diagnostic laboratories to include a culture growth score as part of routine testing, enabling clinicians to make a more informed decision about treatment. For example, light growth (CGS <2) of organisms that can be shed by healthy animals, including *Yersinia* and *Campylobacter* species, indicates that an alternate cause of diarrhoea is more likely and antibiotic treatment may be unnecessary. For these reasons, a combination of traditional bacterial culture, culture growth score and molecular typing was used to investigate outbreaks and describe the epidemiology of yersiniosis in sheep in the current study.

Results from the outbreak investigations demonstrated that both *Y. pseudotuberculosis* and *Y. enterocolitica* played an important role in the scours syndrome (Chapter 3). An outbreak of yersiniosis typically occurred in mobs of sheep in which >10% of animals were shedding either *Yersinia* species in their faeces. However, an outbreak did not inevitably occur under these conditions. For example, the epidemiology study (Chapter 4) found that the prevalence of faecal shedding of virulent *Yersinia* species could be as high as 90% without an outbreak occurring. This was clearly demonstrated on Farm C when an outbreak of yersiniosis occurred when 53% of a mob was shedding *Y. pseudotuberculosis* in 2012. However, no outbreak occurred even though 57% of the trial mob was shedding this organism in August 2013.

A number of risk factors including the time of year, farm, average daily weight gain and WEC, were found to affect the prevalence of faecal shedding of *Yersinia* species. However, in isolation these factors did not inevitably precipitate an outbreak (Chapter 4). It was concluded that the risk factors for an outbreak of yersiniosis, as opposed to shedding of these bacteria in faeces, are likely to be numerous and have very complex interactions which were not evaluated in this study. These risk factors may include rainfall, wind chill index, the time an animal is off feed and nutritional factors, such as feed availability. Further studies on these risk factors and their interactions are warranted.
The risk of an outbreak may also be associated with the contamination of pasture by sheep that are shedding *Yersinia* in their faeces. During winter in south-eastern Australia, ambient temperature is commonly below 20ºC and *Y. pseudotuberculosis* and *Y. enterocolitica* can survive in faeces for more than 40 days in these conditions (Thomson, 2014). It is therefore likely that the risk of an outbreak is also associated with degree of pasture contamination as determined by the quantity of *Yersinia* shed by infected animals, the prevalence of faecal shedding of these organisms within a mob and the time that infected animals graze a particular paddock. Considering this, grazing management strategies may help to manage and prevent yersiniosis by decreasing the risk of ingestion from pasture and minimise the exposure of young, susceptible animals to ‘high risk’ pastures. Strategies may include grazing young sheep at lower stocking rate in winter or during an outbreak, or grazing pastures with mature animals that are less likely to shed large quantities of *Yersinia* ahead of young stock. These strategies have been effectively used to decrease the gastrointestinal parasite burdens of sheep during the winter, and so may also decrease the association between high WEC and the increased risk of *Yersinia* that was identified in Chapter 4.

The manager of farm C used the information gained through this study to more effectively recognise early signs of yersiniosis and alter his management to reduce the risk of an outbreak. This included the targeted treatment of affected animals with oxytetracycline, removing affected animals from the mob, reducing the stocking rate of affected mobs and moving the remaining animals to high quality feed that had not been previously grazed by young animals during winter of that year. It is likely that the targeted treatment, decreased stocking rate and segregation of affected animals reduced the contamination of pastures with *Yersinia* species, and hence decreased the risk of ingestion of these organisms. These interventions may have prevented an outbreak of disease on this farm in 2013 despite a large proportion of animals shedding *Y. pseudotuberculosis*.

*Yersinia enterocolitica* was detected at all times of the year, including during winter. The role of *Y. enterocolitica* in the winter scour syndrome was unexpected, because previous studies had found that outbreaks of diarrhoea in sheep that were associated with this organism were sporadic and often less severe that outbreaks of *Y. pseudotuberculosis*.
The detection of this *Y. enterocolitica* throughout the year also means that it could contribute to breech-soiling (‘dags’) at any time, including the summer when it would increase the risk of fly strike on affected animals. Additional economic losses would also occur with the increased need for crutching (Larsen et al., 1994). Consequently, virulent *Y. enterocolitica* should be included as a differential diagnosis for investigations of diarrhoea in young sheep at all times of the year.

In addition to its effect on animal health, *Y. enterocolitica* can also cause disease in humans (Bancerz-Kisiel and Szweda, 2015). Studies of *Y. enterocolitica* that have been isolated from sheep have not yet found that they are zoonotic, although these studies used less sensitive biochemical and serological typing for the identification of *Yersinia* species (Slee and Button, 1990a). Given the inaccuracies associated with biochemical typing that were demonstrated in the present study, it is possibly that previous studies of the zoonotic potential of these isolates have not been overly sensitive. Therefore, further investigations of the zoonotic potential of virulent *Y. enterocolitica* isolated from sheep using more sensitive molecular techniques are indicated.

Before the series of studies reported in this thesis, the recommendations for the treatment of the winter scours syndrome were imprecise and not evidence based. For example, gastrointestinal parasitism is the most common cause of diarrhoea in young sheep during winter, and so animals with diarrhoea were often first given an anthelmintic treatment. It was only when the diarrhoea did not resolve that animals were subsequently treated with an antimicrobial agent, typically sulphonamide or trimethoprim. Sheep veterinarians and producers do report that affected animals often responded to these treatments, but the results from the antibiotic sensitivity testing question the likely efficacy and wisdom of such approach. The results reported in Chapter 3 clearly demonstrate that there is widespread resistance by both *Y. pseudotuberculosis* and *Y. enterocolitica*, to sulphafurazole (87% and 64% the isolates, respectively).

