Final report

Reducing foetal and lamb losses in young ewes

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Abstract

Improving reproductive performance for maiden ewes is a priority for the Australian sheep industry. This project has determined the extent, timing and causes of lamb mortality between scanning and marking in maiden ewes, including the contribution of losses between scanning and birth (abortion). Infectious diseases were the most common causes of abortion or stillbirth identified in investigations submitted to Australian veterinary laboratories. An on-farm study demonstrated that lamb loss between birth and marking was the largest source of loss after scanning. Mid-pregnancy abortion was an important source of loss on some farms with losses above expected levels (>2%) in approximately one in three ewe lamb flocks. These abortions occurred with no obvious signs of illness that would alert the farmer to the problem. *Chlamydia pecorum* was detected in aborted or stillborn lambs from ewe lamb flocks at multiple properties. Abortions associated with *Campylobacter* were detected on one farm confirming that campylobacteriosis causes losses on some farms in some years. Dystocia, stillbirth and starvation-mismothering were the most important causes of lamb death during or after birth. A monitoring protocol during pregnancy and lambing can be used to determine the timing of foetal or lamb loss and inform strategies to improve maiden ewe reproductive performance.

Executive summary

Background

Improving reproductive performance for maiden ewes lambing for the first time at either 12 months of age (ewe lambs) or 24 months of age (hogget) has been identified as a priority for the Australian sheep industry. It is not clear whether *in utero* losses (between scanning and lambing), poor lamb survival after birth, or a combination of these are responsible for the poorer and more variable reproductive performance for maiden ewes on Australian farms.

Objectives

This project addressed four research questions:

1) Is foetal loss a significant contributor to overall reproductive wastage from young ewes between scanning (day 42 pregnancy) and marking, and if so, when are losses occurring?
2) What is the background prevalence in young ewes for infectious diseases that have potential to cause abortion?
3) Is exposure to infectious disease associated with foetal loss in young ewes, and if so, how what is the relationship between the timing of exposure and outcome for pregnancy?
4) Can management strategies reduce the risk foetal loss between day 42 and lambing, and increase overall marking rates from young ewes?

Methodology

The research questions were addressed using three studies:

1) A survey of sheep producers to determine reproductive performance of maiden ewes relative to mature ewes on the same farm
2) A review of 529 veterinary investigations for abortion or stillbirth submitted to state veterinary laboratories
3) A longitudinal fieldwork study for 30 flocks of maiden ewes that included:
    a. Assessment of timing of foetal and lamb loss
    b. Lamb necropsies to determine cause of death for a subset of 11 flocks
    c. Laboratory investigation for infectious disease in aborted and stillborn lambs for a subset of 7 flocks
    d. Ewe serology to detect antibodies for *Toxoplasma gondii, Neospora caninum, Coxiella burnetii, Campylobacter fetus* fetus and *Campylobacter jejuni*

Results/key findings

Maiden ewe reproductive performance survey

Marking rates in maiden ewes was lower and more variable compared with their mature multiparous ewe counterparts on the same farm, and this was attributable to both lower reproductive rate (determined at scanning) and lower lamb survival (between scanning and marking). The difference in marking rate for non-Merino maiden ewe lambs compared to multiparous ewes was 58%, and this was attributable to 50% difference in reproductive rate and 16% difference in lamb survival. The difference in marking rate for maiden Merino hogget ewes compared to mature multiparous ewes was 22% and this was attributable to 24% difference in reproductive rate and 3% difference for lamb survival. Reproduction traits (reproductive rate, lamb survival and marking rate) were positively correlated between maiden Merino hogget and mature

ewes, but these correlations were weak or non-existent for non-Merino ewe lambs and mature ewe counterparts. Strategies targeting both reproductive rate and lamb survival can address poorer and more variable marking rates in maiden ewes.

Review of submissions to state veterinary laboratories

An aetiological diagnosis was made for 57% of abortion and stillbirth investigations submitted to veterinary laboratories in Western Australia, South Australia, Victoria and Tasmania between 2000 and 2018. Infectious diseases were implicated in 81% of investigations with a diagnosis with *Campylobacter* spp., *Listeria* spp. and *Toxoplasma gondii* the most common disease agents identified. Inclusion of placental tissue samples increased the likelihood that a diagnosis could be made.

