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Investigations into bacterial enteritis ('weaner scours') of Merino weaners in south-eastern Australia

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Abstract

'Weaner scours' causes scouring, illthrift and mortality in 3-16 months old Merino weaners from autumn to early spring. This syndrome, also called bacterial enteritis, causes significant economic loss and is an important animal welfare issue, with up to 80% of a mob affected during an outbreak. Affected sheep often have low worm burdens and the diarrhoea is not stopped by anthelmintic treatment ('drenching'), but does respond to treatment with antibiotics.

Based on clinical investigations, the primary infectious cause of weaner scours are *Yersinia* bacteria. The epidemiology of these organisms has not been investigated recently and appears to have changed, with more severe annual outbreaks on many farms. The main species are *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, both of which can cause disease in humans; these were the focus of this project.

Dr Helen McGregor was recruited as a post-doctoral candidate and responsible for the design and conduct of a 3-year study of weaner scours, from 2012-2015. She co-supervised one Honours student to completion in 2014, and one PhD student due to complete in 2016.

Aspects studied during field trials in 5 flocks, and clinical investigations in 22 flocks, included patterns of faecal shedding, risk factors for transmission and climatic, seasonal and husbandry factors associated with disease outbreaks and shedding of *Yersinia* sp. A laboratory study investigated the potential environmental survival of *Yersinia* isolates from affected flocks.

Key outputs from this project included: 1) the establishment of a library of *Yersinia* isolates from over 30 affected flocks; 2) development of molecular tests for the rapid and more accurate differentiation of *Yersinia* sp.; 3) confirmation that *Y. pseudotuberculosis* is highly seasonal, being isolated from faeces only immediately before or during an outbreak, whereas *Y.enterocolitica* is shed throughout the year; 4) development of standardised method for assessing antibiotic sensitivity in Yersiniae, with 7% of *Y.enterocolitica* and 31% of *Y.pseudotuberculosis* showing resistance to a commonly used antibacterial class (sulphafurazole); 5) *Y.pseudotuberculosis* was more tolerant of low temperatures and desiccation compared to *Y.enterocolitica*; 6) a factor associated with virulence of *Yersinia* sp. was not affected by extremes of temperature or desiccation and 7) development of leadership skills of the post-doctoral candidate and comprehensive training of Honours and PhD students.

Executive summary

Outbreaks of diarrhoea ('scouring') in weaned sheep, often associated with high mortalities, are common in self-replacing Merino flocks in the cool high rainfall regions of south-eastern Australia. One syndrome, 'weaner scours', is defined as scouring, illthrift and mortality in 3-16 months old Merino weaners. These sheep often have low worm egg counts or total worm burdens. The diarrhoea does not respond to anthelmintic treatment ('drenching'), but is successfully treated with antibiotics, typically sulphadimidine, oxytetracycline or a combination of trimethoprim and sulphadiazine. Based on clinical investigations and laboratory submissions, the primary causes of this syndrome are *Yersinia* and *Campylobacter* bacteria.

The patterns of infection ('epidemiology') of these organisms, and their interaction with the host sheep and environment, have not been investigated in great detail since the early 1990s. Increased knowledge about these factors should help understand ways to reduce transmission of these bacteria and control the disease. Both agents have implications for human health, and the widespread and often indiscriminate use of antibiotic preparations to control this syndrome is also of concern due to the potential for development of antimicrobial resistance.

The main species causing disease in sheep and cattle are *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, and these are also the most important in weaner scours. Consequently, the main focus of this post-doctoral study has been these *Yersinia* species. These gut bacteria can cause disase in humans and a wide variety of domestic and wild mammals and birds. *Yersinia enterocolitica* can be isolated from grazing livestock all year round, whereas shedding of *Y. pseudotuberculosis* occurs seasonally.

Aspects studied included risk factors for transmission, the environmental survival of the organism and climatic, seasonal or husbandry factors that are associated with disease outbreaks and shedding of the organism in sheep faeces.

A major outcome of this project has been the establishment of a substantial library of *Yersinia* isolates collected from over 30 affected flocks, mainly in Victoria, but also from NSW and South Australia. A cross section of these isolates have also been identified and tested for antibiotic resistance. Preliminary results for mapping the molecular characteristics ('molecular epidemiology') of these species, all implicated in livestock disease, are also available.

In addition to the appointment of Dr Helen McGregor to the postdoctoral position, from April 2012 to March 2015, a postgraduate student (Kelly Stanger, PhD candidate) was recruited and co-supervised by Drs McGregor and Larsen. Completion and submission of this work is due in 2016 and a number of papers will be submitted to refereed journals. Thus, this work is an integral part of, and important additional output to, the investigations summarised in this report. In 2014, year 3 of the post-doctoral program, an honors student was recruited and co-supervised by Drs McGregor and Larsen. The work undertaken by that student is included in this report and will also be submitted to a peer reviewed journal.

During the on-farm investigations of the epidemiology of the weaner scouring syndrome, faecal samples, live weight and dag score data were collected from trial animals in known affected flocks. This randomised, longitudinal study began as a pilot on 3 farms in year 1 (2012). Based on initial findings, the study was expanded to a full investigation in 5 flocks in year 2 (2013). Tests to detect the presence of *Yersinia*, the relative prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* and to quantify the amount of faecal shedding were applied to these faecal samples.

In year 2, 2800 faecal samples were collected. Bacteria morphologically similar to *Yersinia* sp. were cultured from 530 (19%) of these and analysed further using a molecular (PCR) test developed specifically to detect the Yersinia species of interest to this project. Of these 530 isolates, 42% were identified as *Y. enterocolitica* and 37% as *Y. pseudotuberculosis*, with 95% of the latter identified as serotype O:3. Serotyping of *Y. enterocolitica* is more complex, and these isolates are undergoing further testing, using traditional biochemical techniques and DNA finger printing, to better define the molecular epidemiology of these subgroups. This will provide additional information about the importance of this organism for livestock health and production and its potential as a zoonotic agent.

A standardised method for assessing the sensitivity of field isolates of *Yersinia* to three commonly used antibiotics was developed and applied to a cross section of stored field isolates. Of 282 isolates tested, 7% of *Y. enterocolitica* and 31% of *Y. pseudotuberculosis* were resistant to sulphadimidine, with 4% of the latter also resistant to trimethoprim and 1% of both resitant to tetracycline. Although the number of farms is relatively small, there was a strong link between the frequency of use of antibiotic and the development of resistance. Farms which take a more integrated approach to managing this syndrome, such as using strategic rather than blanket treatment, had less resistance.

A complex analysis of risk factors, including seasonal weather conditions and feed availability, is also being undertaken. This may enable development of a predictive tool for use by owners of flocks in high risk areas or with a high annual incidence of this syndrome. These results, in combination with further antibiotic resistance testing, will also be used to refine treatment and control strategies for affected flocks.

A commercial vaccine against *Yersinia pseudotuberculosis* (serotypes I, II and III; Yersiniavax™) has been widely adopted by the New Zealand deer industry, but is not available in Australia. New Zealand studies show that the vaccine reduces mortalities, from 2.1% to 0.8%, and also the severity of Yersiniosis in deer (Mackintosh, Buddle et al. 1991; Wilson, Mackintosh et al. 1999) The post-doctoral project, through the associated PhD candidate, investigated the efficacy of an autogenous bacterin containing formalin-killed *Y. pseudotuberculosis* (ie. strains isolated from sheep on the affected farm). This study was conducted during the winter of 2014 in merino weaners in two flocks in southern Victoria. Both flocks experience regular outbreaks of weaners scours from yersiniosis during the winter. Results from this study are still being analysed and will be included in the candidate's PhD thesis and refereed journal articles in 2016.

Finally, an honours student was co-supervised by the post-doctoral candidate in year 2 of the project (2013). This student (Clare Thompson) undertook a comprehensive laboratory investigation of the effects of temperature and dessication on the *Yersinia* species implicated in the weaner scours syndrome. The temperature gradients reflected those that typically occur in south-eastern Australia, where this syndrome is most prevalent. The study also examined the effect of temperature and desiccation on the retention or resilience of a virulence factor for these bacteria, hence their capacity to cause disease under a variety of environmental conditions.

Strains of *Y. pseudotuberculosis* and *Y. enterocolitica* isolated from diseased sheep were inoculated into faecal pellets, deposited onto soil in boxes and incubated at different fixed and fluctuating temperatures for up to 40 days. The rate of recovery of organisms from faecal pellets was determined by assessing the density of Yersinia colonies on the media at 36 hours. *Yersinia pseudotuberculosis* was isolated at consistently higher rates compared to *Yersinia enterocolitica* when adjusting for day, water and temperature treatments, and repeated measurements ($P<0.001$). The virulence factor was not affected by desiccation or temperature. This honours project is a first step towards better understanding the effect of climate on the survival of strains of *Y. enterocolitica* and *Y. pseudotuberculosis* that infect livestock.

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1 Background

1.1 Investigations into the epideimiology and control of a weaner scours syndrome in south-eastern Australia

The weaner scours syndrome, also called bacterial enteritis, can cause significant economic loss in affected flocks (Sackett et al 2006). It is also an important animal welfare issue, with up to 50% of affected sheep dying and 80% being affected during an outbreak. The syndrome occurs most often in the cool, high rainfall areas of south-eastern Australia and is typically associated with cold, wet weather, from July to October. In many flocks, particularly on farms with higher fertiliser application, hence improved pastures and higher stocking rates, outbreaks tend to be more regular and more severe compared to sporadic outbreaks on less intensively managed farms.

The agents investigated in this report of a current post-doctoral study are two *Yersinia* species, both of which can be isolated from faeces and intestinal contents (Bin-kun et al 1994, Philbey et al 1991, Slee and Button 1990, Slee and Stillbeck 1992, Soderqvist et al 2012). However, a detailed understanding of the epidemiology of weaner scours has been limited by a predominatly case-based approach to collecting data and reporting outbreaks. Risk factors for this syndrome are likely to be multiple and complex. They are proposed to include climate, seasonal weather patterns, the availability, quality and intake of pasture, live weight and growth rate, intercurrent disease and the presence of a causative bacterial agent in the gut (Philbey et al 1991, Slee and Skilbeck 1992).

Two relatively distinct types of the weaner scours syndrome have been described – a summer and a winter form. The 'summer scour' syndrome is sporadic, but commonly associated with recent weaning, periods of limited feed availability, hence weight loss, and relatively high stocking rates. The winter syndrome occurs annually, with most outbreaks reported when rainfall is high, temperature is low and feed is limiting (Glastonbury 1990; Abbott and Maxwell 2002). It is distinguished from hypersensitivity scouring by the age of affected sheep (<10 months-old compared with > 12-months old in hypersensitivity scours (Larsen et al 1994), response to antimicrobial therapy and occurrence of mortalities.

The epidemiology of the 'winter scours' syndrome has not been fully investigated, but typically presents in young, weaned sheep, as a profuse, watery diarrhoea (Slee and Button 1990). It presents in two ways; acute to subacute enteritis, with high morbidity and moderate mortality, or sub-clinical infections which are thought to cause lost productivity (Jubb and Grant 2008). The syndrome is widely recognised and believed to develop from a complex interaction between infectious agents, stress events and weather conditions (Callinan, Cook et al. 1988; Slee and Skilbeck 1992; Carniel, Autenrieth et al. 2006). Although *Salmonella* and *Campylobacter* have also been implicated, *Yersinia* species have been identified as the primary bacterial agents contributing to this syndrome.

Yersiniosis has significant animal health and welfare implications and can result in increased costs and decreased returns, with the losses to the Australian sheep industry from bacterial enteritis estimated to be at least $29 million annually (Sackett and Holmes 2006). This estimate included mortalities, lost production and the costs of treatment, but was compromised by inadequate data on the prevalence of bacterial enteritis and a lack of knowledge about how much sub-clinical infections could decrease production.

Given these gaps in knowledge, investigations on the epidemiology of weaner scours were initiated in five 'sentinel flocks' in the high rainfall areas of Victoria in 2013 (McGregor et al 2013). *Yersinia pseudotuberculosis* serotype O:3 was identified as the most significant contributor to non-parasitic scouring, comprising 97% of isolates (McGregor et al 2014). The study demonstrated that shedding of *Y, pseudotuberculosis* increased during winter, with the prevalence of scouring averaging 40% and mortality ranging from 0-9%. Very little

quantitative detail is known about risk factors associated with outbreaks of Yersiniosis, or the success of commonly used prevention and control measures, but attempts to identify them are being undertaken with further analyses of climatic and management data collected as part of this post-doctoral study.

Following the observation that *Y. pseudotuberculosis* serotype O:3 was isolated from 97% of cases of Yersiniosis in the sentinel flocks in year 2 (2013), a basic formalin-killed bacterin was developed and evaluated as a potential control measure in year 3 (2014). This was informed by comprehensive studies in the New Zealand deer industry, in which outbreaks of acute enteritis, with mortality rates exceeding 30%, are common. This led to the development of a commercially available vaccine (Yersiniavax™) that is now widely used in NZ, to both decrease mortalities in young deer and reduce the severity of disease in affected animals (Wilson, Mackintosh et al. 1999).

There are differences in the epidemiology of the species of *Yersinia* pathogenic to sheep, with *Y enterocolitica* isolated from grazing livestock all year round, whereas shedding of *Y. pseudotuberculosis* occurs seasonally, mainly in cooler high rainfall seasons, and is rarely found at other times of the year. There is no clear understanding as to why these differences occur. A laboratory study undertaken as part of this post-doctoral project, to better determine the range of environmental conditions under which *Yersinia* is able to survive, will begin to generate information to assist in understanding aspects of the survival of these organisms under Australian pastoral conditions and their transmission within livestock populations.

This report summarises the methods, results and analyses undertaken during a three-year post-doctoral study. The main aim was to investigate the interactions that determine the occurrence and severity of Yersiniosis in merino weaners. It was hoped that this would facilitate development of a cost-benefit analysis for preventative interventions, including husbandry, grazing management, antibiotic treatment and the use of a vaccine. This analysis has not been completed, but will be facilitated with the completion of a PhD candidate (co-supervised through the post-doctoral program) in 2016. This work will include more complex analyses of risk factors and climatic data. Ultimately, it is hoped that this would enable development of a predictive model, for both the occurrence and severity of the weaner scours syndrome. Additional work will be undertaken using a comprehensive library of *Yersinia* isolates that has been collected from sheep in affected flocks. This will include molecular typing of the *Y. enterocolitica* isolates, to identify any association between the severity of disease, zoonotic potential and strain type, and further testing for antibiotic resistance.

2 Projective objectives

1. Ensure that the postdoctoral fellow has opportunity for development of leadership skills by acting as a mentor and by enabling the postdoctoral fellow to receive formal leadership training on an annual basis.

2. Develop the research capability of the postdoctoral fellow to a point where she will be successful in competitive grant applications.

The postdoctoral fellow will develop a program that reduces scouring and the accumulation of dag by:

1. Defining the epidemiology of bacterial causes of scouring in young sheep, mainly *Campylobacter* and/ or *Yersinia spp*., AND

2. Isolation and characterisation of the range of organisms involved

The post doctoral fellow will also:

a) Investigate and define differences in scouring between regions and different aged sheep,
b) Publish 3 scientific publications in high impact journals with the post doctoral fellow as leading author.

3 Methods

3.1 Overview of longitudinal study

3.1.1 Collection of longitudinal data in a 'sentinel flock' trial

A longitudinal sampling framework was applied to 3 properties in 2012 ('year 1') and 5 properties in 2013 ('year 2') of the post-doctoral study. All properties had a history of weaner scours syndrome occurring predominantly or exclusively in the autumn-winter months (from April to September). Animals enrolled in the study were identified with electronic tags to facilitate recording of individual weights and collection of faecal samples.

In year 1, 200 trial animals were tagged on each property and observations taken at approximately 6-week intervals, from April to September. Data collected at each visit included live weight and dag score from all 200 trial weaners, and faecal samples which were collected from up to 100 animals. The same 100 animals were sampled at each collection. Pasture availability and the amount of supplementary feeding, if conducted, was also recorded. Climatic data for use in risk factor analyses was collected from a combination of farm records and the closest available Bureau of Meteorology data site.

Faecal samples were processed in the laboratory to assess faecal consistency, endoparasite burden (worm egg count (WEC) and the presence of coccidia), and in microbiology for the presence and amount of growth of *Yersinia* species. In year 1 traditional microbiological techniques were used to record the presence and amount of growth of *Yersinia* sp. These samples were then tested retrospectively, following development of a polymerase chain reaction (PCR) test, in year 2. This allowed more rapid and more accurate differentiation of the two main specis of *Yersinia* implicated in the weaner scours syndrome, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis.*

The results from year 1 supported the strong association of *Y. pseudotuberculosis* with the weaner scours syndrome. Consequently, in year 2 the study was expanded, with longitudinal sampling occurring within 5 flocks. This enabled the collection of more extensive information about potential risk factors, hence a potentially more powerful analysis of this epidemiological data. Each of the 5 farms was visited ast least 6 times, between weaning in December/January and September. Weaning bodyweights were recorded to calculate growth rates, adjust for differences in weaning weights and investigate any association between low weaning weight and the incidence or severity of disease. Data were collected from 100 animals in each flock, with a total of approximately 4800 faecal samples collected and tested in parasitology and microbiology across this 2 year period. Over 1600 Yersinia isolates have been stored at ultralow temperatures. This library of isolates will facilitate further investigation to map the molecular epidemiology of these Yersinia species and for further antibiotic resistance testing.

