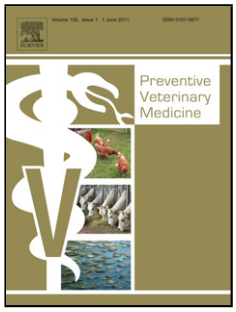

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Original article

Title: Faecal shedding of pathogenic *Yersinia enterocolitica* determined by qPCR for *yst* virulence gene associated with reduced live weight but not diarrhoea in prime lambs

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Highlights

- Longitudinal observational study of healthy crossbred prime (meat) lamb flocks
- qPCR to quantify pathogenic *Yersinia enterocolitica* (based on *yst* virulence gene) in sheep faeces
- Pathogenic *Y. enterocolitica* associated with reduced lamb liveweight
- Higher faecal shedding concentration associated with lower liveweight

- Pathogenic *Y. enterocolitica* associated not associated with increased risk of diarrhoea

Abstract

Associations between faecal shedding of pathogenic *Yersinia enterocolitica* (based on the *yst* virulence gene) with growth, carcass weight and diarrhoea were investigated using an observational longitudinal study of 1,200 crossbred prime (meat) lambs on eight Australian farms. Live weight, breech faecal soiling score (scale 1-5) and faecal consistency score (FCS; scale 1-5) were recorded, and faecal samples collected from each lamb on three sampling occasions; weaning (≈12 weeks of age), post-weaning (≈19 weeks) and pre-slaughter (≈29 weeks). Hot standard carcass weight was measured at slaughter. Faecal samples were screened for presence and concentration of pathogenic *Y. enterocolitica* using quantitative PCR. Associations of pathogenic *Y. enterocolitica* detection and shedding intensity with lamb health and production were assessed using general linear models (carcass weight), linear mixed effects models (live weight, FCS and breech soiling score) and non-parametric tests (FCS and breech soiling score). Prevalence for non-pelleted faeces (FCS ≥3.0) and diarrhoea (FCS ≥4.0) were compared with the two-tailed z-test, odds ratios and relative risk. Lambs shedding pathogenic *Y. enterocolitica* were 3.78kg lighter post-weaning (P<0.001) and 2.61kg lighter pre-slaughter (P=0.035) compared to lambs in which pathogenic *Y. enterocolitica* was not detected. Higher faecal concentration of pathogenic *Y. enterocolitica* was associated with lower live weight (P<0.001). There was no association between pathogenic *Y. enterocolitica* detection and carcass weight. Overall there was no association between pathogenic *Y. enterocolitica* detection and FCS or breech soiling score. Only one flock had increased relative risk for non-pelleted faeces associated with pathogenic *Y. enterocolitica* detection, and one other flock had increased relative risk for diarrhoea associated with pathogenic *Y. enterocolitica* detection. This is the first report of an association between reduced sheep live weight and pathogenic *Y. enterocolitica* based on the presence of the *yst* gene

for heat stable enterotoxin determined by qPCR in sheep. Notably, impacts on live weight were observed in the absence of diarrhoea.

Key words

Yersiniosis; Ill thrift; Sheep; Productivity; Carcass; Carcase; Diarrhoea

1. Introduction

Yersiniosis causes gastrointestinal disease in sheep characterised by diarrhoea, depression, ill thrift and deaths (McSporran et al., 1994; Slee and Button 1990). Clinical manifestation of disease associated with *Y. enterocolitica* has been reported in Australia (Slee and Button, 1990; Slee and Skilbeck, 1992; Stanger 2017), and worldwide (Bin-Kun et al., 1994; Gill, 1996).

The chromosomal *yst* gene encodes a low-molecular-weight, heat-stable enterotoxin belonging to a family of structurally and functionally related enterotoxins produced by several species of diarrheagenic bacteria (Robins-Browne et al., 1979; Delor et al., 1990; Kechagia et al., 2007). As *yst* is confined to pathogenic bioserotypes of *Y. enterocolitica,* it is considered a useful marker of potential virulence (Ibrahim et al., 1997). Quantitative PCR (qPCR) using primers and probe sequences specific to the *Y. enterocolitica yst* virulence gene have been used to quantify "pathogenic *Y. enterocolitica"* in faecal samples from humans (Ibrahim et al., 1997; Zheng et al., 2007) and sheep (Yang et al., 2016). A longitudinal study of prime (meat breed) lambs between weaning and slaughter on eight Australian farms identified pathogenic *Y. enterocolitica* (determined by *yst* virulence gene) in all eight flocks with point prevalence ranging 0-49% (Yang et al., 2016).

Faecal carriage of *Y. enterocolitica* is reported in young sheep in the absence of clinical disease (Slee and Button, 1990; Slee and Skilbeck, 1992; Stanger 2017), but it has not been determined whether faecal shedding of pathogenic *Y. enterocolitica* is associated with impacts on

health and production of lambs in sheep meat production systems. Live weight and carcass weight are important profit drivers for sheep meat production, and live weight is an important determinant of lamb survival and welfare during the post-weaning period (Hatcher et al., 2008; Campbell et al., 2009; Hatcher et al., 2010). Diarrhoea and subsequent faecal soiling of the fleece also have adverse impacts on lamb welfare and productivity, including increasing predisposition to fly strike (Wardhaugh et al., 1989) and risk of carcass contamination with faecal pathogens at slaughter (Hadley et al., 1997).

This study aimed to determine whether faecal shedding of pathogenic *Y. enterocolitica* (determined by *yst* virulence gene using qPCR) was associated with diarrhoea, reduced growth (live weight) or reduced carcass weight in prime lambs.

2. Materials and Methods

2.1 Animals, sample collection and measurements

The study conforms to international cohort study reporting guidelines for strengthening the reporting of observational studies in epidemiology (STROBE) described by von Elm et al. (2008). All procedures were approved by relevant animal ethics committees in each state, with the overall methodology approved and monitored by the Murdoch University Animal Ethics Committee (approval no. R2352/10).