Timing of foetal loss and lamb mortality in maiden ewes

Lamb mortality from birth to marking represented the greatest contributor to foetal and lamb mortality after scanning, but mid-pregnancy abortion was an important contributor to lamb mortality in some ewe lamb flocks. Mid-pregnancy abortion was detected in 5.7% of ewes (range 0–50%) in the ewe lamb flocks and 0.9% of ewes (range 0–4.4%) in the maiden Merino hogget flocks. Mid-pregnancy abortion affecting ≥2% of ewes was observed in 6/19 ewe lamb flocks and 2/11 Merino hogget flocks. Notably, a high frequency of mid-pregnancy abortion was detected using repeat scanning in ewe lamb flocks with no other obvious signs of illness that would trigger investigation. Liveweight and condition score at joining, and liveweight change from joining to Scan 1, had no effect on mid-pregnancy abortion nor overall lamb mortality between scanning and marking in flocks in this study.

Cause of death

Lamb necropsies (*n* = 298) identified starvation-mismothering-exposure (34%), dystocia (24%) and stillbirth (15%) as the most common causes of perinatal death for lambs born to maiden ewes. *Chlamydia pecorum* was detected in 15/35 aborted and stillborn lambs on 5/6 farms in WA. *Chlamydia pecorum* detected in aborted and stillborn lambs were genetically identical to ST23 which has also been associated with arthritis and conjunctivitis in sheep.

Toxoplasma, Neospora and Coxiella

There was no evidence that *T. gondii, N. caninum* or *C. burnetii* were important contributors to *in utero* foetal loss or perinatal lamb mortality observed in the 30 maiden ewe flocks included in this study.

Seropositivity to *T. gondii* was detected using indirect ELISA at 16/28 farms and 8.1% mature ewes (95% confidence interval (CI) 6.0, 10.5). However, seroconversion during pregnancy and lambing period was detected in only 1.0% (95% CI 0.5, 1.7) of ewes that failed to raise a lamb, and 0.6% (95% CI 0.1, 2.9) of ewes with confirmed abortion.

Seropositivity to *N. caninum* was detected using indirect ELISA at 2/28 farms, and for 0.2% (95% CI 0.0, 0.5) of maiden ewes and 0% (95% CI 0.0, 0.5) of mature ewes.

Seropositivity to *C. burnetii* was detected using indirect ELISA at 3/28 farms, and for 0.1% (95% CI 0.0, 0.4) of maiden ewes and 0.4% (95% CI 0.1, 1.1) of mature ewes. Despite low seropositivity to *C. burnetii* detected in this study, sporadic zoonotic transmission from sheep is reported in Australia and overseas so contact with sheep should still be considered a risk factor for Q-fever in humans and precautions taken to reduce the risk of zoonotic *C. burnetii* transmission.

Campylobacter

Seropositivity to *Campylobacter fetus* (titre ≥1:80) was detected for 12% (95% CI 9.6, 15.6) of maiden ewes and 31% (95% CI 25.0, 37.4) of mature ewes. The odds for failing to rear a lamb in *C. fetus*-'exposed' maiden ewes (titre ≥1:10) was 2 times that of seronegative ewes (95% CI 1.09, 3.77; *P* = 0.027), but there was no association between *C. fetus-'*positivity' (titre ≥1:80) and failure to raise a lamb. Abortions associated with *Campylobacter fetus* were detected on one farm confirming that *Campylobacter* cause*s* losses on some farms in some years.

*Campylobacter jejuni-'*positivity' (titre ≥1:80) was detected for 44% (204/462; 95% CI 39.7, 48.7) of maiden ewes, but odds of failing to rear were decreased for *C. jejuni*-'positive' ewes (OR 0.52; 95% CI 0.32, 0.83; *P* = 0.007).

The association between *Campylobacter* serology and reproductive outcome was inconsistent in the study flocks. This highlighted that serology should be considered in the context of other risk factors and used in conjunction with other strategies to investigate the impact of *Campylobacter* exposure on ewe reproductive performance such as monitoring for abortions, lamb necropsies to determine aetiological diagnosis and vaccination trials.

Benefits to industry

This study has provided industry data on the reproductive performance of maiden ewes and new insights into the contribution of *in utero* foetal loss and perinatal lamb mortality to lamb survival for maiden ewes. This will inform improved strategies to address poor reproductive performance in maiden ewes because lamb survival is an important component of maiden ewe reproductive performance, and existing strategies that target lamb survival in the perinatal period are unlikely to address mid- and late-pregnancy abortion.

This study has provided new insight of the role of infectious diseases on ewe reproductive performance, including an emerging role of *Chlamydia pecorum* as a cause of abortion and stillbirths in maiden ewes and especially ewe lambs.

An evidence-based framework for investigating poor reproductive performance to inform strategies to improve reproductive performance has been developed based on the findings from this study.