In years 1 and 2 of the study faecal samples and information about risk factors were also collected from additional 'outbreak' flocks, in which roundworms, predominantly *Teladorsagia* and *Trichostrongylus* spp., had been excluded as the sole or primary cause of

diarrhoea on the basis of low worm egg counts and lack of response to anthelmintic treatment. As far as possible, standardised information on livestock and pasture management, supplementary feeding, estimates of pasture availability and weather data were also collected for these submissions. The preliminary findings from these investigations are presented in this report. However, a more complex analysis of the climatic data and its association with shedding of *Yersinia* and the occurrence of weaner scours is ongoing, with the aim of submitting this for publication in a peer reviewed journal by early 2016.

3.1.2 Statistical analyses in the longitudinal study

3.1.2.1 Overview

Total and direct effects for potential risk factors on risk of shedding, and on risk of 'heavy shedding' (as distinct from 'not heavy', ie. light or no shedding), of each of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* were assessed using multivariable regression models. Factors assessed were farm, day of year when sampled, sheep sex, liveweight, average daily gain, and worm egg count.

Two models were fitted for each potential risk factor, one for each of the total and direct effects. Total and direct effects are based on the concept that a particular factor can affect risk of shedding directly and/or indirectly. Indirect effects are those where the effect is mediated through one or more intervening variables. For example, a risk factor may alter the risk of exposure to an intervening variable which, in turn, alters the risk of shedding. Direct effects are those where no intervening variable was measured. A particular risk factor may have only a direct effect, only indirect effects, or both. The total effect of a risk factor is the sum of its direct and indirect effects.

Total and direct effects were estimated after defining the set of covariates to be fitted in each regression model. These sets (minimal adjustment sets) were identified for each of total and direct effects using a causal diagram (more specifically, a directed acyclic graph). This diagram depicted the hypothesised interrelationships between the potential risk factors, and between potential risk factors and shedding. Pathways were represented by arrows from potential risk factors to shedding. Direct pathways had no intervening variables; indirect pathways were those that could be traced from potential risk factors to shedding through intervening variables.

Faecal consistency was not included in this process as it was considered an effect rather than a cause of shedding. However, to assess the usefulness of faecal consistency as an indicator of shedding, the association between faecal consistency and shedding (heavy or not heavy) was also assessed.

3.1.2.2 Development of the causal diagram

A causal diagram was developed to hypothesise the direct and indirect pathways through which the proposed causal factors influence Yersinia shedding (Fig. 1). Causal variables were only included in this diagram if there was sufficient data to ensure a degree of certainty from the analysis.

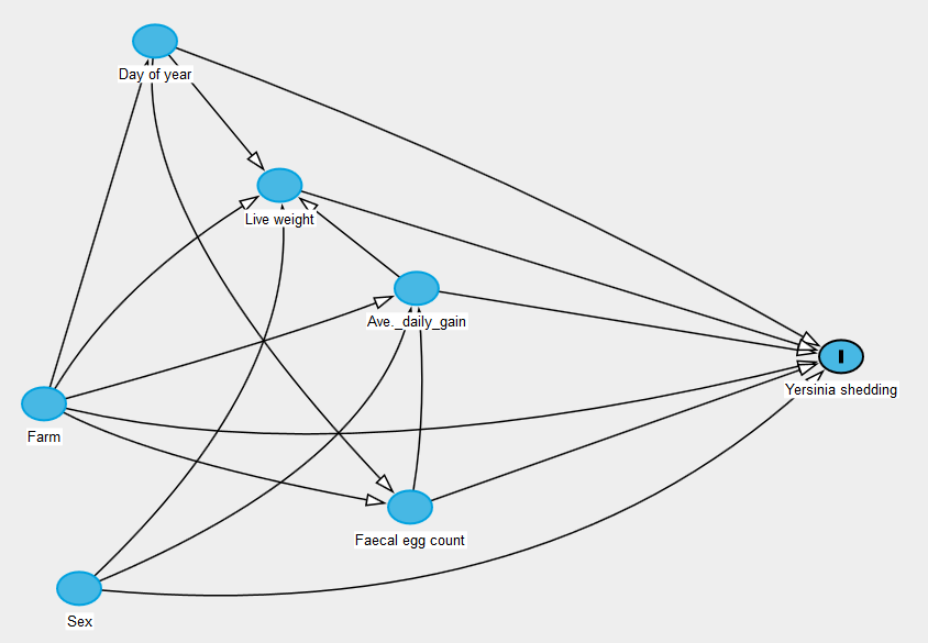


Fig. 1. Direct and indirect factors hypothesised to influence the shedding of *Yersinia* bacteria

3.1.2.3 Minimal adjustment sets

Minimal adjustment sets consisted of a) factors that were potential confounders and b) when estimating direct effects, intervening variables from indirect pathways. Confounders were those factors that had both a pathway to the potential risk factors and a separate pathway to shedding. Confounding can also occur due to conditional association. When a factor is fitted in a regression model, its determinants can become associated with the potential risk factor, and hence can become a confounder for that potential risk factor. Such potential confounders were also identified and included in minimal adjustment sets. Minimal adjustment sets were identified using on-line software (Daggity 2.2, online; Table 1).

Table 1: Total and direct effects of exposure variables for shedding of *Yersinia* sp.

Exposure variable	Total effects	Direct effects
Live weight	ADG, day of year, farm, sex	Total and direct are equal
Average daily gain (ADG)	Farm, WEC, sex	Day of year, farm, WEC, live weight, sex
Worm egg count (WEC)	Day of year, farm,	ADG, farm, day of year, sex
Sex	Farm	ADG, day of year, farm, WEC, live weight
Farm	Day of year	ADG, day of year, WEC, live weight, sex
Day of year	Farm	ADG, farm, WEC, live weight, sex,

3.1.2.4 Regression modelling

For all analyses, the sheep-sampling was the unit of analysis; one sheep sampled at a single point in time contributed one sheep-sampling. Generalised estimating equations (GEE) were used, with sheep as the panel variable, robust standard errors and unstructured correlation structures. The logit link and binomial error distribution were used. Farm was included in all models as a fixed effect, including where not required for the minimal adjustment set to account for clustering of sheep within farm. Analyses were performed separately for 2012 and 2013. Models were fitted using the -xtgee- command in Stata (version 13, StataCorp, College Station, Texas, USA).

Potential risk factors measured as continuous variables (day of year when sampled, liveweight, average daily gain, and worm egg count) were assessed for linearity in the logit (ie. for a linear relationship with the logit of shedding) using visual assessment of lowess plots (locally weighted linear regression), generated using Stata's -lowess- command. Liveweight and worm egg count were approximately linear in the logit and so were subsequently fitted as continuous variables in regression models. However, effect estimates for worm egg count were implausibly precise and close to 0 (ie odds ratios close to 1) even though crude analyses indicated markedly increased risk of shedding at higher worm egg counts. Accordingly, worm egg count was categorised for analyses. Day of year when sampled and average daily gain were curvilinear in the logit so linear and quadratic terms of the grand mean centred variables were used. Relationships between average daily gain and shedding were also assessed using fractional polynomials, fitted with Stata's -fp- command. Forty-four fractional polynomial models were assessed. Two of the following powers were selected for each fractional polynomial: -2, -1, -0.5, 0, 0.5, 1, 2, and 3) and the best fitting model selected. Average daily gain was scaled to be greater than zero.

Interactions between day of year when sampled and farm were hypothesised *a priori* for all outcome variables because differences in microclimate, animal management and husbandry procedures are different and thought to significantly impact the outcome variables This interaction was fitted in subsequent models.

Associations between faecal consistency and shedding were assessed using generalised estimating equations as described above, with faecal consistency fitted as a categorical variable with three categories, 1 (well-formed, firm pellets), 3 (soft faeces with structure) and 5 (watery faeces). These categories were chosen to represent the spread of scores within the system of recording (score range 1-5). Farm was fitted as a fixed effect. Day of year when sampled (linear and quadratic terms of the grand mean centred variables) was also included. Associations were assessed with and without adjustment for worm egg count (categorised).

3.1.3 Species identification, serotyping and biotyping of *Yersinia* isolates

For accurate speciation of *Yersinia,* all isolates cultured on selective media (CIN agar) that were morphologically similar to *Yersinia* (n = 530) were stored at ultra low temperatures for retrospective molecular testing in a multiplex PCR (Stenkova et al 2008). Testing in the PCR identified that 42% were *Y. enterocolitica* and 37% were *Y. pseudotuberculosis*. A modified serotyping methodology was applied to all *Y. pseudotuberculosis* isolates (Hodges et al. 1984; Mair 1965). DNA finger printing and molecular techniques are being investigated to allow concurrent biotype and serotype identification of the stored *Y.enterocolitica* isolates (Lasch et al. 2010; Parisi et al. 2008; Stephan et al. 2011).

3.1.4 Virulence testing

To determine potential pathogenicity associated with the presence of the virulence plasmid, *Y. enterocolitica* isolates were screened to detect the *Yersinia* adherence protein gene ('yadA'). A simplex PCR, to assess virulence associated with the presence of the yadA gene, was applied to samples confirmed as *Y. enterocolitica* in the multiplex species PCR.

3.1.5 Antibiotic sensitivity testing

All isolates tested for antibiotic resistance were field strains recovered and stored from either the sentinel or outbreak farms. Initial testing was focused on isolates from farms with a history of heavy use of antibiotics, typically in response to a high incidence of weaner scours syndrome in most years. The technique used is the standard practice for diagnostic sensitivity testing across a number of antibiotics used in production animal medicine (tetracycline, trimethoprim, sulphafurazole). The technique involves the growth of pure isolate on sheep blood agar, application of antibiotic impregnated discs and measuring inhibition of growth around the disc after 16-18 hours incubated at 30°C. (Plates 1 and 2).

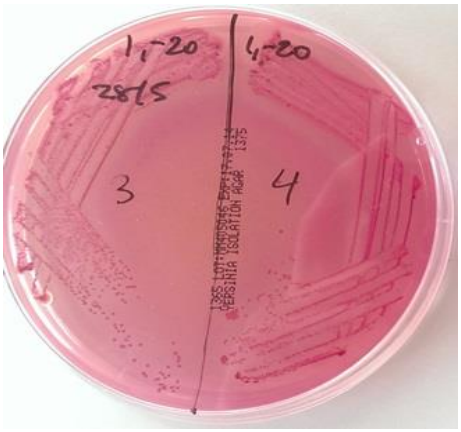


Plate 1. Positive growth for Yersinia on CIN media

Plate 2. Antibiotic sensitivity testing media showing inhibitory zones around bacterial colonies

Testing is continuing to include a greater range of field isolates, from both sentinel and outbreak flocks, and establish the Minimum Inhibitory Concentration (MIC) for the resistant isolates. This is being completed in collaboration with another research group at the University of Melbourne with an interest in antibiotic resistance of organisms causing disease in livestock species. This information will support future recommendations to producers about the selection of animals for treatment, and integration of antibiotic use into a strategic approach to disease management, particularly on properties with a history of heavy use of antibiotics.

Extending the antimicrobial resistance study will facilitate approaches to the dairy industry where Yersiniosis is a recognised cause of production loss and severe disease in dairy calves, often associated with the heavy and frequent use of antibiotics. Whilst a direct consequence of the post-doctoral study, this work falls outside its scope and timeframe and will be published separately in peer reviewed journals and as part of the associated PhD thesis.

3.2 Vaccination trial

An important aim of the post-doctoral study was to provide control and managment options for producers experiencing serious annual losses in their flocks from Yersiniosis. The potential of a vaccine to reduce the prevalence and severity of weaner scours caused by *Y. pseudotuberculosis*, and reduce faecal shedding hence faecal-oral cycling, was highlighted by results from years 1 and 2 of this project (Section 4.1.2). Consequently, in year 3 an autogenous vaccine was developed under a minor use permit from the AVPMA and tested in 2 flocks as part of the PhD progam of Kelly Stanger.

Two flocks which participated in the longitudinal trial, each with a known high prevalence of Yersiniosis, were selected to participate in the vaccine study. A similar longitudinal observation and sampling protocol was applied, with collection of bodyweight and faecal samples across the high risk autumn-winter period in cohorts of vaccinated and non-vaccinated weaners. On each farm, 490 recently weaned merino lambs were randomly selected, tagged with radiofrequency ear tags ('RFID') and allocated to one of three trial mobs. In each mob, 75 animals were vaccinated twice, one month apart, with an autogenous bacterin, containing formalin-killed *Y. pseudotuberculosis* serotype O:3 isolated from that farm. Seventy five weaners were identified as controls. To reproduce the typical management practices within these self-replacing Merino flocks, the size of each mob was increased to 300-400 by the addition of animals not directly enrolled in the vaccine study. Individual live weight and dag score data was collected on four occasions.

Throughout the year, the co-operating farmers collected 50 freshly deposited faecal samples from the ground in the paddock grazed by each of the three trial mobs. Samples were collected at 2-4 week intervals, depending on the level of perceived risk, with the frequency of sampling increased during the winter. They were screened for the presence of Yersinia using CIN selective agar and identified to species using a multiplex PCR developed in year 2 of the study. Bulk worm egg counts and faecal consistency scores were also recorded. Results from this monitoring were used to predict when an outbreak of Yersiniosis was imminent, and dictated the timing of visits to weigh and collect samples from the individually identified animals. This prediction was based on data collected from sentinel farms in year 2 (2013), when it was hypothesised that if *Y. pseudotuberculosis* was isolated from more than 5% of animals the risk of an outbreak of Yersiniosis was high. Animals were closely monitored once shedding of *Y. pseudotuberculosis* reached or exceeded this prevalence, with the aim of collecting individual faecal samples during an outbreak of yersiniosis (ie. animals were clinically ill, had diarrhoea and were shedding *Yersinia* spp. bacteria).

In addition to the faecal samples, serial blood samples were collected from 20 control and 20 vaccinated weaners to evaluate their response to vaccination and natural challenge using an ELISA antibody test. Samples were collected before vaccination, 2 and 4 weeks following vaccination, one month before the high risk period (May) and at the conclusion of the study (September). This testing was designed to monitor the development of immunity following vaccination and natural exposure, providing some insight into when this immunity starts to develop and whether it is protective. This work forms a substantial part of the PhD thesis supervised under the postdoctorate position, and so only an overview of the preliminary results are included in this report.

3.3 Environmental survival of *Yersinia* species

In 2014 an honours student was supervised as part of the capacity building and leadership program for the postdoctoral position. The student participated in the design and conduct of an experiment to investigate the survival of *Y. enterocolitica* and *Y. pseudotuberculosis* under a variety of environmental conditions. There have been no recent investigations into the survival of *Yersinia* sp. under Australian conditions, and so the outcomes from this work

were expected to support the development of grazing management strategies that may help control the weaner scours syndrome.

The isolates used were taken from a library collected from flocks affected by Yersiniosis and stored at -70°C. They included one pathogenic strain of *Y. pseudotuberculosis,* and two field strains of *Y. enterocolitica* isolated from different flocks in Victoria. The field strain of *Y. pseudotuberculosis* was associated with severe enteritis and mortalities in weaner sheep during this project. One of the *Y. enterocolitica* strains was isolated from sheep with overt disease (abortion) and the other was collected from asymptomatic sheep. However, the latter was retrospectively classified as potentially pathogenic after the 70 kb virulence plasmid gene (yadA) was confirmed by PCR. Measured concentrations of these strains were inoculated into individual faecal pellets collected from sheep confirmed not to be shedding *Yersinia* through culture on selective media (CIN agar).

Homogenous solutions of the two strains of *Y. enterocolitica* and one strain of *Y. pseudotuberculosis* were made, diluted to an optical density of 1.0 and then the faecal pellets were soaked in this solution. The innoculated pellets were placed at evenly spaced intervals on top of soil within plastic boxes, with each soil box containing only one strain of *Yersinia* species and exposed to a particular set of temperature and moisture conditions (ie. each box formed one treatment).

The soil for the boxes was collected from a farm in a high rainfall region of south eastern Australia. This farm had a history of weaner scours caused by Yersiniosis, but the soil was collected from an area not grazed by sheep. The use of this soil controlled for soil characteristics experienced by Yersiniae in natural conditions, such as pH, organic matter and microbial populations (Guan and Holley 2003, Tashiro et al. 1991). To preserve the existing biota the soil was not sterilised and no *Yersinia* spp. bacteria were isolated from samples before depositing the spiked faeces.

Sampling of faeces during this experiment was conducted on nine occasions over 40 days. Treatments consisted of watered or dry soil, and four fixed and three fluctuating temperatures; a total of 8 treatments for fixed temperatures, and 6 treatments for fluctuating temperatures, with each treatment having 3 replicates. The fixed temperatures selected were -20°C, 4°C, 20°C and 40°C. Each *Yersinia* sp. had two water treatments in each temperature group; one treatment box was watered every 48 hours in order to maintain a moist environment ('watered'), the other treatment had no additional moisture added and so the faecal pellets and soil were comparatively dry ('standard'). To investigate if a change in temperature influenced survival, compared to a fixed range of temperatures, *Yersinia* sp. were also subjected to temperatures fluctuating between two fixed points. The fluctuations selected were between -20 and 4°C, between 4 and 20°C and between 20 and 40°C. The soil boxes were moved between their respective fixed temperature environments at 48 hour intervals (eg. from fridge at 4°C to room temperature at 20°C).

Faecal pellets were removed from their soil box using sterile forceps. Individual pellets were then macerated with sterile water to produce a standard consistency (a faecal consistency score of 3 on a scale of 1 (firm pellets) to 5 (watery diarrhoea)), then plated onto selective media for *Yersinia* sp. (CIN agar). A colony growth score was recorded after 36 hours incubation at 30°C. This score ranged from 0 (no growth) to 4 (maximum growth), the same scale used in the longitudinal trial.