A prospective observational (cohort) investigation of cross-bred prime (meat) lamb flocks was conducted on eight different farms (one flock per farm) located across four states of Australia (Table 1). Farms were located in states of Western Australia (WA), South Australia (SA), Victoria (VIC) and New South Wales (NSW). Characteristics of the farms (including size of property, total number of sheep on the property, annual rainfall, time of lambing and presence of other livestock on the farm) have been described by Yang et al (2016). Observations for farms WA1 and WA2 were made for lambs born in 2009, and for all other farms observations were made for lambs born

in 2011 (Table 1). Farms were recruited into the study on the basis that at least 100 crossbred female lambs would be available in a single flock, and that lambs would be managed under conditions typical for commercial prime (meat) lamb production in Australia. The sample size (minimum 100 lambs per farm) was determined on the basis of establishing prevalence for a range of protozoan and bacterial pathogens, including *Yersinia* spp. (Yang et al., 2016). Lambs were identified with numbered ear tags. Female lambs were recruited into the study at either marking (approximately 4-6 weeks of age) or weaning (Table 1), and were managed as a single cohort with ewe and wether lamb flockmates until slaughter. Within cohorts (flocks), lamb age was estimated to range up to 8 weeks, depending on duration of lambing.

Each lamb was sampled on three separate occasions, specifically weaning (approximately 12 weeks old), post-weaning (approximately 19 weeks old) and pre-slaughter (approximately 29 weeks old). Lambs were weighed (live weight), assessed for breech faecal soiling and faecal samples were collected at each sampling occasion. Breech faecal soiling score was measured using a scale of 1 (no evidence of breech faecal soiling) to 5 (very severe breech faecal soiling extending down the hind legs to, or below the hocks) as previously described (Sweeny et al., 2012; Australian Wool Innovation, and Meat and Livestock Australia, 2013). Some lambs were lost to follow-up at one or more sample occasions due to incomplete mustering from the paddock or mortality (Table 1).

A total of 3,343 faecal samples were collected directly from the rectum using a sterile swab (weaning sample SA1, SA2 and NSW only) or gloved hand (all other samples) from 1,200 cross-bred lambs (Table 1). Faecal consistency score (FCS) and faecal worm egg counts (WEC) for each lamb were measured where sufficient faecal material was available. Faecal consistency score was measured using a scale of 1-5 (1 = hard pellet, 2 = soft pellets, 3 = firm paste, 4 = soft paste and 5 = liquid diarrhoea). Faecal WEC were performed using a modified McMaster technique using 2.0g

faeces where each egg counted represented 50 eggs per gram (epg) of faeces (Lyndal-Murphy, 1993).

Lambs were consigned for slaughter within 2 weeks of pre-slaughter sample collection. All sheep were classified as "lamb" at slaughter with no eruption of permanent incisor teeth (AUS-MEAT 2005). Hot standard carcass weight was measured for all lambs based on the AUS-MEAT definition (AUS-MEAT 2005).

2.2 DNA isolation and qPCR amplification, quantitation and sequencing

Total DNA was extracted using 200-300 mg of each faecal sample using a Power Soil DNA Kit (Mo Bio, Carlsbad, CA, USA) with some modifications as previously described (Yang et al., 2016).

Pathogenic *Y. enterocolitica* were screened in all samples by qPCR using the forward primer Pr2A AATGCTGTCTTCATTTGGAGCA and reverse primer Pr2c ATCCCAATCACTACTGACTTC specific to the *Y. enterocolitica yst* virulence gene as previously described (Ibrahim et al*.,* 1997). A *yst* gene probe YenPath490: 6xFAM-TTCGGCCAAGAAACAGTTTC-HBQ was designed using software from Biosearch Technologies (https://www.biosearchtech.com/). For the qPCR, each 15 µL PCR mixture contained 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl), 3 mM $MgCl_2$, 1mM deoxynucleotide triphosphates, 1.0 U KAPA DNA polymerase (MolBio), 0.2 µM each forward and reverse primer, and 1 µL sample DNA. The qPCR was performed on a Rotor-gene Q real-time cycler (Qiagen, VIC, Aust). The PCR cycling conditions consisted of 95°C for 3 min, then 45 cycles of 95°C for 20 s and 60°C for 45 s. PCR contamination controls were used, including negative controls (no template and blank extraction controls) and separation of preparation and amplification areas.

A standard curve for quantifying *Y. enterocolitica* DNA was generated by cloning the *yst* PCR product amplified from *Y. enterocolitica* isolate AS-11–2403 (which was originally isolated from a pig in WA), into a pGEMT-vector (Promega) and transforming into *Escherichia coli* competent cells. Plasmid DNA was isolated by alkali–SDS lysis, followed by column purification

using QIAprep Spin Columns (Qiagen) in accordance with the manufacturer's protocol. Plasmid mini-preparations were sequenced using M13F and M13R sequencing primers and clones with the correct sequence then used as positive controls for generating a standard curve.

Template copy numbers were converted to numbers of organisms present on the basis that the targeted gene (*yst*) is a single copy gene (Delor et al., 1990) and the bacterial genomes are haploid, therefore detected plasmid numbers were approximately equivalent to numbers of pathogenic *Y. enterocolitica*.

Randomly selected qPCR amplicons (*n*=5) were sequenced to confirm that *Y. enterocolitica yst* gene was being amplified. All samples that were positive for *Y. enterocolitica yst* gene were also confirmed as positive for *Yersinia* spp. rpoB gene as previously described (Yang et al., 2016).

The analytical specificity of the primers used has been described previously (Ibrahim et al., 1997). In order to determine the sensitivity of the assay, 10-fold serial dilutions (5) of plasmids containing the cloned PCR products amplified from *Y. enterocolitica* isolate AS-11-2403 as described above, were conducted from 1,000,000 copies down to 100 copies of the plasmid template and these were then spiked into faecal samples and the DNA extracted and amplified as described above. Mean minimum detection limit for *Y. enterolitica* was 10 organisms per 1 µl, mean R squared value was 0.98 and % relative standard deviation was 6.4%.

2.3 Statistical analyses

The experimental unit for analyses was individual lambs. Pathogenic *Y. enterocolitica* detection for each lamb at each timepoint was categorised as detected (pathogenic *Y. enterocolitica* detected) or absent (not detected). Shedding concentration and WEC were log-transformed for analyses using Log_{10} (bacteria per gram faeces + 25) and Log_{10} (worm eggs per gram faeces + 25). Faecal consistency score (scale 1-5) was considered as a continuous variable. Faecal consistency was also categorised in two ways; firstly as pelleted (FCS<3.0) or non-pelleted

(FCS ≥3.0), and secondly as not diarrhoea (FCS<4.0) or diarrhoea (FCS ≥4.0). These faecal consistency categories were considered as discrete categorical variables. For longitudinal analyses, missing data for lambs absent at one or more time points were addressed through use of linear mixed effect models which can account for missing data at a single time point without impacting data for other time points from the same animal (West et al., 2007). For analyses of prevalence (z-test), non-parametric tests for FCS and breech soiling (Mann-Whitney U) and carcass weight (general linear models performed separately for each time point), any missing data were excluded (omitted) from analyses.