Future research and recommendations

This report includes recommendations for further research and development activities to address existing gaps to inform management strategies that will improve maiden ewe reproductive performance by reducing in utero foetal loss (abortion) and perinatal lamb mortality.

Chapter abstracts (peer reviewed)

Chapter 3: Survey of reproductive performance in maiden ewes

Suboptimal reproductive performance of maiden (primiparous) ewes remains a source of inefficiency for the Australian sheep industry. However, the extent and causes of the poorer reproductive performance of maiden ewes on Australian sheep farms are not well understood. Here, we show the reproductive performance of maiden ewes relative to their multiparous counterparts on the same farms across Australia using a cohort survey. The difference in marking rate for non-Merino maiden ewe lambs compared to multiparous ewes was 58% (74 vs. 132%; $p < 0.001$), and this was attributable to a 50% difference in reproductive rate (109 vs. 159%; $p < 0.001$) and 16% difference in lamb survival to marking (67 vs. 83%; $p < 0.001$). The difference in marking rate for maiden Merino hogget ewes lambing at approximately 2 years-of-age compared to mature multiparous ewes was 22% (80 vs. 102%; $p < 0.001$) and this was attributable to a 24% difference in reproductive rate (108 vs. 132%; $p < 0.001$) and 3% difference for lamb survival (75 vs. 78%; $p < 0.05$). Positive correlations for reproduction traits (reproductive rate, lamb survival and marking rate) between maidens and multiparous ewes were observed for maiden Merino hogget ewes ($p < 0.001$), but these correlations were weak or non-existent for non-Merino ewe lambs. Strategies to improve both reproductive rate and lamb survival can address the poorer and more variable reproductive performance of maiden ewes.

Chapter 4: Sheep abortion and stillbirth investigations in Australia

Fetal loss and lamb mortality between mid-pregnancy and weaning are important economic and welfare issues for the Australian sheep industry. The aim of this study was to determine common causes of ovine abortion and stillbirths based on sub- missions to veterinary laboratories and identify factors that impact the determination of an aetiological diagnosis. Data for 529 investigations on abortion or stillbirth between 2000 and 2018 were retrieved from four state veterinary laboratories in Western Australia, South Australia, Victoria and Tasmania. An aetiological diagnosis was made for 57% of investigations. Investi- gations that included placental tissue samples were more than twice as likely to have an aetiological diagnosis compared to investigations without placenta (P = 0.017, 95% confidence inter- val 1.1, 4.5). Of the investigations where an aetiological diagnosis was made, 81% involved infectious abortion, with Campylobacter spp. (32%), Listeria spp. (25%) and Toxoplasma gondii (9%) being the three most common abortigenic pathogens implicated. The remaining 19% of investigations with an aetiological diagnosis included a wide range of infectious and non-infectious diseases. Diagnoses made varied year to year and between states. No evi- dence of exotic abortigenic pathogens were reported. Veterinary practitioners can improve the probability of an aetiological diag- nosis by emphasising to farmers the importance of collecting any aborted material, especially placenta, and appropriate storage of the tissues until they can be submitted to the laboratory. Some diseases that cause abortion in Australian sheep have zoonotic potential, and veterinary practitioners play an important role in educating clients about appropriate hygiene when handling preg- nant and lambing ewes or any aborted material.

Chapter 5: Abortion and lamb mortality between pregnancy scanning and lamb marking for maiden ewes in southern Australia

The contribution of abortions to the overall mortality of lambs born to maiden (primiparous) ewes in Australia remains unclear. This cohort study aimed to quantify abortion and lamb mortality for ewe lambs and maiden Merino hogget ewes. Lamb mortality from pregnancy scanning to marking were determined for 19 ewe lamb and 11 Merino hogget ewe flocks across southern Australia. Average lamb mortality from scanning to marking was 35.8% (range 14.3–71.1%) for the ewe lambs and 29.4% (range 19.7–52.7%) for the hogget ewes. Mid-pregnancy abortion was detected in 5.7% of ewes (range 0–50%) in the ewe lamb flocks and 0.9% of ewes (range 0–4.4%) in the hogget ewe flocks. Mid-pregnancy abortion affecting ≥2% of ewes was observed in 6/19 ewe lamb flocks and 2/11 hogget ewe flocks. Lamb mortality from birth to marking represented the greatest contributor to foetal and lamb mortality after scanning, but mid-pregnancy abortion was an important contributor to lamb mortality in some ewe lamb flocks. Variability between the flocks indicates scope to improve the overall reproductive performance for maiden ewes by reducing foetal and lamb losses. Addressing mid-pregnancy abortion may improve the reproductive performance in some flocks.