Where colonies morphologically consistent with *Yersinia* sp. were detected, a pure isolate was removed from the CIN agar and identified to species in the multiplex PCR. To reduce the number of samples submitted for molecular testing, the samples tested were those from each treatment in week one, samples from the week prior to any changes being detected in colony morphology, and then those in subsequent weeks after the colony morphology had changed.

The recovered isolates were also tested for the presence of a virulence plasmid using a molecular test to detect the *Yersinia* adherence protein gene (yadA). YadA is involved in resistance of *Yersinia* sp. to the antimicrobial activity of polypeptides from human granulocytes, and so is partly responsible for resistance to phagocytosis (Cornelis et al. 1998). A simplex PCR, to assess virulence associated with the presence of the yadA gene, was applied to samples confirmed as *Yersinia* species by the multiplex PCR. The samples tested were selected to demonstrate that the plasmid was present at day 0, then to determine if and when it was lost by testing samples from the last point at which the recovered isolate was positively identified as *Yersinia* sp.

3.3.1 Statistical analysis

A mixed effects ordinal logistic regression model (Stata, version 13) was used to compare treatments, based on odds ratios and associated 95% confidence intervals. This model was used because the growth score data was categorical and recorded over time. One model compared *Y. enterocolitica* to *Y. pseudotuberculosis* over all treatments, another compared all temperature and water treatments for *Y. enterocolitica* only, and a third model compared treatments for *Y. pseudotuberculosis* only.

4 Results

4.1 Overview

4.1.1 Longitudinal (sentinel flock) study

The results presented in this report reflect analyses of longitudinal data on the incidence of shedding for *Y. enterocolitica* and *Y. pseudotuberculosis* for the 5 farms tested in year 2 (2013), plus preliminary virulence, serotyping and antibiotic sensitivity data compiled from testing isolates collected in the longitudinal trial. Information about potential risk factors, including climate/weather, feed availability and farm management practices, was also collected for this period. However, these epidemiological data require a more complex analysis to investigate associations between incidence of shedding, occurrence and severity of disease and possible productivity effects. Consequently, the analyses and interpretation of these data are not included in this report but will be published in a peer reviewed journal when completed. These analyses will include investigating whether a risk based predictive tool can be developed to assist producers anticipate the occurence and severity of this syndrome in their weaner sheep. If this is possible, this may allow proactively management to reduce occurence and severity of this syndrome.

4.1.2 Prevalence of faecal shedding of *Yersinia* sp.

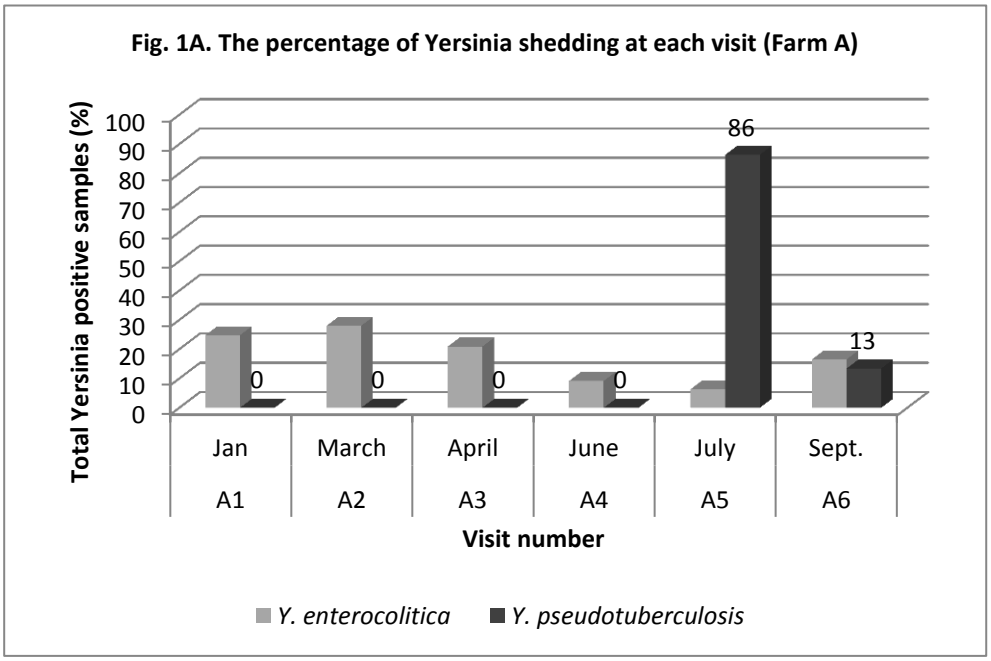
The prevalence of faecal shedding, for both *Y. enterocolitica* and *Y. pseudotuberculosis*, was estimated from samples processed in microbiology and PCR. Longitudinal data for three of the five sentinel flocks in year 2 (2013) are presented below. These flocks recorded outbreaks of weaner scours in year 2 and had detailed prevalence and shedding data. Two of these flocks (A and C) were subsequently recruited as co-operators for the vaccine trial conducted in year 3 (2014) (section 3.2). For the modelling analyses (section 4.2) only 4 farms were included because the data set for the fifth sentinel farm was incomplete due to mixing of mobs of weaners and a mis-muster on 3 of the 6 sampling visits. The excluded farm did not have an outbreak in years 2 or 3 of the trial.

The recovery of *Yersinia* species varied according to the time of year and between flocks (Figures 1A, 2 and 3). However, the frequency of shedding of *Y. enterocolitica* in all flocks was higher than for *Y. pseudotuberculosis*, with from 3-28% of samples classified as positive

across all time points and all flocks. Across all flocks, *Y. enterocolitica* was isolated on 2 to 6 occasions, whereas *Y. pseudotuberculosis* was isolated on only 2 or 3 occasions. The density of growth on selective media was scored using a standardised system (0-4). The degree of shedding of *Y. enterocolitica*, reflected by the growth scores, was also highly variable across all time points and for all years (Figure 1B)

Two of the five sentinel flocks experienced an outbreak of Yersiniosis which coincided with a peak in the number of sheep shedding *Y. pseudotuberculosis* in their faeces. This peak, 86% on Farm A and 49% on Farm C, was significantly higher than at other times. It was expected that during a disease outbreak, the greatest proportion of animals in an affected mob would also shed at a higher rate compared to other times. However, the distribution of growth scores was not consistently increased across outbreaks or flocks. This suggests that the rate of shedding may not necessarily be closely associated with the severity or acute phase of disease in an individual or flock. Alternatively, a high rate of shedding may persist for only a short time and so was not detected within the sampling times undertaken (Figure 1B).

For all flocks the peak number of animals shedding *Y. pseudotuberculosis* occurred in Jul-Aug (the fifth sampling point), regardless of whether an outbreak of disease occurred. The prevalence of shedding of *Y. pseudotuberculosis* was low at all other times of year, in all flocks, supporting previous observations that there is a strong seasonal occurrence of this syndrome. Flocks D and E had a very low proportion of animals shedding either species of Yersinia throughout the year. On farm D, the proportion shedding *Y. enterocolitica* ranged from 0 to 22%, and from 0 to 2% for *Y. pseudotuberculosis*. On Farm E, these ranges were 0 to 13% and 0 to 16%, respectively. Neither of these flocks experienced an outbreak of weaner scours in the trial mobs, or in other mobs on the farm.



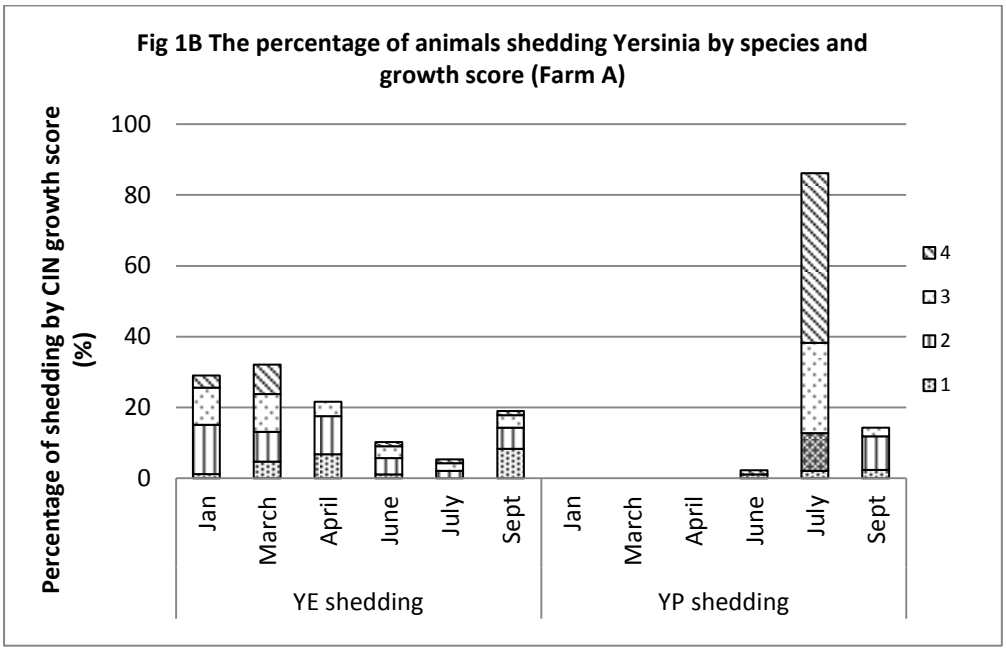


Fig 1B The percentage of animals shedding Yersinia by species and growth score (Farm A)
Percentage of shedding by CIN growth score (%)
100
80
60
40
20
0
4
3
2
1
Jan
March
April
June
July
Sept
Jan
March
April
June
July
Sept
YE shedding
YP shedding

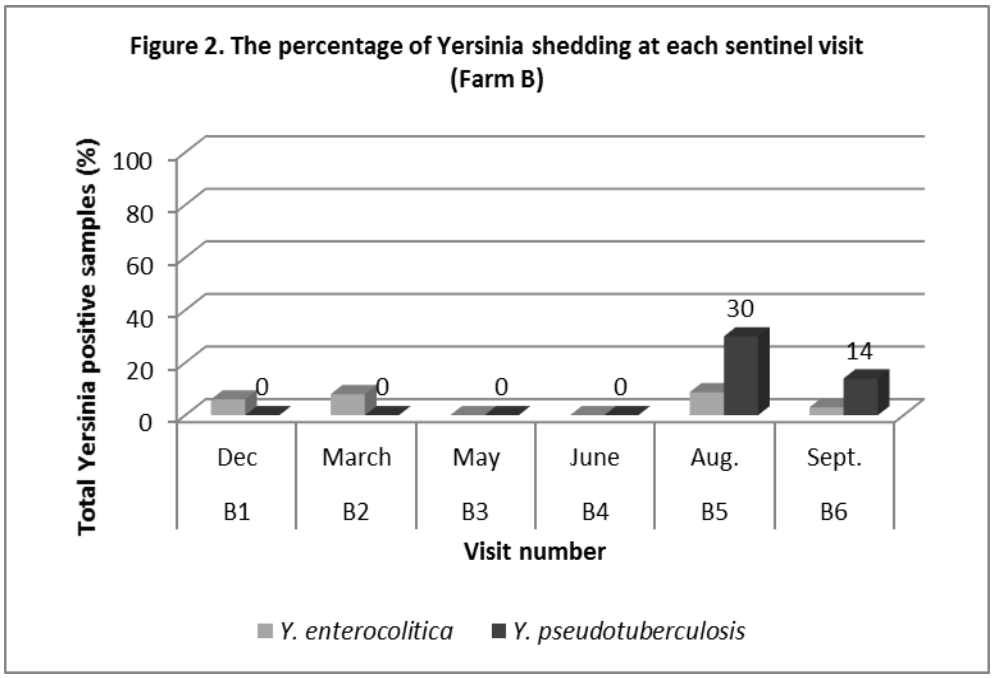
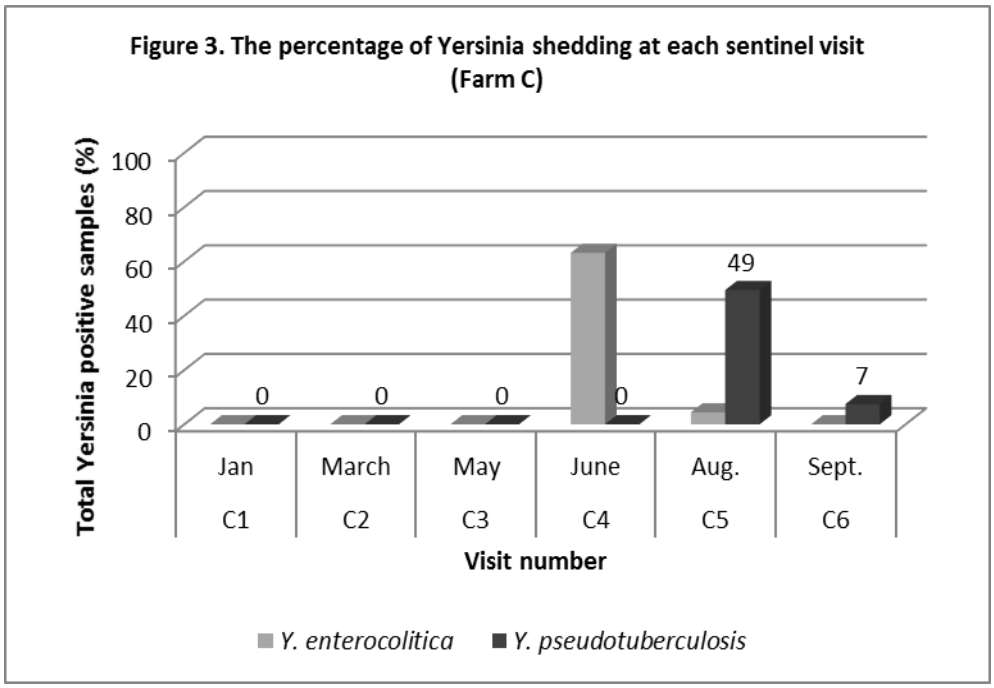


Figure 2. The percentage of Yersinia shedding at each sentinel visit (Farm B)
Total Yersinia positive samples (%)
100
80
60
40
20
0
0
0
0
0
30
14
Dec
B1
March
B2
May
B3
June
B4
Aug.
B5
Sept.
B6
Visit number
Y. enterocolitica
Y. pseudotuberculosis



4.2 Results for modelling of longitudinal data (by exposure variable)

4.2.1 Farm by day of year interaction

Estimation of the interaction between farm and day of year was attempted. The results suggest a very strong interaction between farm and day of year on *Y. enterocolitica* shedding in 2013 (Fig. 4). However, the data were too sparse to allow estimation of the interaction between farm and day of year on absolute shedding of *Y. pseudotuberculosis*, and on heavy shedding of the both organisms. For these, the interaction term was removed and just main effects fitted. These latter models assumed the effects of farm was constant across the year and the effects of day of year were constant within farm.

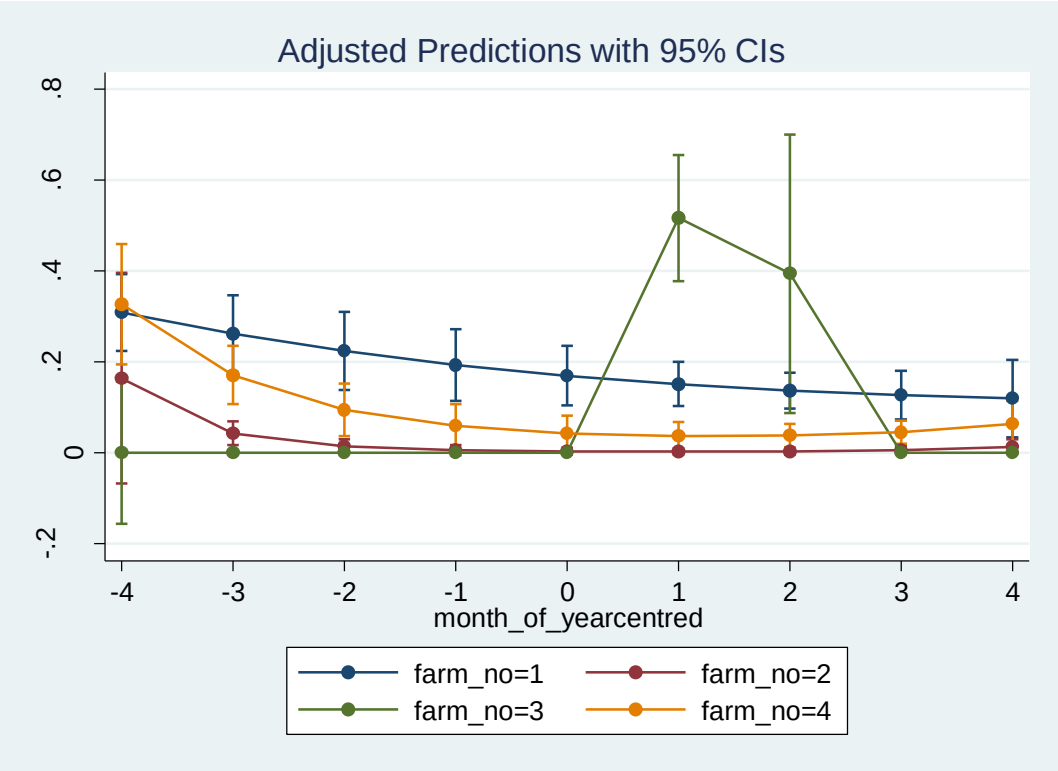


Fig. 4. Adjusted predictions for faecal shedding of *Y.enterocolitica* in 2013 (bars show 95% confidence intervals (CI))[A]

[A] Month is defined as a 30 day block. The farm by day of year interaction term for *Y. enterocolitica* in 2013 is reported, but was not estimated for any other variable. The models for *Y. enterocolitica* shedding and heavy shedding as well as *Y. pseudotuberculosis* shedding and heavy shedding were fitted without the farm by day of year interaction term for both years.