Prevalence 95% confidence intervals were calculated using Jeffrey's interval (Brown et al., 2001; Sergeant, 2016). Period prevalence was calculated by proportion of lambs from which pathogenic *Y. enterocolitica* was detected on at least one sampling occasion. Prevalence of non-pelleted faeces (FCS ≥3.0) and diarrhoea (FCS≥4.0) for lambs with or without pathogenic *Y. enterocolitica* detected were compared within data pooled for all farms, using the 2-tailed z-test (Jekel et al., 2007; Sergeant, 2016). These analyses were repeated within farm (i.e. separately for each farm). Odds ratios with relative risk used to quantify relationships between detection of pathogenic *Y. enterocolitica* and non-pelleted faeces (FCS ≥3.0) or diarrhoea (FCS<4.0). Relative risk were considered significant where 95% confidence interval contained zero.

Normality of residuals was tested using Kolmogorov-Smirnov and Shapiro-Wilk tests, with data (residuals) considered normally distributed where $P>0.05$ (i.e. accept null hypothesis that data are normally distributed). Residuals for live weight and carcass weight were normally distributed ($P>0.150$) and therefore analysed only using linear mixed effect and general linear models (described below). Residuals for FCS and breech soiling score were not normally distributed ($P<0.05$), therefore association for detection of pathogenic *Y. enterocolitica* (detected or absent) with FCS or breech soiling score were also tested with two-tailed Mann-Whitney U (non-parametric test) using IBM SPSS Statistics (SPSS Version 24, IBM Corporation, USA). These

analyses were performed for data pooled for all farms and timepoints, then repeated within farm and time point (i.e. separately for each farm and sampling occasion).

Live weight, breech soiling score and FCS for each sampling occasion (weaning, post-weaning or pre-slaughter), and carcass weight (slaughter only) were expressed as the observed mean (± standard error) for all sites (i.e. not corrected for site). Further, linear mixed effects models (SAS Version 9.2, SAS Institute, Cary, NC, USA) were generated for live weight, breech soiling score and FCS using site (farm location) and sampling occasion (weaning, post-weaning or pre-slaughter) as categorical (discrete) effects. Animal identification code was included as a random term (to account for multiple sampling of individuals across the three time-points). Least square means (± standard error) were generated for the sampling occasion main effect. Similarly, general linear models (SAS Version 9.2, SAS Institute, Cary, NC, USA) were generated for carcass weight using site (farm location) as a categorical effect, and least square means (± standard error) were generated for the sampling occasion main effect.

Trichostrongylid nematodes can cause reduced growth in young sheep (Coop and Angus, 1981; Coop and Holmes, 1996), therefore log transformed WEC was included as a covariate (continuous predictor) in analyses for live weight and carcass weight.

Associations for pathogenic *Y. enterocolitica* with live weight, FCS and breech soiling were analysed using linear mixed effects models (SAS Version 9.2, SAS Institute, Cary, NC, USA). Initially base models were constructed for live weight, FCS or breech soiling score as the dependent variable. These base models included site (farm location) and sampling occasion (weaning, post-weaning or pre-slaughter) as categorical (discrete) effects, and WEC (log transformed) was included as a covariate (continuous predictor). To account for multiple sampling of individuals across the three time-points, animal identification code was included as a random term. Within these base models, pathogenic *Y. enterocolitica* was tested in two ways, firstly as a categorical (discrete) effect (pathogenic *Y. enterocolitica* detected or not detected), and secondly as a

covariate (continuous predictor) effect after log transformation using Log_{10}(pathogenic *Y. enterocolitica* faecal shedding concentration + 25). Hence, this process resulted in six separate linear mixed effects models with the three dependent variables (live weight, FCS and breech soiling score) each tested separately with pathogenic *Y. enterocolitica* as either a categorical (discrete) or covariate (continuous predictor). Interactions between the pathogenic *Y. enterocolitica* term (whether categorical or continuous predictor) and other factors in the base model were tested and non-significant terms (P>0.05) were removed in a step-wise manner.

The prevalence of pathogenic *Y. enterocolitica* was highest for the NSW farm, therefore the process described above for assessing associations for pathogenic *Y. enterocolitica* detection with live weight using linear mixed effects models were repeated including either only the NSW site or all other sites (excluding NSW). These analyses were performed as previously described with sampling occasion (weaning, post-weaning or pre-slaughter) and pathogenic *Y. enterocolitica* detection (detected or not detected) were included as categorical (discrete) effects, WEC (log transformed) was included as a covariate (continuous predictor) and animal identification code included as a random term.

Association between live weight at weaning and pathogenic *Y. enterocolitica* detection post-weaning was analysed using a linear mixed effects model (SAS Version 9.2, SAS Institute, Cary, NC, USA) with site (farm location) and pathogenic *Y. enterocolitica* detection status (detected or not detected) included as categorical (discrete) effects, and log transformed WEC included as a covariate (continuous predictor). Least square means were generated for the pathogenic *Yersinia* main effects.

Associations between pathogenic *Y. enterocolitica* and carcass weight were analysed using general linear models (SAS Version 9.2, SAS Institute, Cary, NC, USA), with site (i.e. farm locations) included as a categorical (discrete) effect and log transformed WEC included as a covariate (continuous) effect. Within this base model, presence of pathogenic *Y. enterocolitica* at the

weaning time-point was tested as a categorical effect (detected or not detected), along with its interactions with other terms in the base model. Non-significant terms were removed in a step-wise manner. This process was repeated twice to assess effect of pathogenic *Y. enterocolitica* presence post-weaning and pre-slaughter time points, resulting in three separate models for carcass weight (i.e. one for each timepoint). A fourth general linear model was developed that included detection of pathogenic *Y. enterocolitica* at any timepoint (i.e. detected on at least one occasion or never detected) and farm included as categorical (discrete) effects, and log transformed WEC included as a continuous (covariate) effect.