Chapter 6: Causes of abortion and perinatal lamb deaths: necropsies and detection of infectious disease

Lamb survival is an important welfare and productivity issue for sheep industries worldwide. Lower lamb survival has been reported for primiparous (maiden) ewes, but the causes of this are not well studied. The aim of this study was to determine causes of perinatal deaths for lambs born to maiden ewes in Western Australia, and identify if infectious diseases are implicated. Lamb mortality from birth to marking were determined for 11 maiden ewe flocks on 10 farms in Western Australia. Lamb mortality from birth to marking averaged 14% for single-born and 26% for multiple-born lambs. Lamb necropsies (*n* = 298) identified starvation–mismothering–exposure (34%), dystocia (24%) and stillbirth (15%) as the most common causes of perinatal lamb death. There was no evidence of exotic abortigenic pathogens in aborted and stillborn lambs (*n* = 35). *Chlamydia pecorum* was detected by qPCR in 15/35 aborted and stillborn lambs on 5/6 farms. Preliminary molecular characterisation of *C. pecorum* detected in samples from aborted and stillborn lambs (*n* = 8) using both Multilocus Sequence Typing and *omp*A genotyping indicated all strains were genetically identical to previously described pathogenic livestock strains, denoted ST23, and dissimilar to gastroin- testinal strains. High frequency of detection of a pathogenic *C. pecorum* strains ST23 associated with ovine abortion and stillbirth on multiple farms located across a wide geographic area has not been previously reported. *Chlamydia pecorum* may contribute to reproductive wastage for maiden ewes in Western Australia. Further investigation to understand *C. pecorum* epidemiology and impact on sheep reproduction is warranted.

Chapter 7: *Toxoplasma gondii* and reproductive performance of maiden ewes

Background: *Toxoplasma gondii* causes reproductive losses in sheep worldwide, including Australia. The reproductive performance of primiparous (maiden) ewes is typically lower than for mature, multiparous ewes, and younger ewes are more likely to be immunologically naïve and therefore more susceptible to reproductive disease if *T. gondii* infection occurs during pregnancy. The aim of this study was to assess the impact of infection with *T. gondii* on the reproductive performance of maiden ewes in southern Australia using a prospective cohort study. This will inform the need for targeted control strategies for *T. gondii* in Australian sheep.

Results: *Toxoplasma gondii* seropositivity using indirect ELISA was detected at 16/28 farms located across southern Australia. Apparent seropositivity to *T. gondii* was lower in maiden ewes (1.1%, 95% confidence interval (CI) 0.6, 1.8) compared to mature, multiparous ewes (8.1%, 95% CI 6.0, 10.5; $P < 0.001$). *Toxoplasma gondii* seroconversion during the gestation and lambing period was confirmed for 11/1097 (1.0%, 95% CI 0.5, 1.7) of pregnant maiden ewes that failed to raise a lamb, and 1/161 (0.6%, 95% CI 0.1, 2.9) maidens ewes with confirmed abortion.

Conclusions: Low frequency of detection of *T. gondii* seroconversion during gestation and low frequency of seropositivity to *T. gondii* suggests that toxoplasmosis was not an important contributor to reproductive losses in maiden ewes on farms located over a wide geographical area in southern Australia.

Chapter 8: *Coxiella burnetti* and reproductive performance of maiden ewes

The role of infectious diseases including coxiellosis in causing poorer reproductive performance of primiparous (maiden) ewes are not well studied. The aims of this study were to determine if natural exposure to *Coxiella burnetii* is widespread in breeding ewes and whether seropositivity is associated with poor reproductive performance of maiden ewes. Seropositivity to *Coxiella burnetii* was 0.08% (95% CI 0.01, 0.36) in maiden ewes and 0.36% (95% CI 0.07, 1.14) in mature ewes. *Coxiella burnetii* was not detected in aborted or stillborn lambs using qPCR. These findings suggest *C. burnetii* infection was unlikely to be an important contributor to abortion and perinatal mortalities observed for maiden ewe flocks, and exposure to *C. burnetii* was not widespread in ewes on farms located over wide geographical region of southern Australia. Whilst ewes on these farms were not an important reservoir for *C. burnetii*, sporadic zoonotic transmission from sheep is reported and has public health implications.