4.2.1.1 *Yersinia enterocolitica*

After removing the farm by day of year interaction term, there was a significant association between farm and *Y. enterocolitica* shedding. Results from 2012 indicated that the prevalence of shedding *Y. enterocolitica* was higher on farm B than farm C. There was a marked reduction in the proportion of animals shedding *Y. enterocolitica* on farm B between 2012 (20.2%) and 2013 (2.1%). In 2013, there was no significant difference between the proportion of animals shedding *Y. enterocolitica* on farms A and C. Farms B and D had a significantly lower proportion of animals shedding this bacteria than farm A.

This pattern was also evident with regard the prevalences of heavy shedding. It was noted that although farm A had the highest total proportion of animals shedding *Y. enterocolitica* (19.2%), less than half of these were heavy shedders (8.2%). This was in contrast to farm C which had a relatively smaller total proportion of shedders (11.8%), but a majority of those were heavy shedders (7.7%).

4.2.1.2 *Yersinia pseudotuberculosis*

In contrast to *Y. enterocolitica* shedding, the total number of animals shedding *Y. pseudotuberculosis* was significantly higher on farm C than on farm B in 2012. Odds of

animals on farm C shedding *Y. pseudotuberculosis* were at least 14 times higher than those on farm B.

In 2013, sheep on farm A were at significantly higher risk of shedding *Y. pseudotuberculosis*, and of being heavy shedders, than sheep on any other study farm.

4.2.2 Day of sampling

4.2.2.1 Yersinia enterocolitica

There appeared to be a significant interaction between farm and the day of sampling on the risk of shedding and also the risk of being a heavy shedder of *Y. enterocolitica* in each study year. The nature of this relationship could not be quantified as described above other than for absolute *Y. enterocolitica* shedding in 2013. .

Although a statistically significant association between shedding and day of year was indicated, the raw data did not illustrate a consistent temporal pattern of shedding *Y. enterocolitica* across farms in either year. There were marked differences in the proportion and timing of shedding between farms. For example, at each sampling on farm A in 2013, there was a moderate proportion of animals shedding *Y. enterocolitica*. In contrast, few animals were shedding this organism on farm C until the 4th sampling, at which time the proportion of weaners shedding *Y enterocolitica* reached 65%. On each of these farms, approximately half of the shedders were heavy shedders.

4.2.2.2 *Yersinia pseudotuberculosis*

The estimated effect of day of year on faecal shedding of *Y. pseudotuberculosis* was significant and large. Nevertheless, there was evidence to suggest that shedding was clustered by day of year and that the odds of shedding increased as day number increased. The raw data from each study year illustrated that most *Y. pseudotuberculosis* shedding was detected after day 200 (July). Farm C had the highest proportion of shedders in 2012 (67%), and farm A had the highest proportion of total and heavy shedders in 2013 (86% and 73%, respectively). Farm C also had a high total number of shedders in 2013 (57%), but only a small proportion of these were heavy shedders (8% overall, or 14% of those shedding).

The proportion of animals shedding *Y. pseudotuberculosis* did not exceed 10% on farm B in either year. An increase in the proportion of animals shedding *Y. pseudotuberculosis* on farm D was noted at the 5th and 6th samplings in 2013 (15% and 30%, respectively), but few animals were heavy shedders (8% and 1%, respectively).

4.2.3 Live weight and average daily gain

4.2.3.1 Live weight

Individual live weights for farms A, B, C and D included in this analysis were recorded on three occasions on 2 farms in 2012 (B and C), and on 6 occasions on all four farms in 2013. The timing of lambing and weaning was similar across each study farm. Where two years of data were collected, the average live weight was slightly higher in 2012 than in 2013 (Table 2). In 2013, animals on farm A were lighter on average compared with all other farms. These animals maintained a positive average daily gain throughout the year, but gains were lower than those achieved on other farms. Sheep on farms C and D had comparable average live weights and tended to have positive average daily gains. Farm B initially had the heaviest animals which lost weight in the early part of the year, but had higher average daily gains later on.

There was no significant relationship between the live weight at sampling and either the odds of shedding or being a heavy shedder of *Y. enterocolitica*. In 2012, live weight at sampling had a seemingly protective effect on the risk of shedding *Y. pseudotuberculosis*. The odds of shedding decreased by 10-40% with every additional kilogram live weight (OR 0.7, P<0.05, 0.6-0.9). There was no significant effect of live weight at sampling on the odds of shedding *Y. pseudotuberculosis*, nor the risk of being a heavy shedder in 2013.

Table 2. Mean (standard deviation, SD) live weight (kg) and average daily gain (g/day) in years 1 (2012) and 2 (2013).

Year	Farm	Mean (SD) live weight at visit:						Average (SD) daily gain since last sampling at visit:				
		1	2	3	4	5	6	2	3	4	5	6
2012	B	-	-	33 (5)		35 (5)	42 (6)	-	-	-	38 (31)	78 (42)
	C	-	-	29 (4)	28 (4)	-	39 (5)	-	-	-21 (44)	-	125 (27)
2013	A	19 (3)	19 (3)	21 (3)	22 (3)	26 (3)	27 (3)	17 (44)	31 (25)	15 (37)	75 (48)	22 (52)
	B	28 (5)	27 (5)	30 (4)	30 (4)	36 (5)	43 (5)	-32 (70)	42 (31)	-7 (48)	157 (54)	161 (55)
	C	23 (3)	22 (3)	25 (3)	26 (3)	29 (4)	36 (5)	-14 (35)	43 (33)	22 (47)	76 (65)	172 (54)
	D	22 (3)	25 (3)	24 (4)	26 (3)	32 (4)	36 (5)	59 (52)	-11 (35)	58 (46)	126 (37)	116 (52)

4.2.3.2 Average daily gain.

4.2.3.2.1 *Yersinia enterocolitica*

There was evidence of a significant relationship between average daily gain and the odds of shedding *Y. enterocolitica* in 2012, but the nature of the relationship was unclear. Graphical illustration of the direct and total effects, of both linear and quadratic terms for *Y. enterocolitica*, suggested that animals losing weight and those gaining weight quickly (>100 g/ day) were at increased risk of shedding (Figures 5 and 6). To ascertain if the bidirectional relationship was accurate, a fractional polynomial model was fitted (Figure 7) which found that only animals losing weight were at increased risk of shedding.

In 2013 a similar relationship was observed for total shedding and the odds of being a heavy shedder or shedding at all. Although significant, the associations were small and are not considered biologically important (Figures 8-11).

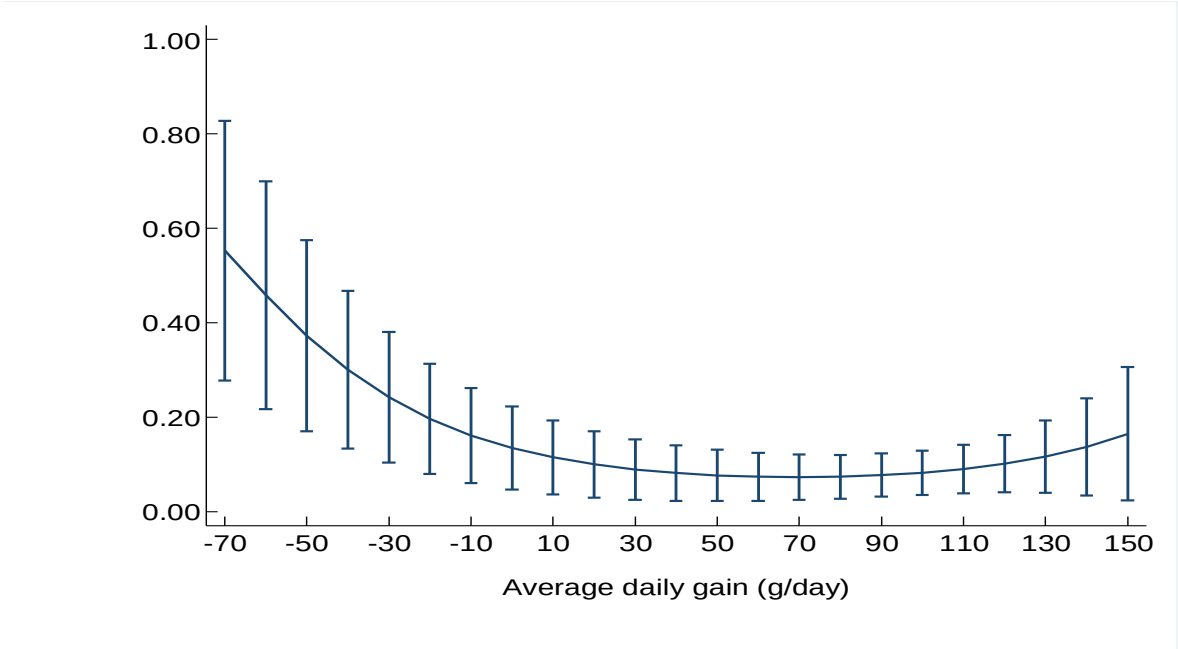


Fig. 5. Faecal shedding of *Yersinia enterocolitica* (2012); total effects, linear and quadratic terms modelled

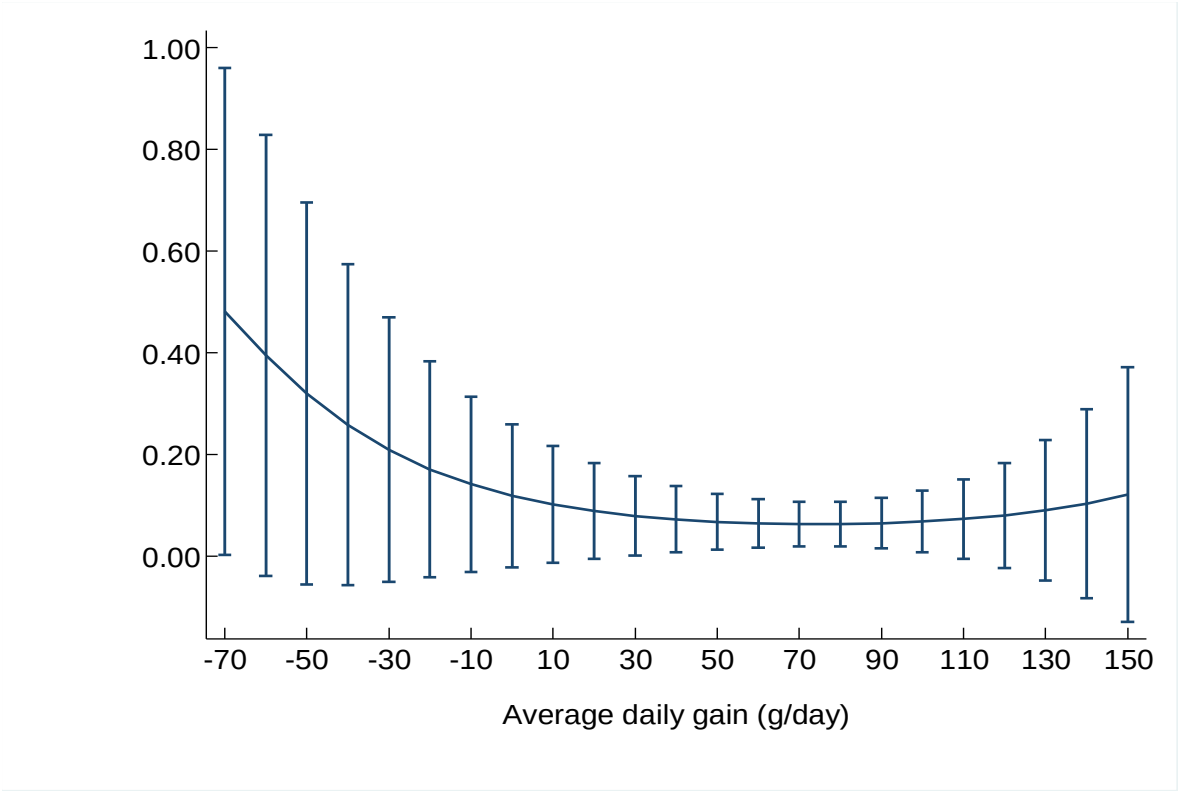


Fig. 6. Faecal shedding of *Yersinia enterocolitica* (2013); direct effects, linear and quadratic terms modelled

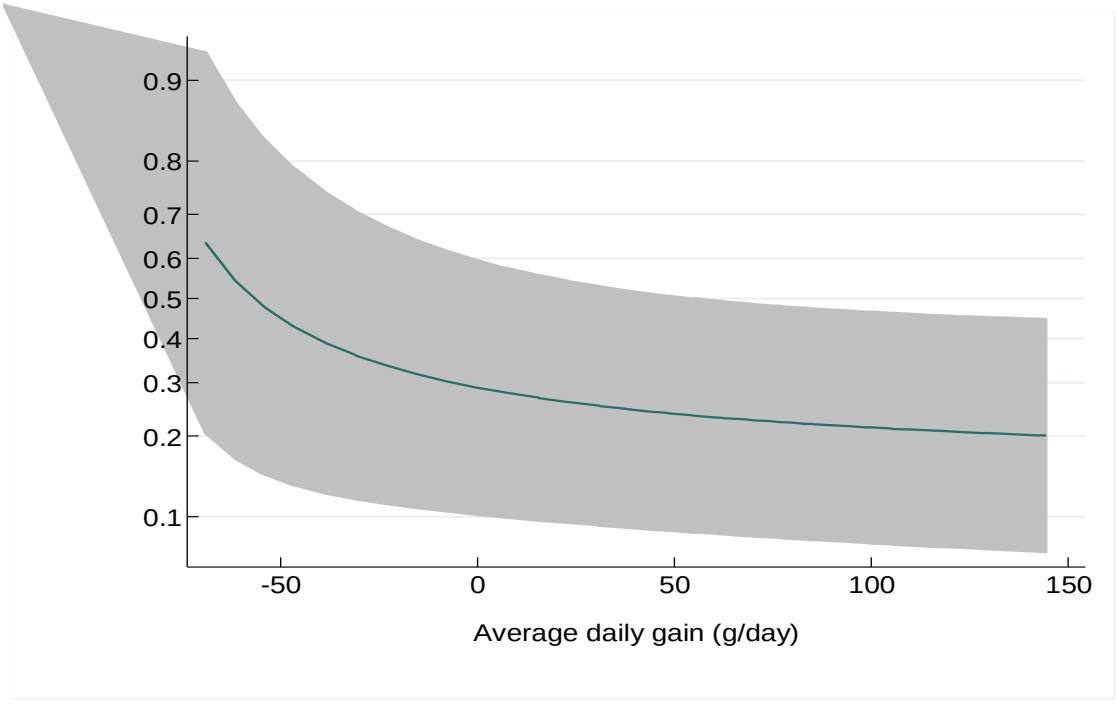


Fig.7. Faecal shedding of *Yersinia enterocolitica* (2012); total effects, fractional polynomial model

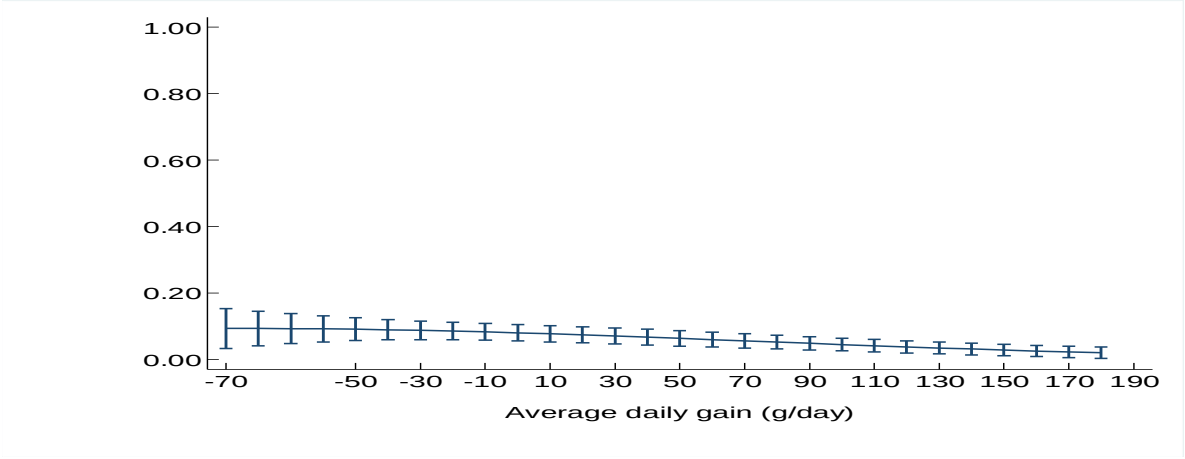


Fig. 8. Faecal shedding of *Yersinia enterocolitica* (2013); total effects, linear and quadratic terms modelled