3. Results

3.1 Prevalence of pathogenic Y. enterocolitica *in lambs*

Prevalence and faecal shedding concentration of pathogenic *Y. enterocolitica* are shown in Table 1. Pathogenic *Y. enterocolitica* was identified in 5.8% of faecal samples, and was identified in all eight flocks sampled (Table 1). Period prevalence (proportion of animals positive on at least one sampling occasion) for pathogenic *Y. enterocolitica* ranged from 0.8 – 66.3% across the eight farms. Pathogenic *Y. enterocolitica* prevalence for the NSW farm was higher than for the other seven farms. Prevalence was low (<8%) for all farms pre-slaughter.

3.2 Association between pathogenic Y. enterocolitica *and live weight*

Live weights at each sampling occasion are shown in Table 2. Associations between detection of pathogenic *Y. enterocolitica* and live weight are shown in Table 3. Lambs in which pathogenic *Y. enterocolitica* was detected were 3.78kg lighter post-weaning (P<0.001) and 2.61kg lighter pre-slaughter (P=0.035) compared to lambs in which pathogenic *Y. enterocolitica* was not detected (Table 3). When analysed separately, lambs from NSW flock with pathogenic *Y. enterocolitica* detected were 4.43kg lighter than lambs without pathogenic *Y. enterocolitica* post-weaning (P<0.001). For all other farms (i.e. excluding NSW), lambs with pathogenic *Y. enterocolitica* detected were 2.99kg lighter post-weaning (P<0.007) and 2.70kg lighter pre-slaughter (P<0.020) than lambs without pathogenic *Y. enterocolitica*. Results for pre-slaughter sampling need to be interpreted with caution due to the low prevalence observed at that time point (n=9). Lambs positive for pathogenic *Y. enterocolitica* post-weaning were lighter (29.1kg) at the previous sampling (weaning) compared with lambs not shedding pathogenic *Y. enterocolitica* (31.7kg; P<0.001).

Higher concentration of faecal pathogenic *Y. enterocolitica* shedding was associated with lower live weight (main effect P<0.001). Associations between faecal shedding concentration and live weight for lambs in which pathogenic *Y. enterocolitica* were identified are shown in Figure 1. Data for pre-slaughter sampling are not shown in Figure 1 due to the small number of positive samples at that time point (n=9), but an association between higher faecal shedding concentration and lower live weight (P<0.001) was observed.

3.3 Association between pathogenic Y. enterocolitica *and carcass weight*

Carcass weights are shown in Table 2. There was no association between carcass weight (at slaughter) and detection of pathogenic *Y. enterocolitica* at either weaning (*P*=0.151), post-weaning (*P*=0.307) or pre-slaughter (*P*=0.795). There was no association (*P*=0.483) between carcass weight and pathogenic *Y. enterocolitica* being detected at any occasion (i.e. ever positive).

3.4 Associations between pathogenic Y. enterocolitica *and faecal consistency*

Faecal consistency scores are shown in Table 2, and data for each farm and sampling occasion are shown in Supplementary Material Appendix 1. There was wide variation in FCS between sites (farms) and time points.

Associations between detection of pathogenic *Y. enterocolitica* and faecal consistency are shown in Table 3. Whilst the association between pathogenic *Y. enterocolitica* and FCS was significant (main effect *P*=0.023), a difference in FCS was only evident pre-slaughter, at which time lambs in which pathogenic *Y. enterocolitica* was detected had firmed faeces (lower FCS) than those in which pathogenic *Y. enterocolitica* was not detected (Table 3). This observation (tested with linear mixed effect model) was consistent with non-parametric tests that showed overall (all farms and timepoints combined), detection of pathogenic *Y. enterocolitica* was associated with lower FCS (Mann-Whitney U *P*=0.002). When each farm was analysed separately for each timepoint, there was no association between detection of pathogenic *Y. enterocolitica* and FCS (*P*>0.05).

Overall (all farms and all timepoints combined), detection of pathogenic *Y. enterocolitica* was associated with lower prevalence of non-pelleted faeces (FCS ≥3.0; Table 4). Faecal samples with pathogenic *Y. enterocolitica* detected were 1.8 (relative risk 95% confidence interval 1.2, 2.8) times more likely to be classified as pelleted compared to samples without pathogenic *Y. enterocolitica.* Within farms, prevalence of pathogenic *Y. enterocolitica* detection was associated with higher prevalence for non-pelleted faeces for two farms (NSW and Vic1) and lower prevalence of non-pelleted faeces for two farms (Vic2 and WA2; Table 4). Samples from NSW with pathogenic *Y. enterocolitica* detected were 2.7 (95% CI 1.3, 5.8) times more likely to be classified as non-pelleted compared to samples without pathogenic *Y. enterocolitica* detected. Relative risk for non-pelleted faeces for Vic1 was not significant (95% CI 0.96, 10.2).

Detection of pathogenic *Y. enterocolitica* was associated with higher prevalence of diarrhoea (FCS ≥4.0) only for one farm (Vic 1) where samples with pathogenic *Y. enterocolitica* detected were 5.6 (95% CI 1.2, 27.6) times more likely to be classified as diarrhoea compared to samples without pathogenic *Y. enterocolitica* detected (Table 4).

Pathogenic *Y. enterocolitica* shedding concentration was not associated with FCS (main effect *P*=0.996).

3.5 Associations between pathogenic Y. enterocolitica *and breech faecal soiling*

Breech faecal soiling scores are shown in Table 2, with the data for each farm and sampling occasion shown in Supplementary Material Appendix 2. There was a variation observed in breech soiling scores between sites.

There was no association between breech soiling score and either detection of pathogenic *Y. enterocolitica* (*P*=0.971) or faecal pathogenic *Y. enterocolitica* shedding concentration (*P*=0.991). Non-parametric tests demonstrated that overall (all farms and timepoints combined), detection of pathogenic *Y. enterocolitica* was associated with less breech faecal soiling (lower breech soiling

score) (Mann-Whitney U $P<0.001$). When each farm was analysed separately for each timepoint, there was no association between detection of pathogenic *Y. enterocolitica* and breech soiling score (Mann-Whitney U $P>0.05$).

3.6 Associations between WEC and live weight, carcass weight, faecal consistency or breech faecal soiling

Observed WEC (mean ± standard error) were 285 epg ± 28 (weaning), 115 epg ± 14 (post-weaning) and 185 epg ± 73 (pre-slaughter). No association (main effect $P>0.05$) between WEC (as a co-variate) and either live weight, carcass weight, faecal consistency or breech faecal soiling were identified.