Chapter 9: *Neospora caninum* and reproductive performance of maiden ewes

Neospora caninum has been implicated as a sporadic cause of abortion and perinatal deaths in sheep flocks globally. However, its significance as a reproductive pathogen for sheep in Australia remains unknown. The aims of this study were to (i) determine the seroprevalence of *N. caninum* in Australian breeding ewes and (ii) examine if natural exposure to *N. caninum* is associated with poor reproductive performance of primiparous (maiden) ewes in southern Australia. Thirty flocks of maiden ewes (aged 1–2 years old at lambing) from 28 farms in three states (Western Australia, South Australia and Victoria) were monitored between joining and lamb marking. Blood samples were also collected from multiparous mature ewes (aged 3 years or older) at each farm. Seroprevalence for anti-*N. caninum* IgG using indirect ELISA was determined for a subset of maiden ewes that were predominantly determined to be pregnant and subsequently failed to rear a lamb (*n* = 1279) and randomly selected mature multiparous ewes with unknown reproductive status (*n* = 558). *Neopsora caninum* apparent seroprevalence was 0.16% (95% confidence interval 0.03%, 0.5%) in maiden ewes, with seropositivity identified in two ewes from farms located in South Australia and Victoria. There was no evidence of seropositivity in mature ewes with apparent seroprevalence 0% (0%, 0.45%). These findings suggest that *N. caninum* infection was not widespread in maiden ewes or mature multiparous ewes on these farms, and exposure to *N. caninum* infection was unlikely to explain abortion and perinatal mortalities observed for maiden ewes.

Chapter 10: *Campylobacte*r spp. and reproductive performance of maiden ewes

This case-control study investigated associations between *Campylobacter fetus* or *Campylobacter jejuni* titre and reproductive outcomes in 22 flocks of Merino and non-Merino maiden ewes aged 1–2 years old. *Campylobacter* titres were also determined for multiparous ewes aged 3 years or older on the same farms. *Campylobacter fetus* 'positivity' (titre ≥1:80) was detected for 12% (57/462; 95% confidence interval (95%CI) 9.6, 15.6) of maiden ewes and 31% (65/210; 95%CI 25.0, 37.4) of mature ewes. The odds for failing to rear a lamb in *C. fetus*-'exposed' maiden ewes (titre ≥1:10) was 2.01 times that of seronegative ewes (95%CI 1.09, 3.77; *P* = 0.027), but there was no association between *C. fetus*-'positivity' (titre ≥1:80) and failure to raise (OR 1.69; 95%CI 0.77, 3.76; *P* = 0.191). *Campylobacter fetus* abortions were confirmed with microbial culture in one maiden ewe flock. In this flock, *C. fetus* titres fluctuated and often waned by lamb marking, highlighting the value of necropsies during abortion investigations. *Campylobacter jejuni*-'positivity' (titre ≥1:80) was detected for 44% (204/462; 95% CI 39.7, 48.7) maiden ewes, but odds of failing to rear were decreased for *C. jejuni*-'positive'ewes (OR 0.52; 95%CI 0.32, 0.83; *P* = 0.007). The association between *Campylobacter* serology and reproductive outcome was inconsistent in these flocks. Serology should be considered in the context of other risk factors, and used in conjunction with other strategies to investigate the impact of *Campylobacter* exposure on ewe reproductive performance such as monitoring for abortions, lamb necropsies to determine aetiological diagnosis, and vaccination trials.

Abbreviations

BLAST	basic local alignment search tool
CI	confidence interval
CrI	credible interval
CS	condition score
DM	dry matter
ELISA	enzyme-linked immunosorbent assay
FOO	Feed-on-offer
H&E	haematoxylin and eosin
Ha	hectare
IHC	immunohistochemistry
LW	live weight
MLST	multilocus sequence typing
NA	not available
NATA	National Association of Testing Authorities
NSW	New South Wales
OD	optical density
OR	odds ratio
ompA	outer membrane protein A
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
SA	South Australia
SE	standard error
SME	starvation-mismothering-exposure complex
S/P	sample/positive value
ST	sequence type
TAS	Tasmania
VIC	Victoria
WA	Western Australia

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Chapter 3

Co-authors for this chapter include Bronwyn Clarke (Murdoch University), Dayna Hutchison (honours student, Murdoch University), and Elise Bowen (higher degree candidate, Murdoch University).

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Chapter 4

Co-authors for this chapter include Shane Besier (Department of Primary Industries & Regional Development, Western Australia), Graeme Knowles (Department of Primary Industries, Parks, Water and Environment, Tasmania), Roger Paskin (OMNI Animal Health Consultancy, South Australia), Grant Rawlin (Agriculture Victoria, Department of Jobs, Precincts and Regions) and Robert Suter (Agriculture Victoria, Department of Jobs, Precincts and Regions).