The discrepancy between the clinical response to treatment reported by sheep veterinarians and producers, and the *in vitro* efficacy of the antimicrobials used for treatment, may be explained by the natural progression of the disease within a flock.
Infected animals shed *Yersinia* species in faeces, and these organisms can survive on pasture during winter. This contamination is likely to be cumulative, with an outbreak occurring when the proportion of infected animals and the degree of pasture contamination reaches a critical point. Results from the investigation in the sentinel flocks in Chapter 4 support this explanation. It was found that there was an acute but transient rise in the number of animals shedding pathogenic *Yersinia* species, with these numbers declining rapidly following an outbreak. In some cases, this was associated with the resolution of the outbreak without any treatment. If an antibiotic treatment was administered during the period preceding an outbreak or immediately after it, the natural resolution of this disease may be confused with a response to the antimicrobial compound administered. For these reasons, it is likely that yersiniosis in sheep has been grossly underdiagnosed because very few outbreaks of scouring in winter are investigated. If animals respond to treatment or the disease resolves, veterinary intervention is rarely sought.

Based on the high proportion of isolates that were found to be resistant to sulphonamides, it is unlikely that this compound will continue to be an effective treatment against bacterial enteritis caused by *Yersinia* species. Instead, the targeted use of oxytetracycline to treat affected animals is more likely to be effective. The results of the current study emphasise the need to routinely assess the sensitivity of *Yersinia* isolates to common antibiotics, especially on farms that have had frequent outbreaks of yersiniosis and a history of heavy use of antibiotics for treating this disease.

There were also major discrepancies between the detection of sulphonamide resistance when using disc diffusion compared with the agar dilution method. Consequently, although the agar dilution method is more laborious, it is necessary if a meaningful test for sulphonamide resistance is indicated, before this compound is used to treat yersiniosis.

The use of an autogenous vaccine to prevent outbreaks of yersiniosis requires further assessment. In the absence of an outbreak, the autogenous *Y. pseudotuberculosis* bacterin used in this study gave no production advantages. However, a multi-strain vaccine developed in New Zealand has successfully decreased the mortality and
prevalence of clinical cases of yersiniosis in deer, although it also has no detectable production benefits in the absence of an outbreak.

As discussed above, the occurrence of yersiniosis in sheep is probably significantly underestimated. As such, the benefit from an effective vaccine which reduces mortalities and clinical disease may be greater than previously estimated. Further research is necessary to determine if an autogenous bacterin vaccine, similar to that trialled in this study, is effective during an outbreak of yersiniosis or when animals are ingesting high numbers of *Yersinia* species. In addition, the investigation of vaccines using alternate adjuvants or formulations, such as a live-attenuated vaccine, would be useful.

The series of studies reported in this thesis have confirmed that the causative agents of the winter scours syndrome in young Merino sheep are *Y. pseudotuberculosis* and *Y. enterocolitica*. It has added to the understanding of the epidemiology and risk factors for this syndrome, but identified that further studies of these complex interactions are needed. It has also identified widespread resistance of *Yersinia* species to sulphonamides which are still used to treat the syndrome, and pointed to a need for further investigation of vaccination as a strategy to control this disease.

Based on results reported in this thesis, recommendations for the improved diagnosis and more effective management and treatment of yersiniosis in sheep include:

1. **Integrating molecular testing into diagnostic laboratories** - The specificity and repeatability of molecular tests, including PCRs, far exceed the traditional biochemical typing techniques used currently. The inaccuracies associated with biochemical typing may have contributed to the under-diagnosis of this syndrome.

2. **Increasing the use of culture growth scoring by diagnostic laboratories** – Adoption of this rapid, cost-effective method would enhance the ability of clinicians to interpret laboratory results and make informed, practical decisions about treatment.

3. **Including *Y. enterocolitica* as a differential diagnosis for all investigations of scours in sheep** – This pathogen was shed in faeces throughout the year (up to 30% shedding). Thus this organism could cause diarrhoea at any time, but confirmation of primary causation without pathology and tissue culture would be difficult.
4. The targeted use of an effective antibiotic to control an outbreak – Clinically affected animals should be removed from an affected mob and only these animals should be treated with antibiotics. In most cases, oxytetracycline should be the first line of therapy because there was widespread resistance of Yersinia species to sulphadimidine. Agar dilution rather than disc diffusion techniques should be used for the in vitro assessment of the efficacy of sulphonamides for Yersinia species.

5. The integration of grazing management strategies to manage and prevent this disease – Decreasing the stocking rate of affected mobs and moving them onto a pasture that had not been grazed by young animals during that winter should help to decrease the number of new clinical cases of yersiniosis and mitigate the risk of an outbreak.

Based on the results in this thesis, recommendations for future research include;

1. Determining the importance of Y. enterocolitica as a cause of scouring in sheep during spring, summer and autumn

2. Evaluating how environmental, management and animal factors such as weather, stocking rate, grazing management strategies or previous infection with Yersinia spp. affect the prevalence and severity of disease

3. Evaluate the efficacy of the autogenous vaccine and other vaccine formulations (i.e. live-attenuated) in the face of an outbreak of disease or following experimental challenge with Yersinia spp.
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