When analysed as a bi-variate correlation, no association between WEC and FCS were identified, but there was a weak correlation between WEC and breech soiling score (Pearson correlation 0.120; $P=0.037$).

3.7 Associations between other factors included in statistical models and measures of sheep productivity

Within each linear mixed effect model for live weight, faecal consistency and breech faecal soiling, significant main effects were identified for farm ($P<0.001$) and sampling occasion ($P<0.001$), and significant interactions for farm x sampling occasion ($P<0.001$), confirming that lamb weight, faecal consistency and breech faecal soiling varied between locations and time points. Significant interactions between sampling occasion x pathogenic *Y. enterocolitica* were noted only for live weight ($P<0.001$) and faecal consistency ($P=0.016$ for detection and $P=0.018$ for shedding concentration). This confirmed that the association between live weight or faecal consistency and pathogenic *Y. enterocolitica* varied between sampling occasions.

Within the general linear models for carcass weight at each time point, significant main effects were noted for farm (P<0.001). No significant interactions were identified.

Across all models (linear mixed effect models and general linear model) there were no interactions (P>0.100) between site and pathogenic *Y. enterocolitica* (tested either as a covariate or as a categorical term).

4. Discussion

Both presence and increased shedding concentration of pathogenic *Y. enterocolitica* were associated with reduced live weight in prime lambs with no other evidence of clinical Yersiniosis, including diarrhoea. *Yersinia enterocolitica* has been associated with outbreaks of clinical yersiniosis in Australian sheep (Slee and Button, 1990; Philbey et al. 1991; Stanger 2017), but is also isolated from clinically normal sheep (Slee and Skilbeck, 1992; Stanger, 2017). To the authors' knowledge, associations between pathogenic *Y. enterocolitica* shedding concentration (based on qPCR detection of *yst* virulence gene) and either live weight or diarrhoea in sheep under field conditions have not been previously reported.

Within *Y. enterocolitica*, the *yst* gene is generally confined to pathogenic bioserotypes, and hence considered a useful marker of potential virulence. A homologous gene is found in some isolates of *Y. intermedia* and *Y. kristensenii* (Ibraham et al., 1997; Kechagia et al., 2008) which are considered non-pathogenic, however primers used in this study were designed to be specific to the *yst* gene in *Y. enterocolitica* (Ibraham et al., 1997). Other studies have used *yst*A in combination with other genes including YadA (Kechagia et al., 2007; McGregor et al., 2015; Stanger, 2017), *ail*, *inv* and *myf*A (Joutsen et al., 2016) as virulence markers for *Y. enterocolitica* from sheep, with Stanger (2017) reporting that whilst faecal carriage of virulent *Y. enterocolitica* (based on presence of YadA plasmid virulence gene, with or without YstA) occurred both in presence and absence of clinical Yersiniosis, faecal carriage was lower in Merino lambs with higher growth rates.

Diarrhoea and breech faecal soiling is commonly reported in lambs raised for meat production in southern Australia (Sweeny et al., 2012). This study was conducted using flocks of prime (meat) lambs managed from birth until slaughter under conditions representative of commercial lamb meat production in Australia, and located over a wide geographical area. Lambs grew according to expectation based on genotype and level of nutrition provided, with no

evidence of overt clinical disease observed; that is, these were considered "healthy" typical crossbred lamb flocks. This was reflected in the generally low prevalence for diarrhoea (FCS ≥4.0) observed. The prevalence of pathogenic *Y. enterocolitica* shedding was generally low in these flocks, with highest rate of detection in the flock from New South Wales. It could not be determined whether the degree of reduced live weight associated with pathogenic *Y. enterocolitica* shedding would be similar in flocks with evidence of clinical yersiniosis outbreak (e.g. depression, diarrhoea, illthrift, enteritis associated with microabscessation). Reduced live weight and growth in the weaning and post-weaning periods can be associated with adverse impacts on sheep welfare and survival (Hatcher et al., 2008; Campbell et al., 2009; Hatcher et al., 2010), and whilst critical growth rates are better described for Merinos than crossbred lambs, high post-weaning mortalities have been reported in Australian crossbred flocks (Campbell et al., 2014). Further studies to confirm the association between pathogenic *Y. enterocolitica* and reduced live weight, and to better characterise prevalence for pathogenic *Y. enterocolitica* for farms across sheep producing environments are required to determine likely impacts at an industry level, if indeed the relationship is shown to be causative.

Greater impacts on live weight were associated with higher faecal shedding concentration (Fig 1). This was consistent with observations by Slee and Button (1990) where intestinal lesions were often associated with heavy *Yersinia* growth, but not with light-moderate *Yersinia* growth. It should be noted that the association between faecal shedding of pathogenic *Y. enterocolitica* and reduced live weight could not be confirmed as causative (i.e. infection causing reduced growth) in this observational study. It is possible that lambs that otherwise had restricted growth were more susceptible to infection, as evidenced by lambs shedding pathogenic *Y. enterocolitica* post-weaning being 2.5kg lighter at the weaning sample collection (approximately 2 months earlier). Other studies have noted that Merino sheep (aged 8-9 months at start of the study) that gained

weight had moderately lower risk of shedding *Y. enterocolitica* than those not gaining weight (Stanger, 2017).

The observation that *Y. enterocolitica* shedding post-weaning was associated with lower live weight contrasts with a longitudinal study in south eastern Australia that showed no relationship between live weight and *Y. enterocolitica* shedding in Merinos post-weaning for four farms in south-eastern Australia (Stanger, 2017). There were a number of differences between the study by Stanger (2017) and the present study, including *Y. enterocoltica* virulence factors (YadA plasmid virulence gene with or without YstA, versus *yst* for the present study), host animal differences including sheep genotype (Merinos versus cross-bred meat lambs for the present study), and environmental (farm) variation.