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Chapter 6

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Chapter 7

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Chapter 8

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Chapter 9

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Chapter 10

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Molecular diagnostic testing for aborted and stillborn lambs was performed under the Western Australian Ewe Abortion and Newborn Lamb Death Surveillance Program (Department of Primary Industries and Regional development, Western Australia).

Table of contents

1. Background

Improving reproductive performance for primiparous (maiden) ewes lambing for the first time at either 12 months of age (ewe lambs) or 24 months of age (hogget) has been identified as a priority for the Australian sheep industry (Young et al., 2014). Breeding (joining) ewe lambs can increase farm profitability and lamb supply (Young et al 2010; Young et al 2012). However, poor and variable reproductive success of ewes lambs is a barrier to adoption (Kenyon et al., 2014b). Similarly, reproductive success of maiden hogget ewes is typically poorer compared to mature multiparous ewes and whilst the 'gap' in reproductive performance relative to mature ewes across different genotypes and production systems is not well quantified, weaning rates of 50-60% are not uncommon in some genotypes and environments. Current management recommendations for maiden hoggets are based on limited evidence, are generic across genotypes and fail to consider the full impacts of better management during their first two years of life. It is important to understand the causes for poorer and more variable reproductive performance for maiden ewes to inform strategies to improve reproductive success. This is turn can address barriers to adoption and increase the number of ewe lambs joined.

Reproductive performance is commonly assessed using marking rate. This refers to the number of lambs marked as a proportion of the number of ewes mated (joined). Marking rate is a function of reproductive rate (the number of foetuses identified at pregnancy scanning) and losses between scanning and lamb marking. Factors that impact reproductive rate in ewe lambs include genotype, age at joining, liveweight at joining and response to teasers. Survival of the foetus/lamb between scanning and marking is impacted by *in utero* mortality (mid- and late-pregnancy abortion), mortality in the perinatal period (during or soon after birth) or in the neonatal period up until lamb marking. Australian studies have shown that lamb mortality in the perinatal period are the main source of loss between scanning and marking (Hinch and Brien, 2014b, Refshauge et al., 2016). However, most studies reporting timing of foetal/lamb mortality were conducted predominantly in mature or mixed age ewes. Consequently, it is not clear whether the *in utero* foetal losses (between scanning and lambing) are an important contributor to poorer and more variable reproductive performance for maiden ewes on Australian farms. Improved understanding of the role of *in utero* losses is important for informing strategies to improve reproductive performance because tactics targeting perinatal losses may not adequately address *in utero* losses occurring due to mid- and late-pregnancy abortion.

There is perception amongst Australian sheep producers that ewe lambs have greater *in utero* foetal losses compared to mature ewes, with this presenting as maiden ewes that are scanned pregnant but subsequently fail to lamb. Overseas studies have shown that *in utero* foetal losses may be an important contributor to the reproductive inefficiencies of maiden ewe lambs (Howe et al., 2008, Lafi et al., 2009, Ridler, 2015, West et al., 2006, Kenyon et al., 2014b). However, the timing of foetal and lamb mortality and relative importance of *in utero* and perinatal mortality for ewe lambs on Australian farms is not well studied, and even less is known about the contribution of *in utero* foetal losses to reproductive performance for maiden ewe hoggets.

If *in utero* foetal losses due to mid- and late-pregnancy abortion are an important contributor to reproductive performance in maiden ewes, then understanding the causes (aetiology) is important to inform strategies to improve reproductive performance. There are several disease agents (bacteria, viruses, parasites) that are endemic in Australian sheep and may cause foetal loss or birth of lambs that have poor viability. There is limited data on the distribution of a number of these infectious diseases, and describing the role of infection on reproductive outcome is often complicated because the disease agents or antibodies produced in response to infection may also be

detected in normal, healthy ewes with successful pregnancies. There are also gaps in understanding how these infections interact with other factors (such as nutrition, environmental stress or a concurrent infection) to impact pregnancy outcomes.

The studies outlined in this report seek to determine the extent, timing and causes of *in utero* foetal loss in maiden ewes, and the contribution of *in utero* foetal loss to reproductive performance of maiden ewes on Australian farms.

2. Objectives

This project aimed to address four research questions:

1) Is foetal loss is a significant contributor to overall reproductive wastage from young ewes between scanning (day 42 pregnancy) and marking, and if so, when are losses occurring?
2) What is the background prevalence in young ewes for infectious diseases that have potential to cause abortion?
3) Is exposure to infectious disease associated with foetal loss in young ewes, and if so, how what is the relationship between the timing of exposure and outcome for pregnancy?
4) Can management strategies reduce the risk foetal loss between day 42 and lambing, and increase overall marking rates from young ewes?