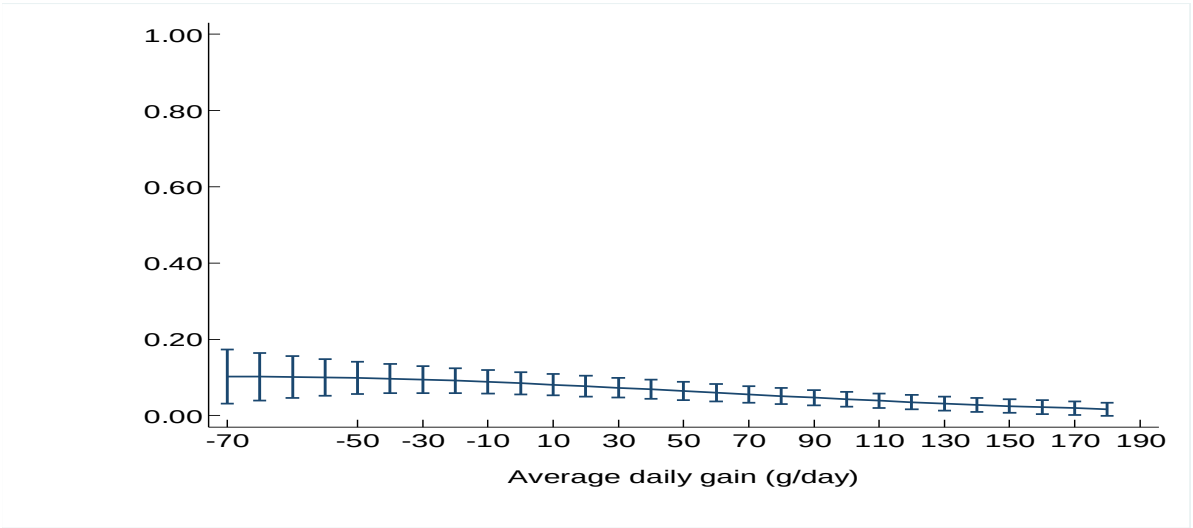


Fig. 9. Faecal shedding of *Yersinia enterocolitica* (2013); direct effects, linear and quadratic terms modelled

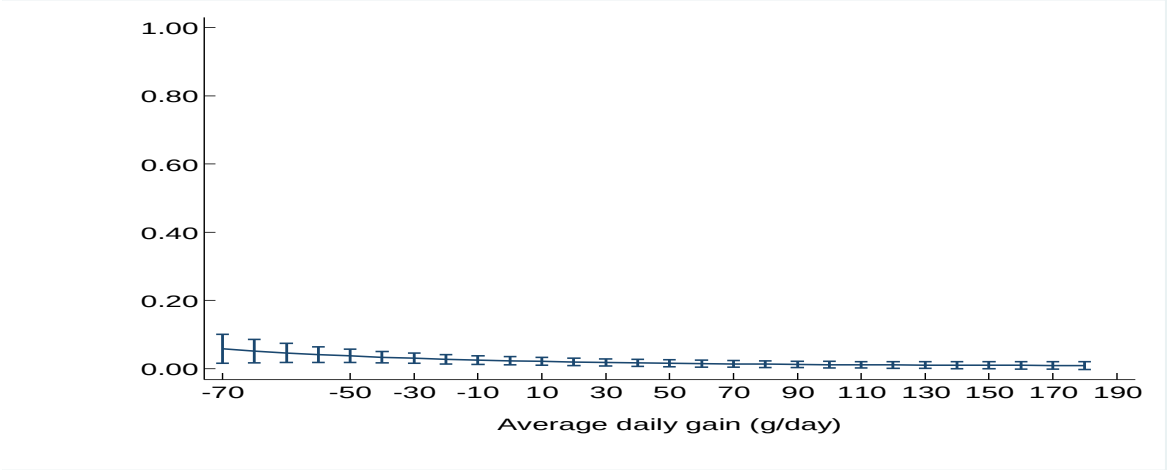


Fig. 10. Heavy faecal shedding of *Yersinia enterocolitica* (2013); total effects, linear and quadratic terms modelled

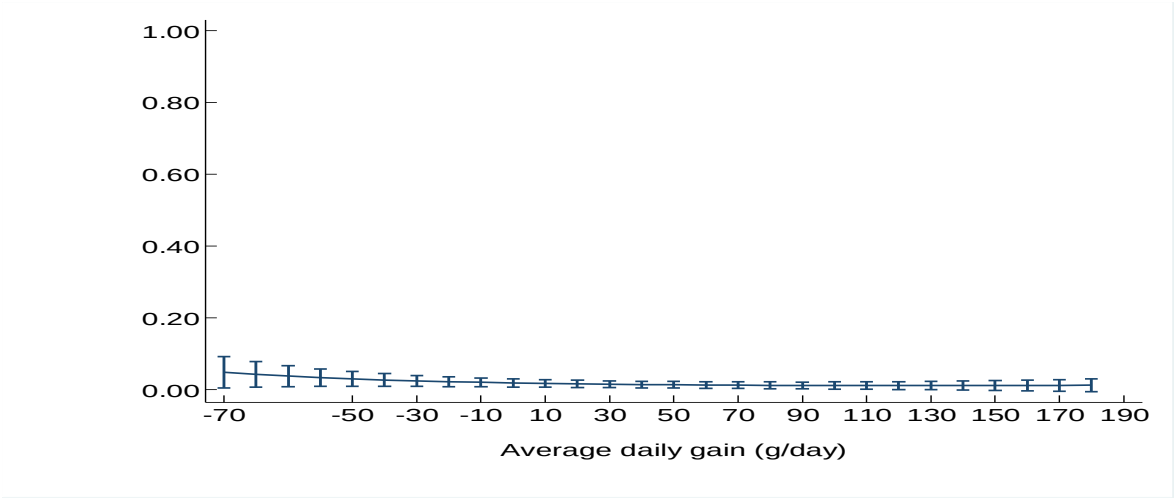


Fig. 11. Heavy faecal shedding of *Yersinia enterocolitica* (2013); direct effects, linear and quadratic terms modelled

4.2.3.2.2 *Yersinia pseudotuberculosis*

Estimates of the total effects of average daily gain on *Y. pseudotuberculosis* shedding were imprecise and inconsistent between the two study years. In 2012, the odds of shedding decreased by 60-90% if animals were gaining weight, but risk increased by up to 40% if positive gains were made in 2013. Despite a smaller sample size in 2012, there was strong evidence to support that animals gaining weight were at a markedly lower risk of shedding than those losing weight (Figure 12). A small, but opposing relationship was observed in 2013 (Figures 13 and 14).

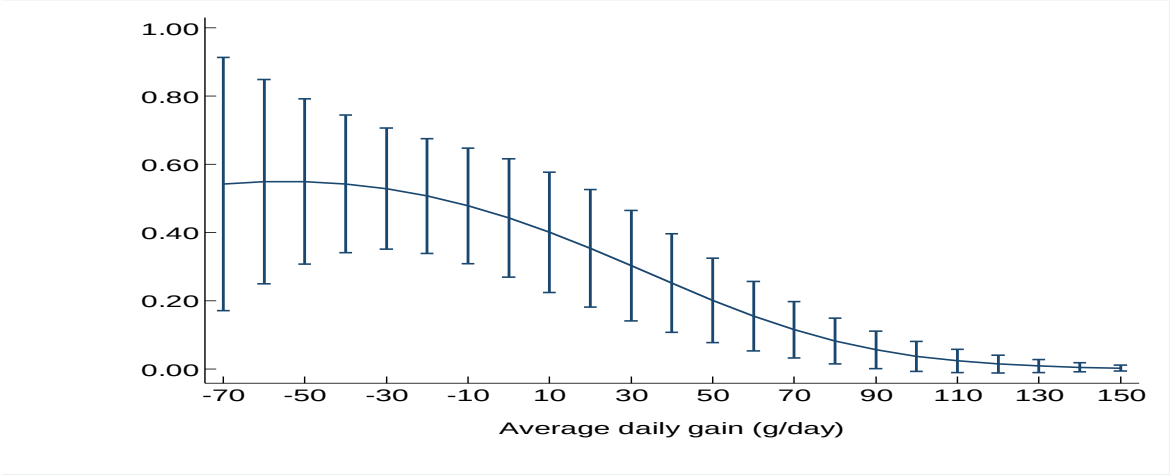


Fig. 12. Faecal shedding of *Yersinia pseudotuberculosis* (2012); total effects, linear and quadratic terms modelled

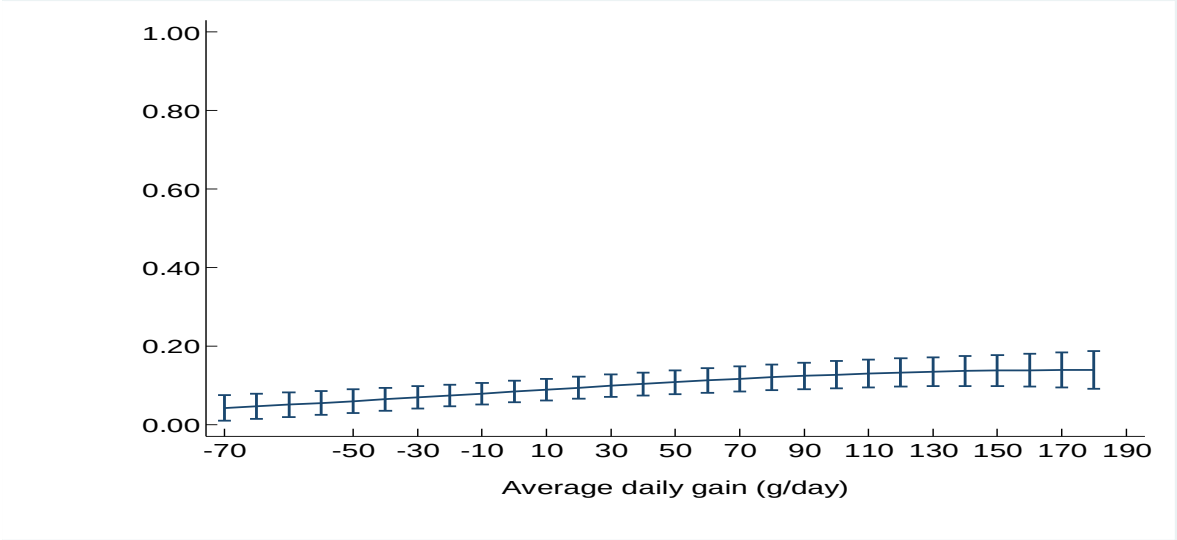


Fig. 13. Faecal shedding of *Yersinia pseudotuberculosis* (2013); total effects, linear and quadratic terms modelled

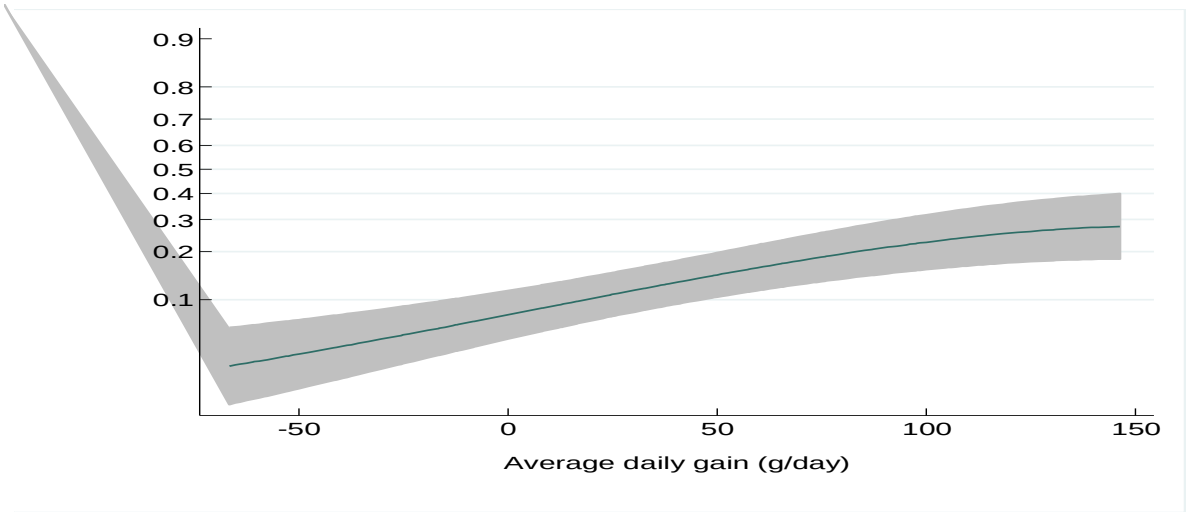


Fig. 14. Faecal shedding of *Yersinia pseudotuberculosis* (2013); total effects, fractional polynomial model

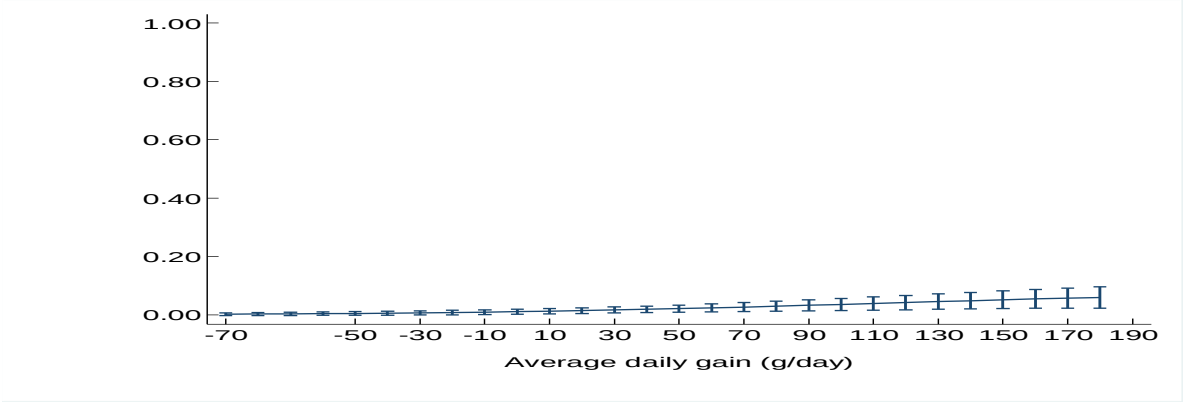


Fig. 15. Heavy shedding of *Yersinia pseudotuberculosis* (2013); total effects, linear and quadratic terms modelled

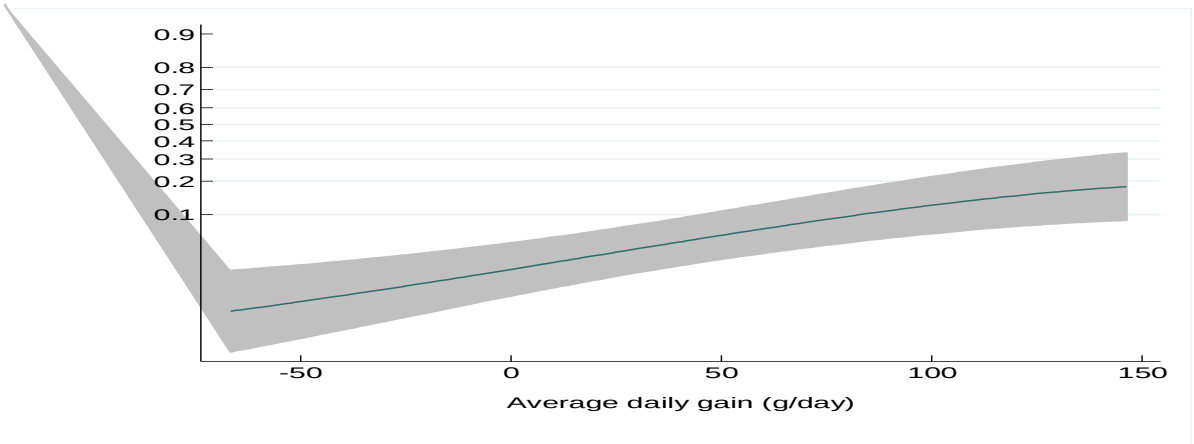


Fig. 16. 2013 – Heavy shedding of *Yersinia pseudotuberculosis* (2013); total effects, fractional polynomial model.

The total effects of average daily gain on the odds of being a heavy shedder suggested that animals gaining weight were at higher risk than animals maintaining or losing weight. Although significant, the relationship was small and considered inconsequential. Further, after adjusting for sampling day number, farm, sex, worm egg count and weight, the direct effects of average daily gain were not significant.

4.2.3.3 Worm egg counts

Worm egg counts varied considerably between farms and years, with a tendency to increase as the year progressed on all farms (Table 3). Farm B had much higher counts in 2012 than in 2013. Information about the worm egg counts of the trial mobs was provided to the managers of the farm and used to determine the timing of anthelmintic treatments.

Table 3. Mean (standard deviation, SD) worm egg counts for each farm at each sampling in Years 1 (2012) and 2 (2013).[1]

Year	Farm	Mean (SD) worm egg count (eggs per gram) at sampling number:					
		1	2	3	4	5	6
2012	B			641 (589)		879 (1012)	581 (636)
	C			3 (8)	820 (952)		98 (142)
2013	A	1 (6)	0 (0)	9 (15)	2 (22)	543 (497)	502 (396)
	B	21 (83)	9 (25)	21 (35)	181 (132)	51 (105)	400 (367)
	C	0 (0)	13 (33)	658 (522)	103 (296)	1 (8)	559 (582)
	D	16 (45)	16 (31)	.	.	263 (343)	1377 (1204)

4.2.3.3.1 *Yersinia enterocolitica*

In Year 1 (2012) there was no significant association between worm egg counts and faecal shedding of *Y. enterocolitica*, but the analysis of total effects suggested that the odds of shedding increased when animals had moderate or high worm egg counts (OR = 2.2, 95% CI 0.8-5.9; P=0.11). The direct effects of worm egg count on shedding implied that animals with moderate or high worm egg counts were less likely to shed *Y. enterocolitica* compared to animals with low counts but this was mitigated by factors in the minimal adjustment set.