It was not possible to determine if differences in observations between sampling occasions reflect an effect of age on risk of shedding and live weight loss, seasonal variation in *Y. enterocolitica* shedding, impacts of stress associated with weaning, or other factors such as inclement weather (Slee and Skilbeck, 1992; Gill, 1996). The greatest impact on live weight associated with pathogenic *Y. enterocolitica* was observed post-weaning. By pre-slaughter, pathogenic *Y. enterocolitica* prevalence was very low (Table 1), and there was no association between pathogenic *Y. enterocolitica* (at any time point) with carcass weight. This suggests affected lambs had recovered by slaughter such that effects on carcass weight were not evident. Results for pre-slaughter sampling need to be interpreted with caution due to the low prevalence observed at that timepoint. Other studies have reported that faecal carriage of *Y. enterocolitica* is more common in younger sheep (Slee and Button, 1990; Slee and Skilbeck 1992).

Pathogenic *Y. enterocolitica* was not associated with higher FCS (looser faeces) or greater faecal soiling of the breech, suggesting that the live weight effects observed were in the absence diarrhoea. Fleece faecal soiling is more likely to occur when faeces are not in pelleted form (Waghorn et al., 1998), and soiling is associated with predisposition to flystrike (Watts and

Marchent, 1977) and faecal microbial contamination of carcasses (Hadley et al. 1997). Factors that increase risk of non-pelleted faeces (FCS ≥3.0), and especially diarrhoea (FCS ≥4.0) therefore have implications for the health and welfare of sheep, meat quality (i.e. shelf life) and food safety. Increased risk of non-pelleted faeces for samples with pathogenic *Y. enterocolita* was identified only for one farm (NSW), and increased risk of or diarrhoea was identified only for one farm (Vic1). This was consistent with other reports that morbidity and mortality of clinical yersiniosis, with enteritis considered an unusual manifestation of *Y. enterocolitica* infection (Slee and Button, 1990), and faecal carriage of *Y. enterocolitica* occurring in healthy populations. Other longitudinal studies monitoring lamb flocks have noted absence of clinical disease in lambs identified as shedding *Y. enterocolitica* (Slee and Skilbeck, 1992), and Stanger (2017) reported shedding of *Y. enterocolitica* by Merino lambs in the absence of clinical disease.

Observations from this study suggest the need for further work to explore the role of *Y. enterolitica* in sheep as a potential cause for reduced growth in young sheep that may occur in the absence of obvious disease outbreaks evidenced by diarrhoea. The prevalence of pathogenic *Y. enterocolitica* varied across the eight farms included in this observational study, with the New South Wales flock having higher prevalence. Molecular tools offer opportunity to determine pathogenic *Y. enterocolitica* faecal carriage (prevalence) across different geographical areas and different seasons and establish potential impact at industry level. Experiments that include experimental infections could be used to confirm whether the observation that of pathogenic *Y. enterocolitica* is associated with reduced sheep live weight whilst addressing potential biases and confounding factors that exist in observational studies. Statistical analyses demonstrated that "farm" was a significant main effect for all of the sheep health and productivity measures (live weight, carcass weight, faecal consistency and breech soiling). However, there were a number of differences between the eight farm sites (apart from location) that are likely to have impacted these measures, for example sheep genotype, weather conditions (especially temperature and

rainfall) throughout study period, management and husbandry procedures, nutrition, and concurrent infections. Further investigation would be required to define impact of these specific factors on expression of disease in sheep shedding pathogenic *Y. enterolitica*. The three WA farms were sampled in different years to the other sites, but it is likely the aforementioned factors are likely to outweigh temporal variation within farms likely to occur independent of these that may impact expression of disease. Furthermore, there were potential biases in the study that may have impacted lamb live weight independent of infection (e.g. date of birth, dam parity, litter size and procedures performed in pre-weaning period), and assumptions of similarity of factors (such as distribution of litter size) between flocks was made. This could be addressed in future studies to better define associations between sub-clinical or clinical infection and growth in sheep across different farming systems and seasons. Finally, the methodology for the present study did not include faecal culture and isolation of *Yersinia* spp.. The primers and probe used in this study were highly specific (Ibrahim et al., 1997) and positive samples from this study were also positive for *Yersinia* spp. at rpoB using a nested PCR (Yang et al., 2016), suggesting the qPCR had acceptable specificity for screening faecal samples. It has been suggested that *yst*B virulence may not be expressed in some human and swine *Y. enterocoltica* isolates, signifying potential for an inactive *yst* gene or presence of a non-specific homologous region (Singh and Virdi 2004). A combination of qPCR and conventional microbial techniques in parallel would be required to confirm expression of virulence (enterotoxin production) in association with *yst* for *Y. enterocolitica* strains isolated from sheep.

5. Conclusion

The presence and increased shedding concentration of pathogenic *Y. enterocolitica* (determined by *yst* virulence gene detected by qPCR) were associated with reduced live weight in prime lambs. These observations were made in flocks of crossbred lambs raised for slaughter

under typical commercial conditions located over a wide geographical area, and that were otherwise normal with no evidence of widespread clinical disease, including diarrhoea, that would normally trigger flock disease investigation for enteritis. The prevalence of pathogenic *Y. enterocolitica* varied across the eight farms included in this observational study, with one farm having higher prevalence, and the repeatability of these observations should be confirmed. Further investigation into the role of *Y. enterocolitica* on lamb health and productivity are warranted.

Conflict of Interest

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper. The funding bodies (Meat and Livestock Australia and Australian Wool Innovation) approved the experimental design of the overall project (B.AHE.0027) of which this data formed one part, and Meat and Livestock Australia approved the manuscript for submission. Neither funding body was involved in the collection, analysis or interpretation of data, or in the writing of the manuscript beyond minor editorial advice to improve clarity of presentation provided by Johann Schroder (Meat and Livestock Australia).