These objectives have been addressed through a series of studies described in this report (Table 1). The studies described in Chapters 3-10 have undergone peer-review and accepted for publication in international scientific journals.

Table 1: Delivery on key research questions and project objectives

Objective	Report reference	Description of report contents	Scientific journal DOI
1	Chapter 3	Survey quantifying reproductive wastage from young ewes between scanning (day 42 pregnancy) and marking in maiden ewes	https://doi.org/10.3390/ani12040513
	Chapter 5	Quantification of *in utero* and perinatal lamb mortality between scanning and marking in 30 maiden ewe flocks	https://doi.org/10.3390/ani12010010
2	Chapter 4	Evaluation of 529 investigations for abortion or stillbirth submitted to state veterinary laboratories 2000 - 2018	https://doi.org/10.1111/avj.13040
	Chapter 7	Determination of seropositivity to *Toxoplasma gondii* in 30 flocks of maiden ewes and mature ewes on 28 farms	https://doi.org/10.1186/s12917-022-03211-w
	Chapter 8	Determination of seropositivity to *Neospora caninum* in 30 flocks of maiden ewes and mature ewes on 28 farms	https://doi.org/10.1007/s00436-021-07328-z
	Chapter 9	Determination of seropositivity to *Coxiella burnetii* (Q fever) in 30 flocks of maiden ewes and mature ewes on 28 farms	https://doi.org/10.1016/j.cimid.2021.101727
	Chapter 10	Determination of seropositivity to *Campylobacter fetus* and *Campylobacter jejuni* in maiden and mature ewes on 22 farms	https://doi.org/10.1111/avj.13173
3	Chapter 6	Detection of infectious disease in aborted and stillborn lambs from 7 maiden ewe flocks	https://doi.org/10.1186/s13567-021-00950-w
	Chapter 7	Investigation of the association between seropositivity to *Toxoplasma gondii* and foetal/lamb mortality in 30 flocks of maiden ewes	https://doi.org/10.1186/s12917-022-03211-w
	Chapter 8	Investigation of the association between seropositivity to *Neospora caninum* and foetal/lamb mortality in 30 flocks of maiden ewes	https://doi.org/10.1007/s00436-021-07328-z
	Chapter 9	Investigation of the association between seropositivity to *Coxiella burnetii* (Q fever) and foetal/lamb mortality in 30 flocks of maiden ewes	https://doi.org/10.1016/j.cimid.2021.101727
	Chapter 10	Investigation of the association between seropositivity to *Campylobacter fetus* or *Campylobacter jejuni* and foetal/lamb mortality in 22 flocks of maiden ewes	https://doi.org/10.1111/avj.13173
4	Chapter 11	Recommendations for investigating poor reproductive performance in maiden ewes to inform mitigation strategies	-

3. Survey of reproductive performance of maiden ewes

Co-authors contributing to this study:

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This section has undergone peer-review and published (open access) in the journal *Animals*:

Hutchison D, Clarke BE, Hancock S, Thompson AN, Bowen E and Jacobson C (2022). Lower reproductive rate and lamb survival contribute to lower marking rate in maiden ewes compared to multiparous ewes. *Animals*, 12(4): 513

DOI: https://doi.org/10.3390/ani1204051

3.1 Introduction

Improving the reproductive performance of primiparous (maiden) ewes lambing for the first time at either approximately 12 months of age (ewe lambs) or 24 months of age (hogget ewes) has been identified as a priority for the Australian sheep industry (Young et al., 2014). It is widely accepted that the reproductive performance of maiden ewes is generally poorer and more variable compared to multiparous ewes. However, the reproductive performance of maiden ewes on commercial farms in Australia has not been well quantified. Marking rate, which describes the number of lambs marked (tailed and/or tagged) relative to the number of ewes joined (mated) to rams, has increased in Australia by about 15% over the last 30 years (Australian Bureau of Agricultural and Resource Economics, 2022, Trompf et al., 2018). This has been attributed to changes in flock structure and greater adoption of husbandry practices, including differential management of single- and multiple-bearing ewes to optimise the condition score (Trompf et al., 2011, Thompson et al., 2020). However, industry data for marking rates are based predominantly on mature multiparous and mixed-age ewes and do not differentiate between maidens and multiparous ewes (Australian Bureau of Agricultural and Resource Economics, 2022, Trompf et al., 2018). Improved understanding of the reproductive performance of maiden ewes can inform benchmarks for reproduction and strategies aimed at improving whole-flock reproductive performance. Apart from the economic benefits from improved reproductive performance, improving ewe and lamb survival can improve animal welfare and address societal demands for ethical livestock production (Ferguson et al., 2014).