The same relationship was not observed in Year 2 (2013), when animals with moderate worm egg counts had increased odds of faecal shedding compared to those with low counts (OR 1.3, 95% CI:0.8-2.1). Further, animals with high worm egg counts had reduced odds of faecal shedding compared to animals with low worm egg counts (or 0.7, 95% CI: 0.5-1.0). The estimate for the effect of worm egg count on the risk of shedding *Y. enterocolitica* was imprecise and so no firm conclusions were possible. This may have been due to the strongly negatively skewed worm egg counts, with a large number of low and zero counts.

There was little evidence of a relationship between the odds of being a heavy shedder of *Y. enterocolitica* and worm egg count. These odds appeared to be highest with moderate worm egg counts, but this estimate was imprecise. Similar to total *Y. enterocolitica* shedding, a high worm egg count seemingly decreased the odds of being a heavy shedder. Only a relatively small proportion (33%) of animals in this cohort had a worm egg count greater than 100 eggs per gram, and so this may have contributed to this unexpected result.

4.2.3.3.2 *Yersinia pseudotuberculosis*

There was no significant difference between the odds of shedding *Y. pseudotuberculosis* amongst sheep with low or moderate worm egg counts in either year (both P>0.05). In 2012 the confidence intervals around this estimate were large, probably due to the relatively small sample size. In both years animals with high worm egg counts were at significantly higher risk of shedding than those with low counts, with this relationship stronger in 2012 than in 2013.

Animals with moderate or high worm egg counts had a significantly higher risk of being a heavy shedder of *Y. pseudotuberculosis* compared to animals with low counts (moderate WEC OR = 2.9, 95% CI 1.3-6.7; high WEC OR 2.9, 95% CI 1.2-7.1). Although there was some variation in the confidence around the odds ratio, there was strong evidence to indicate that as worm egg count exceeded 100 epg, the odds of heavy shedding also increased by more than 20% (CI: 1.2-7.1).

For all models, the direct and indirect effects of worm egg count were comparable, indicating that worm egg count was responsible for the relationships observed.

4.2.3.4 Sex

There were approximately equal numbers of male and female sheep in all cohorts on all farms in both years.

4.2.3.4.1 *Yersinia enterocolitica*

In 2012, males had higher odds of shedding *Yersinia enterocolitica* than females. However, after adjusting for the indirect effects of average daily gain, day of year, farm and live weight, there was no evidence of a sex effect. There were no significant effects of sex on the odds of shedding *Y. enterocolitica* in 2013, and no significant or biologically important relationship between sex and the risk of being a heavy shedder was evident after accounting for variables within the minimal adjustment set.

4.2.3.4.2 *Yersinia pseudotuberculosis*

Sex did not affect the odds of shedding *Y. pseudotuberculosis* nor the odds of being a heavy shedder. There was some evidence to suggest that males were at increased risk of shedding compared with females, but this was offset by differences in average daily gain, day of year, farm, live weight and worm egg count.

4.2.3.5 Faecal consistency

Faecal consistency was scored on a scale of 1 to 5 to determine whether it may be used, in conjunction with other indicators of management, as a tool to predict heavy shedding of *Yersinia*. Worm egg count was identified as a potential confounding factor and so two models were built to determine this relationship – one including worm egg count and one excluding this variable. No confounding relationship was detected, and so worm egg count was included in the minimal adjustment set.

Table 4: Mean (standard deviation, SD) faecal consistency scores at each visit in Years 1 (2012) and 2 (2013).[A]

Year	Farm	Mean (SD) faecal consistency scores at sampling visit:					
		1	2	3	4	5	6
2012	B	-	-	4.1 (1.3)	-	2 (1.2)	4.4 (1)
	C	-	-	1.2 (0.6)	3.6 (1.8)	-	2.3 (1.3)
2013	A	1.8 (1.2)	2.1 (1.3)	2 (1.3)	1.8 (1.1)	2.7 (1.6)	2.8 (1.3)
	B	1.6 (0.9)	2.3 (1.1)	1.6 (1)	3.5 (1.4)	4.5 (1)	3.7 (1.5)
	C	1.2 (0.7)	3.9 (1.5)	1.7 (1.1)	3.8 (1.2)	2.9 (1.5)	2.9 (1.2)
	D	2.9 (1.6)	2.9 (1.6)	-	-	4 (1.3)	4.9 (0.7)

[A] Faecal consistency scored on a scale of 1 (hard pellets) to 5 (watery diarrhoea)

Examining the raw data indicated that average faecal consistency scores varied markedly between sampling time, farm and year. Additionally, the high variation (SD) indicates that there is considerable variation between animals at each sampling.

Table 5: Number and percentage of animals shedding each *Yersinia* sp. at each faecal consistency score, and estimated total effects (adjusted odds ratios) of the relationship between faecal consistency score and heavy faecal shedding of *Y. enterocolitica* and *Y. pseudotuberculosis* in year 2 (2013).

Measure	No. shedding/ total samples (crude %)	Adjusted odds ratio* (95% CI)	P value
***Y. enterocolitica* – heavy faecal shedding compared to not heavy**			
Effect of year			*<0.001*
Faecal consistency – score 1	18/ 699 (2.6%)	Reference group	
Faecal consistency –score 3	32/ 631 (5.1%)	2.1 (1.2,3.8)	0.009
Faecal consistency –score 5	25/ 495 (5.1%)	3.0 (1.7,5.4)	<0.001
***Y. pseudotuberculosis* – heavy faecal shedding compared to not heavy**			
Effect of year			*0.17*
Faecal consistency – score 1	30/ 699 (4.3%)	Reference group	
Faecal consistency – score 3	28/ 631 (4.4%)	0.8 (0.4,1.4)	0.430
Faecal consistency – score 5	30/ 496 (6.0%)	1.4 (0.8,2.7)	0.244

*Adjusted for sampling day number (linear and squared terms), farm, worm egg count and sheep (panel variable)

4.2.3.5.1 *Yersinia enterocolitica*

There was evidence of a strong association between faecal consistency score and heavy shedding of *Y. enterocolitica.* The adjusted odds ratios (Table 5) suggest that animals with a faecal consistency score greater than 3 have significantly higher odds of being a heavy shedder than animals with a score 1. There was no significant difference between the odds ratio of heavy faecal shedding for animals with a faecal consistency score of 3 and 5. This finding suggests that assessing faecal consistency score could be a useful component of a predictive model for rate of shedding of *Y. enterocolitica*.

4.2.3.5.2 *Yersinia pseudotuberculosis*

There was no apparent relationship between faecal consistency score and heavy *Y. pseudotuberculosis* shedding (Table 5). The estimations of the relationship were centred around unity and so no firm conclusions were possible. There was some evidence that animals with a faecal consistency score of 5 (ie. diarrhoea) were at increased odds of heavy shedding, but this relationship was not significant. It is concluded that faecal consistency score is unlikely to be a useful component of a model to predict heavy faecal shedding of *Y. pseudotuberculosis*.

4.2.4 Climatic data

Weather data were collected for all farms both from outbreak submissions and those in the longitudinal study. Factors recorded included rainfall, minimum and maximum temperature, and wind speed and direction.

The strong seasonal occurrence of this disease, specifically the sudden onset of cold, wet weather, was confirmed during this study. However, the data collected suggest a much more complex interaction between the host, environment and organism than simply chilling or exposure to adverse weather.

Consequently, an index combining the effects of wind, rainfall and temperature will be calculated for all observation points from this raw data and included in a model to determine the strength and significance of the association between disease outbreaks, occurence and prevalence of faecal shedding of each Yersinia species and a measure of 'weather stress' or 'chilling'. The model, which is still under development, will facilitate a more complex analysis to investigate the strength of association between weather over the high risk period for the weaner scours syndrome (June-September), and the incidence and severity of disease.

4.2.5 Serotyping and biotyping *Yersinia* isolates

Serotyping has been completed for 301 field isolates of *Y. pseudotuberculosis* (56%) with serotype O:3 identified in 97% (292) of those tested. Biotyping *of Y. enterocolitica* is more complex, as it was determined that the accuracy and consistency of results obtained in traditional bench-top serotyping were unsatisfactory. Initial testing using pulse field gel electrophoresis (PFGE) has been superceded by pulse field and DNA finger printing methods. This testing is ongoing at the time of preparing this final report. This information will be finalised as part of the ongoing work for the PhD thesis, and subsequently published in a peer reviewed journal article.

4.2.6 Virulence testing

To date, 64% (283) of the field isolates of *Y. enterocolitica* have been assessed for virulence by probing the presence of the *Yersinia* adherence protein gene (yadA). Of these, 85% (241) were assessed as virulent and 15% (42) of isolates were negative for the adherence protein gene. Isolates negative in this test will also be screened for a chromosomal virulence gene. Testing of the remaining isolates is continuing at the time of preparing this report. A

comparison of *Y. enterocolitica* isolates associated with severe disease (scouring and abortion), and those recovered incidentally from asymptomatic animals, has to date shown no association with detection of the adherence protein gene and the severity of disease.

4.2.7 Antimicrobial sensitivity testing

To date 282 field isolates, 145 *Y. enterocolitica* and 137 *Y. pseudotuberculosis*, from both outbreak and sentinel properties, have been tested to determine the rate of antibiotic resistance. The results so far have identified emerging resistance to the antimicrobials commonly used for treatment of this syndrome, in particular a commonly used oral drench (sulphafurazole). These chemicals are also used more broadly in livestock production (Table 6). Mean inhibitory concentrations will also be deterimed for the isolates tested. This further work is ongoing at the time of writing of this report and will form part of a short paper to be submitted for publication towards the end of 2015. These preliminary results will also inform the potential for future work, including further utilising this catalogue of isolates.

Table 6. Number and proportion of Yersinia isolates showing resistance to 3 antimicrobials commonly used to treat weaner scours

Flock	*Y. enterocolitica*				*Y. pseudotuberculosis*			
	No. isolates	Tetracycline no (%)	Trimethoprim no. (%)	Sulphafurazole no.(%)	No. isolates	Tetracycline no. (%)	Trimethoprim no.(%)	Sulphafurazole no. (%)
A	20	0	0	2 (10.0)	23	0	0	8(34.8)
B	20	1 (5.0)	0	1 (5.0)	19	1 (5.3)	0	3(15.7)
C	20	0	0	0	19	0	2(10.5)	0
D	20	0	0	2 (10.0)	21	0	0	8(38.1)
Outbreaks	65	0	0	5 (7.7)	55	1(1.8)	3(5.4)	23(41.8)
Total	145	1 (0.7)	0	10 (6.9)	137	2 (1.5)	5 (3.6)	42(30.7)

To date, flock A has demonstrated the highest occurrence of antibiotic resistance with 10.0% (2/20) and 34.8% (8/23) resistance to sulphafurazole for *Y. enterocolitica* and *Y. pseudotuberculosis* isolates, respectively. Sentinel flock B, found to have relatively high shedding rates of *Yersinia* (30%) and an outbreak of weaner scours in year 2, also has a high prevalence of resistance in *Y. pseudotuberculosis*, with 15.7% of isolates resistant to sulphafurazole and 5.3% resistant to tetracyclines. Interestingly, flock C, which has also experienced annual outbreaks and a high incidence of disease associated with *Yersinia* sp., had a lower proportion of resistant isolates detected. For all isolates from all flocks (both sentinel and outbreak), the highest prevalence of resistance was to the sulphafurazole class of antibiotics, with 30.7% and 6.9% showing resistance amongst *Y. pseudotuberculosis* and *Y. enterocolitica* isolates, respectively.

Oxytetracycline is now used in many flocks in which resistance to sulphafurazole and related antimicrobials is suspected, or response to sulphafurazole treatment was poor. For all flocks, 1.5% of *Y. pseudotuberculosis* isolates were found to be resistant to oxytetracycline. This is of concern and may signify an emerging resistance to this antimicrobial class, particularly where a poor response to treatment with sulphadimidine has been previously observed.

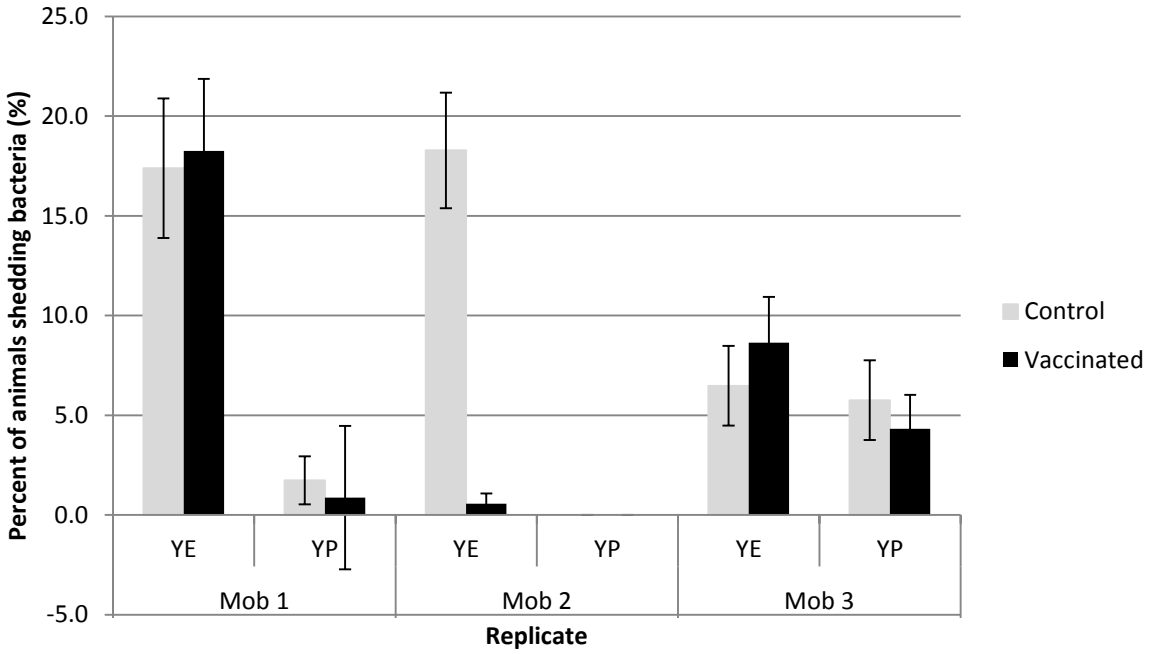
4.3 Autogenous vaccine trial; preliminary results

In July 2013 (year 2), a significant peak in the proportion of weaners shedding *Y. pseudotuberculosis* occurred in flock A (Figure 1A, 86%) and was associated with a mortality rate of 8%. This flock was selected for inclusion in the vaccine trial in 2014 based on high rates of faecal shedding of *Y. pseudotuberculosis* and outbreaks of weaner scours in years 1 and 2. Unfortunately, in year 3 the proportion of weaners shedding *Y. pseudotuberculosis* peaked at only 5.8% in September, and no outbreak of bacterial enteritis due to yersiniosis occurred.

In flock A, at the final sample collection in September, more control animals were shedding *Y. enterocolitica* than vaccinated animals in mob 2, but this was not observed in the other mobs.It was not clear why this occurred, but factors associated with the paddock grazed by the mob 1 may have contributed to this difference (Figure 17).

In mobs 1 and 3, more control animals were shedding *Y. pseudotuberculosis* when compared to the rate of shedding in vaccinated animals. However, these differences were not statistically significant and within the context of the other observations in this trial are unlikely to be of any biological significance.

Fig. 17. The percentage of control and vaccinated weaner merinos shedding *Yersinia enterocolitica* or *Y. pseudotuberculosis* in September (Flock A)



Flock C was also selected for inclusion in the vaccine trial based upon a peak prevalence of faecal shedding of *Y. pseudotuberculosis* in August of year 2 of 49%. This was associated with a typical outbreak of bacterial enteritis with a 9% mortality incidence (Figure 3). During the vaccine trial in year 3 (2014), the prevalence of faecal shedding of *Y. pseudotuberculosis* peaked at over 60%, but no outbreak of weaner scours occurred.

Similar to flock A, no clear association between bacterial shedding and vaccination was observed in flock C. For mobs 1 and 2, there was little difference in the percentage of vaccinated and control animals shedding *Y. pseudotuberculosis* and, for mob 3, more

vaccinated animals were shedding this organism compared to the controls. No clear pattern for the faecal shedding of *Y. enterocolitica* was apparent between the treatment groups.

As part of the PhD program, co-supervised by the postdoctoral position, an indirect antibody ELISA for *Y. pseudotuberculosis* was developed. The testing and analysis of these results has not been completed at the time of preparing this report, but will be included in the PhD thesis and submitted to a peer reviewed journal when finalised. These results will provide additional information on the development and longevity of immunity, from both vaccination and natural exposure to Yersiniae, and the potential of a vaccine to control the weaner scours syndrome.

4.4 Environmental survival of *Yersinia* species

At day 0, all CIN plates for both fixed and fluctuating temperature treatment groups had high growth scores (3 or 4). Over the course of the trial, higher scores were observed in all treatments for *Y. pseudotuberculosis*, compared to *Y. enterocolitica*, after adjusting for day, water, temperature and repeated measurements ($P<0.001$).

Over all treatments, the effect of water on the survival of either species of *Yersinia* was not statistically significant. The relative effect of water on survival for *Y. pseudotuberculosis* and *Y. enterocolitica* was 58% ($P=0.54$) and 20% ($P=0.29$), respectively. However, at 20°C the survival of *Y. pseudotuberculosis* was strongly associated with water treatment ($P<0.001$), whereas for *Y. enterocolitica* it was not.