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Table 1: Prevalence (with Jeffrey's 95% confidence interval) and faecal shedding of pathogenic *Y. enterocolitica* (*yst* virulence gene by qPCR) in sheep faecal samples collected from 8 Australian farms

				Shedding concentration in positive samples (organisms/g faeces)	
Farm	**Sampling occasion**	**Samples** (n)	**Prevalence*** % (95% CI)	**Range**	**Median**
NSW	Weaning	156	50.0 (42.2-57.8)	$4.2x10^3$-$7.2x10^7$	$1.08x10^5$
	Post-weaning	160	29.4 (22.7-36.8)	$8.5x10^3$-$3.6 x10^6$	$6.68x10^4$
	Pre-slaughter	140	0.0 (0.0-1.8)	-	-
SA1	Weaning	165	5.4 (2.7-9.7)	$4.2x10^3$-$8.6x10^5$	$3.08x10^4$
	Post-weaning	154	3.9 (1.6-7.8)	$1.1x10^4$-$1.4x10^5$	$2.74x10^4$
	Pre-slaughter	158	0.6 (0.0-2.9)	-	$4.38x10^4$
SA2	Weaning	158	5.7 (2.9-10.1)	$1.1x10^4$-$1.4x10^6$	$4.14 x10^5$
	Post-weaning	151	2.0 (0.6-5.2)	$6.4 x10^5$-$9.4x10^6$	$1.86 x10^6$
	Pre-slaughter	123	0.0 (0.0-3.0)	-	-
Vic1	Weaning	177	8.5 (5.0-13.2)	$1.4 x10^4$-$1.4 x10^6$	$8.10 x10^4$
	Post-weaning	172	0.0 (0.0-1.4)	-	-
	Pre-slaughter	159	0.0 (0.0-1.6)	-	-
Vic2	Weaning	176	6.2 (3.4-10.6)	$3.6 x10^4$-$1.4 x10^6$	$5.92x10^4$
	Post-weaning	175	0.0 (0.0-1.4)	-	-
	Pre-slaughter	128	0.0 (0.0-1.9)	-	-
WA1	Weaning	123	0.8 (0.0-3.7)	-	$4.58x10^4$
	Post-weaning	122	0.0 (0.0-2.0)	-	-
	Pre-slaughter	121	0.0 (0.0-2.0)	-	-
WA2	Weaning	107	0.0 (0.0-2.3)	-	-
	Post-weaning	109	0.0 (0.0-2.3)	-	-
	Pre-slaughter	107	7.5 (3.6-13.6)	$3.4x10^4$-$15.6x10^5$	$8.94x10^4$
WA3	Weaning	101	2.0 (0.4-6.2)	$2.9 x10^4$-$4.5x10^4$	$3.71x10^4$
	Post-weaning	101	1.0 (0.1-4.5)	-	$8.95x10^4$
	Pre-slaughter	100	0.0 (0.0-2.5)	-	-
All farms	Total (n)	3343	5.7 (5.0-6.5)	$4.2 x10^3$-$7.2x10^7$	$8.55x10^4$
	Period prevalence	**1200**	**14.3 (12.4-16.4)**		

NSW: New South Wales SA: South Australia Vic: Victoria WA: Western Australia

* Adapted from Yang et al. (2016)

Table 2: Live weight, carcass weight, faecal consistency score and breech soiling score (mean ± standard error) for lambs (*n*=1200) on 8 Australian farms

	Sampling occasion			
	Weaning	**Post-weaning**	**Pre-slaughter**	**Slaughter**
Live weight (kg)				
Observed	31.30±0.15	39.85±0.21	47.66±0.22	-
Adjusted for site*	31.12±0.17	39.73±0.17	47.17±0.18	-
Carcass weight (kg)				
Observed	-	-	-	22.0±0.13
Adjusted for site*	-	-	-	21.62±0.08
Faecal consistency score[1]				
Observed	1.74±0.03	2.47±0.03	2.24±0.03	-
Adjusted for site*#	1.77±0.03	2.49±0.02	2.25±0.02	-
Breech soiling score[2]				
Observed	1.42±0.02	1.64±0.03	1.31±0.02	-
Adjusted for site*#	1.44±0.02	1.67±0.02	1.27±0.03	-

*least square mean for sampling occasion main effect determined using linear mixed effect model

residuals not normally distributed

[1] Faecal consistency score measured on scale of 1 (hard dry faecal pellet) to 5 (liquid diarrhoea). Distribution of data shown in Supplementary Material Appendix 1

[2] Breech faecal soiling score measured on scale of 1 (no evidence of breech faecal soiling) to 5 (extensive faecal soiling of the breech and hind limbs). Distribution of data shown in Supplementary Material Appendix 2

Table 3: Associations for pathogenic *Y. enterocolitica* detection (*yst* virulence gene by qPCR) with live weight and faecal consistency score (FCS) for lambs on 8 Australian farms, showing count (number of lambs per category), pathogenic *Y. enterocolitica* detection main effect (*F* and *P* value) and least square means (LSM ± standard error) for linear mixed effects models.

				Least square means ± standard error	
Sites included in model	**Sampling occasion**	**Detection category and main effect**	**Count** (*n*)	**Live weight** (kg) LSM ± SE	**FCS** LSM ± SE
All farms	Weaning	Detected	125	31.00 ± 0.40	n/a
		Not detected	1038	31.63 ± 0.15	n/a
	Post-weaning	Detected	57	36.22 ± 0.57^{A}	2.44 ± 0.02
		Not detected	1087	40.00 ± 0.15^{B}	2.50 ± 0.11
	Pre-slaughter	Detected	9	44.18 ± 1.24^{A}	1.57 ± 0.24^{A}
		Not detected	1027	46.79 ± 0.15^{B}	2.28 ± 0.02^{B}
		F value		*24.37*	*5.17*
		P value		*<0.001*	*0.023*
NSW only	Weaning	Detected	78	27.57 ± 0.58	n/a
		Not detected	78	27.49 ± 0.56	n/a
	Post-weaning	Detected	47	39.19 ± 0.72^{A}	1.89 ± 0.07
		Not detected	113	43.62 ± 0.49^{B}	1.78 ± 0.08
	Pre-slaughter	Detected	0	-	-
		Not detected	140	52.11 ± 0.43	1.13 ± 0.07
		F value		*13.99*	*0.55*
		P value		*<0.001*	*0.459*
Excluding NSW	Weaning	Detected	47	30.99 ± 0.52^{A}	n/a
		Not detected	960	32.18 ± 0.16^{B}	n/a
	Post-weaning	Detected	10	36.48 ± 1.09^{A}	2.39 ± 0.23
		Not detected	974	39.47 ± 0.16^{B}	2.55 ± 0.02
	Pre-slaughter	Detected	9	43.39 ± 1.16^{A}	1.73 ± 0.24^{A}
		Not detected	887	46.09 ± 0.16^{B}	2.44 ± 0.02^{B}
		F value		*16.71*	*5.43*
		P value		*<0.001*	*0.020*

FCS: faecal consistency score (continuous variable with 1 = hard dry faecal pellet and 5 = liquid diarrhoea)

^{AB} Values within sampling occasion and outcome (live weight or FCS) with different superscripts are significantly different (*P*<0.05).

Values within sampling occasion/outcome without superscripts are not different (*P*>0.05)

n/a Least square means not generated – insufficient faecal material available for 3 farms (including NSW) to conduct FCS

Table 4: Prevalence for non-pelleted faeces (faecal consistency score 3.0 or higher) and diarrhoea (faecal consistency score 3.0 or higher) in lambs with and without pathogenic *Y. enterocolitica* detected (*yst* virulence gene by qPCR) on 8 Australian farms, with z test (2-tailed) for significance

	Prevalence % (95% confidence interval)		
Farm	Pathogenic *Y. enterocolitica* detected	Pathogenic *Y. enterocolitica* not detected	*P*-value
Non-pelleted faeces (FCS ≥ 3.0)			
Overall (all farms)	30.4 (22.1, 39.8)	44.0 (42.2, 45.8)	0.006
NSW*	28.9 (17.3, 43.1)	12.9 (9.1, 17.7)	0.007
SA1*	100 (64.6, 100)	91.1 (87.5, 93.9)	0.409
SA2*	25.0 (7.6, 52.9)	44.3 (39.6, 49.0)	0.185
Vic1	26.7 (9.7, 51.7)	10.4 (7.9, 13.3)	0.046
Vic2	18.2 (4.0, 46.7)	55.1 (50.5, 59.6)	0.015
WA1	0 (0, 85.3)	38.4 (33.5, 43.4)	0.431
WA2	0 (0, 26.2)	37.8 (32.6, 43.2)	0.029
WA3	66.7 (17.7, 96.1)	71.2 (65.9, 76.1)	0.862
Diarrhoea (FCS ≥ 4.0)			
Overall (all farms)	4.9 (1.9, 10.4)	10.6 (9.5, 11.8)	0.064
NSW*	6.7 (1.9, 16.7)	3.9 (1.9, 7.0)	0.401
SA1*	0 (0, 29.2)	12.5 (9.1, 16.6)	0.318
SA2*	0 (0, 18.5)	4.5 (2.9, 7.0)	0.450
Vic1	13.3 (2.9, 36.3)	2.7 (1.5, 4.4)	0.016
Vic2	0 (0, 20.0)	35.4 (31.2, 39.9)	0.015
WA1	0 (0, 85.3)	6.0 (3.9, 8.8)	0.800
WA2	0, (0, 26.2)	7.9 (5.3, 11.3)	0.407
WA3	0 (0, 53.6)	5.4 (3.2, 8.3)	0.681

FCS: faecal consistency score (continuous variable with 1 = hard dry faecal pellet and 5 = liquid diarrhoea)

*No faecal consistency scores available at weaning sampling (insufficient material)

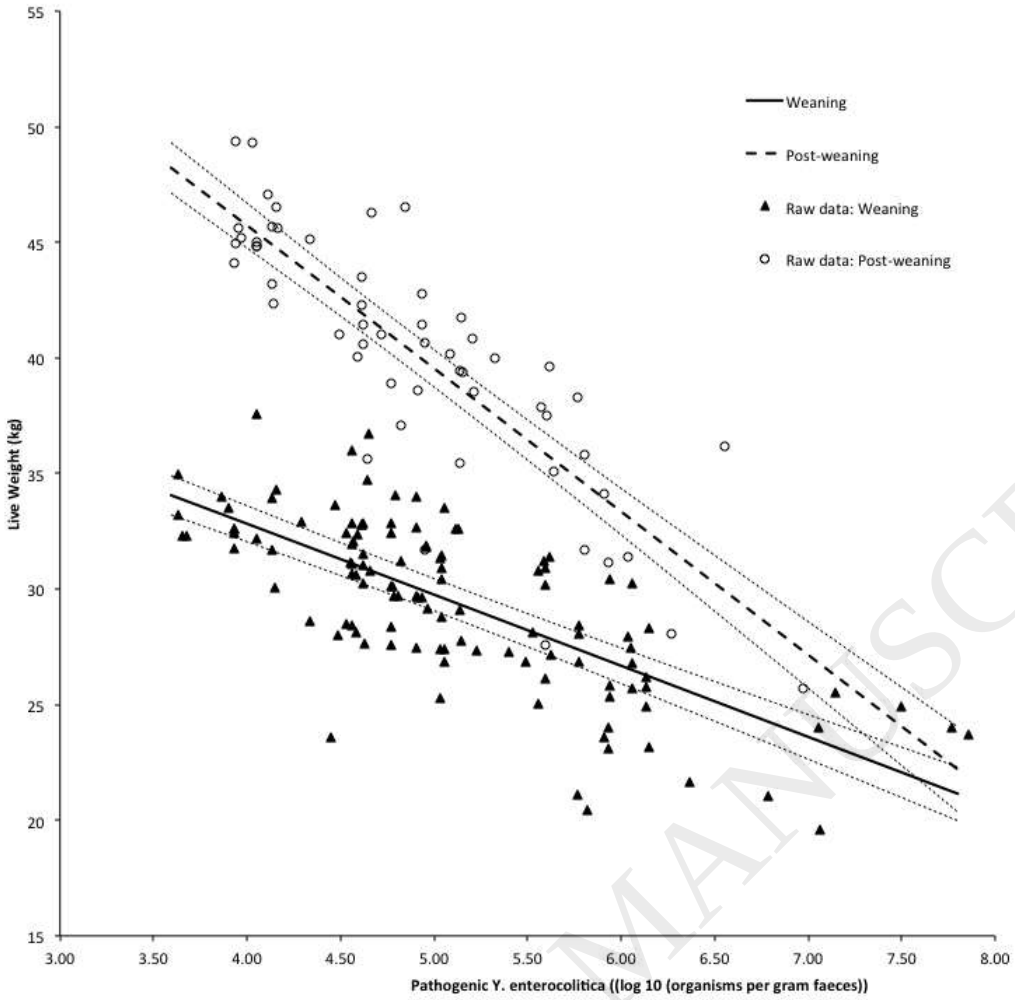

Figure 1: Relationship between intensity of pathogenic *Y. enterocolitica* faecal shedding and live weight (least square means) determined using linear mixed effect model for pathogenic *Y. enterocolitica*-positive faecal samples at weaning (*n*=38) and post-weaning (*n*=55) for lambs on eight Australian farms