The most pronounced difference between the species occurred when the organisms were exposed to temperatures fluctuating between -20°C and 4°C. Under these conditions, survival of *Y. pseudotuberculosis* was significantly greater than for *Y. enterocolitica* over all water treatments, when adjusted for day and repeated measurements ($P<0.001$).

Compared to all other treatments, the fixed 40°C and fluctuating 20°C to 40°C temperature treatments had the greatest rate of decline in survival of organisms, with the addition of water having no effect on the survival of either species. *Y. pseudotuberculosis* was recovered for 5 more days when fluctuating to 40°C compared to the fixed treatment at 40°C (Figure 18).

Fixed temperatures of 4°C and -20°C, and temperature fluctuations from 4°C to 20°C, both with and without water, supported the greatest survival of both *Yersinia* species. *Y. pseudotuberculosis* survived until the end of the study (day 40) when fluctuated between -20°C and 4°C and when watered at 20°C.

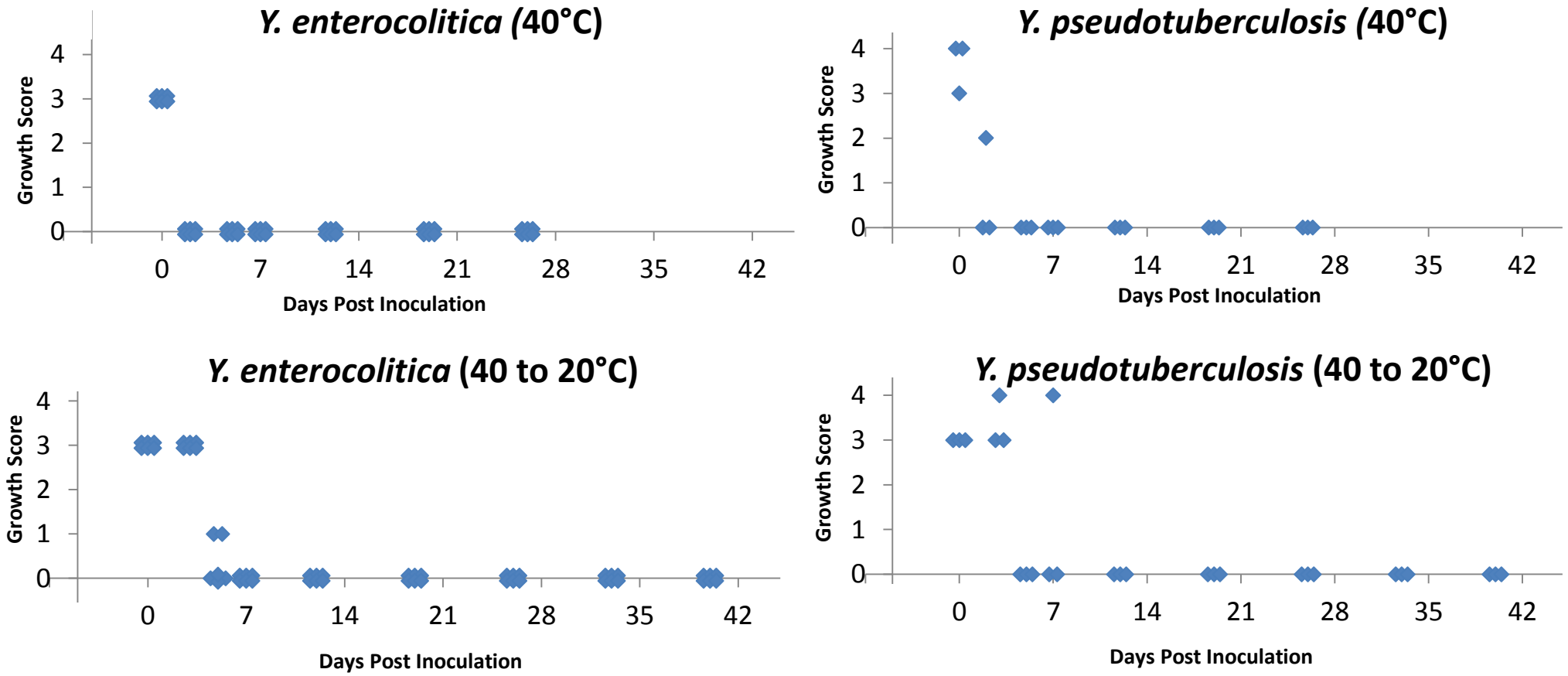


Fig 18. Growth scores for watered treatments of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* incubated at fixed temperature of 40°C and fluctuating temperatures of 20°C to 40°C. Plots jittered on x and y axis.

4.4.1 Detection of *Yersinia* sp. and the virulence plasmid (yadA gene)

DNA collected from *Yersinia* sp. at the last sample point of the study confirmed that the recovered *Yersinia* isolates did not lose the virulence plasmid gene (yadA).

5 Discussion

5.1 Summary of project objectives and outcomes from postdoctorate program to date.

1. Ensure that the postdoctoral fellow has opportunity for development of leadership skills by acting as a mentor and by enabling the postdoctoral fellow to receive formal leadership training on an annual basis.

During the postdoctoral appointment, from 2012-2015, Dr McGregor demonstrated considerable independence, developing and consolidating her leadership skills by helping train and mentor junior staff within the Mackinnon Project. For example, she assisted a junior staff member plan a research project on atypical balanitis of beef bulls, which was a successful grant application to MLA in 2013 (MLA B.AHE.0227). She also facilitated the development of whole-farm consultancy skills by accompanying junior staff on visits to client's farms, overseeing the delivery of services to those clients and allowing the junior consultant to develop increasing independence, confidence and capacity. This responsibility continues as she is now employed by the Mackinnon group within the Faculty of Veterinary and Agricultural Sciences at the University on Melbourne. She contributed to the development of graduate scientists by teaching agriculture and veterinary students enrolled in the Bachelor of Agricultural Science (BAgSc) and Doctor of Veterinary Medicine (DVM) degrees, and developed the curriculum and content for a production animal major in the 2nd and 3rd years of the BAgSci degree. During the postdoctoral appointment she supervised one Bioscience Honours student to completion in 2014 and is co-supervisor of a PhD student, recruited to work on elements of the weaner scours syndrome, expected to complete in 2016 (Dr Kelly Stanger). As part of these responsiblities she was able to attend several teaching and supervisory workshops and seminars offered by the University of Melbourne. Since completing the postdoctoral position she is also involved in mentoring and supervising two new junior residents, appointed as part of an MLA donor company residency training project lead by the postdoctoral mentor, A/Prof John Larsen (MLA P.PSH.0719). The trainee consultants, employed in April 2015, are also enrolled in Masters research degrees and will be co-supervised by Dr McGregor.

2. Develop the research capability of the postdoctoral fellow to a point where he/she will be successful in competitive grant applications.

The data and library of samples generated throughout this project will facilitate continuing work in this area, both for those already involved and for future postgraduate students. Project proposals and grant submissions are currently being drafted for further work in a number of areas, including: a) studying the development of antibiotic resistance in *Yersinia* and *Campylobacter* species, in both the sheep and dairy industries; b) investigating the potential for vaccines to reduce shedding of *Campylobacter* species from grazing livestock, hence reduce the zoonotic risk and improve food safety and human health, and c) investigating the significance of Yersiniosis as a cause of mortality and productivity loss in dairy calves, which can cause significant financial losses in the dairy industry by increasing the cost of rearing dairy heifers. Work is also continuing to complete the PhD program supervised through this postdoctoral position.

3. The postdoctoral fellow will develop a program that reduces scouring and the accumulation of dag.

The research summarised in this report has identified that both *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are major causes of the 'weaner scours' syndrome of weaned Merino sheep. There is strong evidence that acute disease and high mortalities are associated with *Y. pseudotuberculosis* infections, whereas *Y. enterocolitica* is associated with chronic infections and persistent, less severe diarrhoea that can cause breech soiling ('dag') and an increased risk of breech strike.

Two other important findings were:

a. *Y. enterocolitica* is shed in sheep faeces for much of the year, and so weaners may scour and accumulate dag at high risk times for fly strike (from October through to March). This risk is emphasised by the correlation between faecal consistency and both the prevalence and the severity of faecal shedding of *Y. enterocolitica*.
b. The finding that there is a strong association between worm egg count and *Y. pseudotuberculosis* faecal shedding and infection also highlights the importance of regular monitoring of worm burdens. This integrated approach to management of this syndrome and endoparasites in weaner mobs will reduce the risk of chronic scouring, accumulation of dag accumulation and increased risk of flystrike.

These key findings will be communicated to producers within the context of a grazing management strategy, similar to that proven to prepare pastures of reduced infectivity for internal parasites for weaned sheep. Known as the 'Smart Grazing' strategy (Niven et al. 2002a, 2002b), this approach integrates strategic anthelmintic treatment with modified grazing to combat the effects and reduce the impact of both worms and bacterial enteritis in mobs of Merino weaners.

4. Defining the epidemiology of bacterial causes of scouring in young sheep, mainly Campylobacter and/ or Yersinia sp.

Preliminary results and analyses outlining aspects of the epidemiology of Yersiniosis, including farm, organism and animal factors, are presented in this report. This work is continuing, with the development of a complex model to assess the association between the occurrence and severity of this syndrome and weather data across a number of regions in SE Australia where the weaner scouring syndrome commonly occurs. The work undertaken as part of the Honours project, to investigate the environmental survival of the two main *Yersinia* spp., will be important in defining risk periods for the transmission of these organisms in grazing systems. It is hoped that this analysis will facilitate the development of a predictive tool for producers, allowing them to assess the risk and severity of an outbreak of weaner scours within their farm, perhaps even down to a mob basis. This would allow the management recommendations, developed as part of this postdoctoral project, to be more proactively adopted and help prevent severe disease in susceptible mobs on high risk farms.

4.1. Isolation and characterisation of the range of organisms involved

A traditional microbiological approach was taken to the isolation and identification of *Yersinia* sp. in the first year of the study. Due to the enormous workload, this was refined and eventually replaced by molecular techniques in years 2 and 3. The subtyping of the stored *Yersinia* isolates is continuing. Completion of this will provide additional information about the molecular epidemiology of this syndrome and the importance of each organism in livestock health, their potential zoonotic potential and food safety.

5. The post doctoral fellow will also Investigate and define differences in scouring between regions and different aged sheep and publish 3 scientific publications in high impact journals with the post doctoral fellow as leading author.

At least 3 publications are in preparation and will be submitted to appropriate journals in 2015-2016. Samples were collected from sentinel and outbreak flocks across Victoria and southern NSW. There is a strong association between the winter scouring syndrome and *Yersinia pseudotuberculosis* infections. *Y. enterocolitica* has also been implicated in a more chronic scouring syndrome, in both weaners and young adult sheep, across these regions. Other infectious causes of acute scouring (*Campylobacter*, *Salmonella*) were also investigated in the submissions for disease outbreaks. From this data it appears that there are distinct differences in the presentation of yersiniosis and other possible causes of scouring, and owners or managers of flocks that are affected by weaner scouring syndrome are aware of these differences. The role of gastrointestinal roundworms in scouring in all age classes is well established. Preliminary analysis of data from the current study also suggests a strong association between elevated worm egg counts and infection with *Y. pseudotuberculosis* in weaners. A more definitive analysis will be possible once subtyping of all isolates is completed. However, the analysis so far suggests no correlation between regions, organisms or subtype.

5.2 Longitudinal study

Data collected from the sentinel flocks and outbreaks of scouring demonstrated a clear association between the weaner scouring syndrome and faecal shedding of *Y. pseudotuberculosis* (both prevalence and severity). Two sentinel flocks experienced an outbreak in year 2 (2013). Both had a marked increase in the proportion of sheep excreting *Y. pseudotuberculosis* at this time and no other cause of scouring was identified. This was also the case for outbreaks investigated in other flocks during years 1 and 2. Thus, there is a clear association between amount of faecal shedding of this organism and the occurrence and severity of disease in a proportion of the population. However, it is not yet clear what role the level of challenge has in precipitating an outbreak.

Some insights about this were gained in year 2 of the longitudinal study, and used in the design of the vaccine trial in year 3 (2014). It appeared that a threshold of 5% of animals shedding high numbers of organisms (a growth score 3 or 4 in microbiology) was required to precipitate an outbreak. However, during the vaccine trial this threshold was exceeded on flock C but no outbreak ocurred. In this case the numbers of animals shedding *Y. pseudotuberculosis* reached 61%, but no animals presented with signs of Yersiniosis. This was unexpected given observations at the same time of year in year 2, when a shedding rate of 54% of sheep precipitated an outbreak with 9% mortalities. The amount of shedding detected in sheep sampled in year 3 of the project was high, but no signs of Yersiniosis were detected.

There is also a strong association between an increased prevalence of faecal shedding of *Y. pseudotuberculosis* and time of year, with all flocks in year 2 having a peak in July/August. Analysis of the 4 sentinel flocks determined that there was a significant association between day of sampling, risk of shedding and risk of heavy shedding of *Y. pseudotuberculosis*. The most shedding occurred after day 200 of the trial, coinciding with mid-July. This supports, but does not explain, previous evidence of the highly seasonal nature of outbreaks of the scouring caused by this organism (Slee, Slee & Button 1990). The strong association between farm, and both the rate and degree of shedding, suggests that there may be farm- or region-specific factors that contribute to the occurrence and severity of disease. The fact that day of the year had such a strong association with the occurence and degree of faecal shedding suggests that level of challenge, a carrier status or the cumulative effect of weather and management factors may be important in precipitating an outbreak.

From the climate data there do appear to be subtle differences between years in the weather patterns at this time, even though the range of temperatures and total rainfall appear similar. For example, in year 2 there was a pattern of increasing rainfall during the high risk period, whereas in year 3 there was a pattern of decreasing rainfall during the vaccine trial. Consequently, a more detailed analysis is needed to assess the relative importance of weather and its interactions with the host animal, the organism, environment and management factors. A model is being developed to analyse these relationships. When completed, the outcomes from this analysis will be submitted to a peer reviewed journal. The results will also indicate the viability of a predictive tool for producers in regions where this syndrome causes significant losses on an annual basis. The implications for producers may be contrary to what has been assumed for this syndrome, with weather previously considered the most important factor in determining the timing of outbreaks. This should lead to a more integrated approach to assessing risk and enable a more proactive approach to managing this syndrome, especially on farms where outbreaks are common. An additional benefit would be to reduce the the indiscriminate use of antibiotic treatments, hence decrease selection for antimicrobial resistance.

On farms where annual outbreaks of Yersiniosis occur, producers have the view that light weaners are more susceptible. Contrary to this anecdotal evidence, no association was observed between liveweight and the prevalence of faecal shedding and occurrence of disease. However, there was an association between heavy shedding and a negative daily weight gain. This means that weaners which were either maintaining or gaining weight during the high risk period for this disease were at lower risk of becoming heavy shedders.

There was also an association between the risk of shedding *Y. pseudotuberculosis* and worm egg count (WEC). Weaners with either a high or moderate WEC had a significantly increased risk, of both faecal shedding and heavy shedding of this organism, compared to those with low counts. More specifically, if weaners had a WEC ≥ 100 eggs per gram, their odds of being a heavy shedder increased by more than 20%. The management implications for this are clear, as the high risk period for Yersiniosis coincides with a high risk for and regular monitoring of nematode infecions. Consequently, if producers monitor WECs and maintain worm burdens at an acceptable level, this should decrease the prevalence of heavy shedders and contamination of pasture with Yersinia. The physiological basis of this association is not clear from this work. However, it is possible that changed appetite may lead to a change in conditions in the intestine such that this facilitates the establishment of infection with *Y. pseudotuberculosis.* These effects and relationships require further investigation.

Y. enterocolitica was detected at 30-100% of the collection periods in year 2. This, combined with the fact that 85% of these isolates were assessed as potentially virulent, suggests that this organism may play a more important role in scouring weaners than previously thought. However, the analysis found no strong association between farm factors, reduced live weight and faecal shedding of *Y. enterocolitica*. There was some indication that weaners losing weight may be at greater risk of shedding *Y. enterocolitica*. However, this was not strong, nor present in all years or all flocks, and so it is likely that the productivity penalties from infection with this organism are small and quite sporadic. Nevertheless, the information collected from a disease outbreak in year 2 did identify that *Y. enterocolitica* was associated with severe disease in 16-month-old hogget sheep, causing both abortions and scouring. However, these were isolated and infrequent cases compared to outbreaks caused by *Y. pseudotuberculosis* that consistently had a high morbidity and high mortalities.

The fact that such a large proportion of *Y. enterocolitica* isolates were not associated with disease suggests that the triggers for precipitation of severe disease associated with this organism may be even more complex than for *Y. pseudotuberculosis.* Thus, further work is required to define the role and understand the epidemiology of *Y. enterocolitica* infection, in particular to identify if subclinical production loss occurs in some populations or at certain

times. A more detailed investigation of risk factors and the host-organism interactions that determine when these bacteria cause disease is also needed. At this stage the major significance of this organism is its' association with a low grade scouring syndrome. *Y. enterocolitica* was detected throughout the year, and there was no consistent pattern of shedding of this organism between flocks or years. The most signiticant impact of this organism is likely to be its contribution to an increased prevalence of breech soiling ('dag') due to chronic, low grade scouring, hence increasing the risk of breech strike, in both spring/ early summer and the autumn risk period. The strong association between less formed faeces and rate of faecal shedding (the isolation of *Y. enterocolitica* organisms) strongly supports the association with low grade scouring at various times of the year. This was particularly evident for sheep with a faecal consistency score ≥ 3 (on a scale of 1-5), where animals had a significantly higher odds (OR 2.1, 95% CI 1.2-3.8) of being classified as a heavy shedder (growth score 3 or greater (scale 0-4)) compared to animals with a faecal consistency score of 1 (OR 3.0, 95% CI 1.7-5.4). This relationship was not found with *Y. pseudotuberculosis*, and so using faecal consistency as a predictive tool for inceased risk of infection will not be of possible with his organism.

The zoonotic importance, of both *Y. pseudotuberculosis* and *Y. enterocolitica*, will also be more clearly defined as this work continues past the postdoctoral appointment. This will explore the well documented correlation between certain biotypes or serotypes and the potential to cause human disease (Soderqvist et al 2012, Hodges et al 1984, Logue et al 1996). Investigating determinants of virulence for each of the species and subspecies of Yersinia isolated during this project will also provide further insights into the relative importance of these pathogens for the weaner scours syndrome, food safety and human disease. This would inform decisions regarding likely benefits from univalent or multivalent vaccines developed for the control or prevention of Yersiniosis. In addition, this work could have important implications for control of the disease in other livestock species, such as dairy calves.

A major aim of this work was to develop management strategies for flocks in which outbreaks of the weaner scours syndrome occur on an annual basis. To this end, we will investigate if a predictive modelling tool could be developed from the epidemiological data. In addition, the fact that 97% of *Y. pseudotuberculosis* isolates collected during this study were serotype O:3 supports the notion of developing and evaluating the efficacy of a vaccine. This would include investigating whether vaccination reduces the severity or prevalence of disease or faecal shedding of organisms. If vaccination was effective, this may be a strategy equally or more applicable to the dairy industry, where calf-rearing and management is more intensive, animals have higher individual value and outbreaks of disease may have a stronger association with management and nutrition.

The antibiotic sensitivity testing suggests that resistance is more of a problem for flocks in which antimicrobials are used more frequently to treat weaners affected by yersiniosis. This correlates with the management information collected regarding the approach to treatment in the face of a disease outbreak. Farms A, B and C consistently experience a high prevalence of disease due to *Y. pseudotuberculosis*. However, on farm C, a more strategic approach is taken to identify and treat only those sheep visibly affected with Yersiniosis from a 'tail' of the mob that appears unwell and is drafted off for treatment. This may reduce the selection pressure for resistance in two ways; only about 30% of the mob are treated at any one time, plus under-dosing is less likely with more attention given to correctly estimating liveweight in the fewer sheep treated. Farm A, with the higher rate of antimicrobial resistance, typically treats on multiple occasions each year, between June and September, and does not draft animals based on their clinical signs or bodyweight. This leads to a higher rate and frequency of use of antimicrobials, and the possibility that some heavier animals may not receive an adequate dose.

Testing more isolates, including the more specific minimum inhibitory concentration (MIC) test in a subgroup of resistant isolates, will increase our understanding of the importance of antimicrobial resistance and its implications for food safety and human health. This will also help develop more sustainable strategies for the use of antibiotics in the face of outbreaks of this disease. These results have important implications for other livestock species, particularly dairy calves, in which *Y. pseudotuberculosis* is thought to play an important role in a scouring syndrome of weaned heifer calves and antibiotic use is higher in these more intensive livestock systems. These preliminary results should facilitate collaborative work with other veterinary and 'One-Health' personnel, and approaches to explore collaboration with another group at Melbourne University is underway.

5.3 Environmental survival for transmission of *Yersinia sp*

Results from this study show that *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* do not survive well in faecal pellets at temperatures over 20°C. Neither species survived more than 7 days when incubated at 40°C, nor when subjected to temperature fluctuations between 40°C and 20°C, even when watered. This suggests *Yersinia* sp. are unlikely to survive for over a week in faeces on soil or bare ground if ambient temperature remains above 20°C.

These results were unexpected because it has been previously reported that *Y. pseudotuberculosis* had an optimal growth temperature of 22°C, and a maximum temperature tolerance to 33.7°C (Bhaduri & Phillips, 2011), whilst *Yersinia* species could multiply at temperatures reaching 43°C (Moxley 2012). However, the current study design inoculated organisms into faecal pellets placed onto soil, which was in contrast to many previous studies which used nutrient rich broth or media. It is possible that such nutrient rich environments may have facilitated the longer survival. Alternatively, in nutrient broths there may have been less competition for available nutrient compared to the potentially less nutrient rich environment of a faecal pellet in which many other microorganisms are present. A previous study demonstrated that survival of *Yersinia enterocolitica* in soil and water was greater at 4°C, compared to at 20°C, because other enterobacteria were unable to survive at temperatures below 4°C (Tashiro et al. 1991). Conversely, *Yersinia* sp. are poor competitors with other psychotropic bacteria at temperatures above 4°C (Stern, Pierson, & Kotula, 1980). Thus, at higher temperatures, the rapid clearance of *Y. enterocolitica* and *Y. pseudotuberculosis* may be partly explained by other bacteria being better able to survive and compete for available nutrients within the faecal pellet.

As already discussed, infection with *Y. pseudotuberculosis* is highly seasonal, occurring mainly in cool, wet months, whereas infection with *Y. enterocolitica* occurs throughout the year (Slee & Skilbeck, 1992). The laboratory study of survival demonstrated that, when incubated at 20°C and watered, *Y. enterocolitica* and *Y. pseudotuberculosis* survived for 19 and 40 days, respectively. The increased environmental survival of *Y. pseudotuberculosis* makes it potentially less reliant on persisting in the gastrointestinal environment of a host animal for effective transmission. However, the opposite was observed for *Y. enterocolitica*, and so it is potentially more reliant on a host carrier status for long term survival. This suggests that *Y. enterocolitica* may need to cycle through a number of host animals in a flock in order to build sufficient environmental contamination to initiate transmission to new hosts, and this is consistent with the pattern of faecal shedding observed in the longitudinal study.

Water enhanced the odds of survival of *Y. pseudotuberculosis* when it was incubated at 20°C. This highlights that additional moisture, in the form of rainfall or increased retention of moisture in soil and vegetation, may assist its environmental survival and is consistent with the winter outbreaks of Yersiniosis due to this bacterium. This is also supported by the observation that in temperate regions, greater numbers of *Y. enterocolitica* were found in soil from deciduous forests compared with soil from grasslands (Botzler, 1987). *Y.*

pseudotuberculosis survived for 40 days when watered and incubated at 20°C, confirming that moist environments favour environmental survival of this *Yersinia* species. It also highlights the difficulty in decontaminating pasture following an outbreak of yersiniosis the previous winter, especially if frequent rainfall occurs during the summer or an early autumn break make conditions more favourable for survival and transmission.

Temperature fluctuations were included in the study design to test the hypothesis that if the bacteria were not exposed to continually hostile conditions (i.e. extremes of fixed temperatures) they would survive for longer. The results suggest that pathogenic *Yersinia* may be able survive in faeces throughout much of the year in temperate climates.

The temperature fluctuation of 20°C to 4°C was chosen to be representative of winter and early spring in south east Australia, a region that experiences severe seasonal outbreaks of yersiniosis. The results confirm that pathogenic *Yersinia* has the capacity to survive for at least 40 days in faecal material under simulated 'natural' conditions in this area, with the potential to survive much longer under field conditions. This makes the implementation of management procedures to decrease transmission quite a challenge, with less natural decontamination of pasture likely to occur due to high temperatures compared to other areas with less summer rainfall and hotter temperatures.

Exposure to high temperatures (37°C) can lead to the loss of the 70 kb virulence plasmid carried by *Yersinia*, whereas it persists between 1 and 31°C (Goverde, Kusters et al. 1994). Consequently, it was important to investigate if exposure to high temperatures, and subsequent desiccation, had damaged the virulence plasmid in the isolates used in the current study. Contrary to expectations, the yadA gene within the virulence plasmid was not lost. However, although the plasmid was still present it may not necessarily be functional and capable of causing disease, and so this aspect requires further investigation.

Shade from grass, rocks and trees, and damp areas around dams and sheep camps, could allow increased survival of *Yersinia* bacteria. These factors need to be considered in future studies, especially when investigating attempts at decontamination or management strategies that aim to decrease transmission and the expression of disease. For example, to reduce transmission of infection and the likelihood of disease outbreaks it may be advisable to avoid grazing susceptible animals, such as weaned lambs, on pastures grazed by affected sheep the previous season. It is unlikely that pasture can be made 'free' of *Yersinia* species, but systems to decontaminate pasture could also be devised. In general these will be consistent with current recommendations for internal parasite control in this region, such as 'Smart Grazing' (Niven et al, 2002a), or reducing the transmission of other diseases transmitted by the faecal oral route, most notably ovine Johne's disease (OJD). Preparation of lower risk pastures should use mature animals that are likely to shed fewer *Yersinia* (Slee & Skilbeck, 1992), with pastures grazed to a shorter length enabling increased mortality of organisms due to increased exposure to ultraviolet light and desiccation.

5.4 Vaccine trial

The high rainfall areas in which the trial properties were located experienced an unusually good spring in 2013 and an early autumn break in 2014. Feed quality and availability was exceptional, with the farm managers reporting that weaners were in excellent health coming into the winter. Whilst each property experienced short periods of cold temperatures and heavy rainfall, conditions were generally milder with altered (decreasing) rainfall patterns compared with the 2013 winter.

The absence of an outbreak in either study flock may have also been associated with insufficient bacterial challenge (faecal shedding) from animals with *Y. pseudotuberculosis*. When only small numbers of animals are shedding, pasture contamination is reduced and faecal-oral infections within the mob potentially far less. Results from Farm A support this

hypothesis, with bacterial shedding remaining low throughout the year. Farm C, however, recorded a higher rate of *Y. pseudotuberculosis* shedding in 2014 than the previous year, but this was not associated with an outbreak. On this farm, natural exposure to the bacteria was presumably high, but other risk factors were presumably not sufficient to cause disease. These observations again highlight the complex and multifactorial nature of the weaner scours syndrome.

Since the seasonal conditions were not conducive to an outbreak of Yersiniosis, an estimate of vaccine efficacy will be made by comparing the humoral response to both natural exposure and vaccination. Research from the deer industry has shown that a basic, multi-strain formalin-killed bacterin vaccine against *Y. pseudotuberculosis* was effective in decreasing the severity of Yersiniosis in vaccinated animals (Mackintosh, Buddle et al. 1991). Should an outbreak occur in 2015, vaccinated and control animals from 2014 will be sampled to compare faecal shedding and antibody titres. Animals representing a cross section of age groups will also be bled for assessment of antibody level to determine the longevity of antibodies produced in response to natural exposure.

Results from this trial indicate that a vaccine against *Y. pseudotuberculosis* may not be a viable option for control due to the sporadic nature of the disease and difficulties predicting outbreaks. A cost-benefit analysis will be undertaken to determine the level of protection required for a vaccine to be financially viable. We will also investigate the potential value of this vaccine to other industries. Most notably, Yersiniosis is frequently implicated in the calf scours syndrome, particularly of dairy heifers that are intensively reared.

6 Conclusions/recommendations

The results from this study strongly support the association between the weaner scours syndrome and presence of *Yersinia* sp. Faecal shedding and disease caused by *Y. pseudotuberculosis* was more severe and confirmed as being strongly seasonal, being detected almost exclusively in the high risk winter period and associated with outbreaks of severe disease, characterised by a high morbidity (up to 80% of sheep affected) and high mortalities. Contrary to anecdotal belief, *Y. enterocolitica* was quite often associated with disease and confirmed as being shed in faeces throughout the year. Consequently, further work is needed to determine the relative importance of these two *Yersinia* sp., both for their ability to cause disease in sheep and cattle and their potential to contaminate food and infect humans.

The development of resistance to antibacterials commonly used in the sheep and other food animal industries, associated with the frequent and relatively intensive use of these compounds to control bacterial enteritis caused by *Yersinia* sp., also deserves wider monitoring and investigation.

The postdoctoral candidate is now employed by the Mackinnon Project, and so further work using the data collected during this project will include a more complex analysis of risk factors, including seasonal conditions, live weight and faecal shedding of *Yersinia* sp. From this analysis it is hoped to develop firmer recommendations for the control and treatment of the weaner scours syndrome, including assessing the viability of a predictive tool for use by producers in high risk flocks to more accurately assess disease risk in various mobs.

An autogenous vaccine was developed for two flocks and trialled in 2014. However, in the absence of an outbreak of yersiniosis in that year it is difficult to accurately assess the efficacy of this pilot vaccine. Results from the natural challenge that occurred during 2014 demonstrated no beneficial effect on the bodyweight of animals, nor did it decrease faecal shedding of *Y. pseudotuberculosis*. Additional work is being undertaken by the PhD student

(Dr Kelly Stanger), including measuring the antibody response of sheep, to both vaccination and natural challenge. When completed, this will give further insights into the feasibility of vaccination and the potential of incorporating a vaccine into an integrated control strategy for Yersiniosis. Future work is planned to study *Yersinia* sp. infection in dairy replacement heifers, and so this will give additional information on the potential use of a vaccine in animals raised under more intensive conditions.

The environmental survival study demonstrated that it is highly unlikely that pasture can ever be made 'free' from *Yersinia* species. However, a system to decontaminate pasture is proposed, and this is consistent with research based recommendations for internal parasite control in the commonly affected regions, known as 'Smart Grazing' (Niven et al, 2002a), and methods to reduce environmental challenge of other organisms transmitted by the faecal-oral route, most notably *Mycobacterium paratuberculosis*, the infectious cause of OJD. Preparation of lower risk pastures would preferably use mature animals which are likely to shed fewer *Yersinia* sp. (Slee & Skilbeck, 1992). Grazing pastures to a relatively short length (say ≤ 700-900 kg dry matter per hectare, or 2.0-2.5 cm) should reduce the numbers of organisms on pasture by increasing exposure to the lethal effects of ultraviolet light and heat.

Further work, to support the implementation of these practices and collect case-control data to evaluate the efficacy and cost-benefit these programs, is strongly recommended so as to give producers more confidence in the proposed strategies. If demonstrated to be cost-effective, this would provide a more integrated approach to controlling weaner scours compared to the current approach, which incurs considerable production losses and relies almost solely on antibiotic treatment, with the associated risks of selection for resistance to these valuable compunds. The proposed strategies are relatively labour efficient, and so this should facilitate adoption in large flocks which are often those more severely affected with weaner scours.

Considerable communication of the results from this postdoctoral project, to both producers and veterinary advisors, has already been undertaken through conference and seminar presentations in Australia and New Zealand. This process will continue through producer networks, including the Southwest Victorian High Rainfall Animal Health group that was engaged with this project in early 2013, and Mackinnon Project newsletters and seminars. A focus on the development and adoption of the strategies proposed to reduce the risk of outbreaks occurring will be the primary focus of this material. These will demonstrate that this syndrome is not wholly driven by seasonal weather conditions, and provide practical tools for producers to proactively manage their weaner mobs. This will include suggestions for regular worm egg count (WEC) monitoring and weighing of weaner mobs, which addresses the major risk factors of increased worm burdens and the need for consistent growth to achieve live weight targets to reduce the risk of severe disease.

7 Key messages

1. The factors that lead to an outbreak of 'weaner scours' (bacterial enteritis caused by *Yersinia* sp.) are complex and multiple. Inclement weather is a necessary factor, but not sufficient by itself to induce this syndrome. Consequently, a combination of management interventions, both preceding and at the time of greatest risk, will reduce the likelihood of a severe outbreak.
2. Outbreaks of Yersiniosis, with high mortalities and a high proportion of sheep scouring, are most likely to occur in mid- to late-winter. However, an early autumn break or a wet summer may precipitate outbreaks at other times of the year, as these seasonal conditions may lead to greater contamination of pastures with *Yersinia* bacteria during

the summer or autumn. This means that mobs of weaners considered at risk of disease should be monitored regularly, typically from June to September.

3. An important component of monitoring mobs of weaners are regular (6-weekly) worm egg counts (WEC). There is a strong association between WECs >100epg and heavy shedding of *Yersinia* sp. Thus, in addition to the direct production benefits from reduced worm burdens, there will be additional benefits, from a reduced risk of yersiniosis, associated with low or moderate WECs in weaners from late June to September.
4. The preparation of 'Smart Grazed' paddocks for weaners to graze during winter will decrease this risk, for both Yersinia outbreaks and of unacceptably high worm burdens. Smart Grazed paddocks are prepared by grazing those paddocks selected for weaners to graze during the next winter for a maximum of 30 days after each of the preceding summer anthelmintic drenches (ie. the preparation occurs during the preceding December and February, grazing the paddocks with weaners subsequently occurs during May to September).
5. The laboratory-based environmental survival study indicates that *Yersinia* sp. can survive for prolonged periods under environmental conditions common in the high rainfall regions of south-eastern Australia. For those flocks with a high prevalence of weaner scours, summer destocking of paddocks that are heavily contaminated with *Yersinia* sp. (eg. those in which severe outbreaks have recently occurred), or heavy stocking with adult animals to reduce herbage to around 700-800 kg dry matter/ hectare (approximately 2 cm), should reduce the numbers of bacteria surviving in faeces and on the pasture the following winter. This is consistent with the 'Smart Grazing' approach discussed in point 4.
6. Weaners that are losing weight during winter, the highest risk time of year for weaner scours, are more likely to be shedding *Yersinia* bacteria compared to those maintaining or increasing their weight. Consequently, another important component of monitoring is to regularly weigh weaners and proactively manage their nutrition, such as drafting off light weaners for preferential supplementary feeding. This should achieve growth rate and bodyweight targets, hence reduce the prevalence of heavy shedding of *Yersinia* bacteria and the risk of an outbreak.

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