Marking rate is a function of reproductive rate or the number of foetuses identified at pregnancy scanning during mid-pregnancy and foetal and lamb survival between scanning and lamb marking. A range of factors is known to contribute to lower marking rates in ewe lambs compared to multiparous ewes, including lower fertility and ovulation rates, higher embryo loss between conception and scanning and lower foetus and lamb survival between scanning and marking (reviewed by Kenyon et al. (2014b)). There has been less work on the performance of ewe lambs in Australia compared to New Zealand. Thompson et al. (Thompson et al., 2021) and Clune et al (Chapter 5) recently reported considerable variation in reproductive rate and or marking rates between different flocks of ewe lambs. In these studies, the average reproductive rate was 108%, and foetal and lamb losses between scanning and marking exceeded 35%. Lamb mortality in the

perinatal period was the primary cause of lamb mortality after scanning, although abortions were an important contributor in some flocks (Chapter 5). However, neither study compared the performance of ewe lambs and multiparous ewes or focussed on the relative contributions of reproductive rate and foetus and lamb survival to the poorer and more variable marking rates for ewe lambs.

Surprisingly, even less is known about the reproductive rate and lamb survival for maiden hogget ewes compared to multiparous ewes. A large study on 43 farms over 4 years in South Australia found that reproductive rate was approximately 13% lower for maiden hogget ewes, and survival of both single and twin lambs was approximately 10% lower compared to multiparous ewes on the same farm (Kleemann and Walker, 2005). Other work has reported no significant differences in lamb survival between maiden hogget ewes and multiparous ewes across eight flocks around Australia over five years (Paganoni et al., 2014b). Lockwood et al. (2020) reported no differences in lamb survival between maiden hogget ewes and multiparous ewes from survey respondents in south-eastern Australia, whereas, in New Zealand, survival was reported to be 6% lower for maiden hogget ewes than mature multiparous ewes. Whilst differences in lamb survival between maiden hogget ewes and mature multiparous ewes may be less than observed between maiden ewe lambs and multiparous ewes, even small differences impact national marking rates due to the large number of maiden hogget ewes joined annually.

This study aimed to determine the difference in reproductive performance between maiden and multiparous ewes across major sheep producing regions of Australia to inform strategies to improve reproductive performance in maiden ewes. We hypothesised that (i) maiden ewes joined either as ewe lambs or hogget ewes will have lower marking rates than multiparous ewes, and (ii) this will be due to a combination of lower reproductive rate and lower lamb survival between scanning and marking.

3.2 Methodology

3.2.1 Study design and setting

This study surveyed sheep producers from Western Australia, South Australia, Victoria, New South Wales and Tasmania. Sheep producers were recruited for the survey between 2019 and 2021 and completed a questionnaire focused on reproductive performance for ewes that lambed between 2018 and 2020. This included data recorded at pregnancy scanning (typically conducted 70–90 days from the start of joining period) and lamb marking (tail docking).

3.2.2 Study participants

Sheep producers were contacted by the project team via phone or e-mail. Contact details for producers were obtained through their participation in other sheep reproduction studies, or from commercial providers of sheep pregnancy scanning and livestock production advisors. Livestock production advisors and pregnancy scanning providers also contacted clients on behalf of the project team to confirm interest and eligibility for participation before providing contact details to the project team. The survey was also promoted to sheep producers using social media and in newsletters by sheep breeders' societies, sheep production extension organisations, grower groups and livestock production advisors.

Participants were provided with a cover letter outlining the aims of the project, requirements for eligibility to participate and information relevant for providing informed consent to participate. Respondents were selected for inclusion in the survey on the basis that: (a) they separately managed maiden ewes mated as ewe lambs (7–10 months at start of joining period) or hogget ewes (16–22 months at start of joining period), (b) utilised pregnancy scanning by transabdominal ultrasonography to determine the number of foetuses for maiden and multiparous ewes and (c) were able to determine lamb survival to marking for maiden and multiparous ewes on the same property, which generally required managing maiden ewes separately from multiparous ewes during lambing. Very few responses were received for Merino ewe lambs and non-Merino maiden hogget ewes during the first 12 months of the study, so they were subsequently excluded from the survey, and only non-Merino ewe lambs and Merino hogget ewes were targeted thereafter.

The most common reasons for exclusion from study were: (a) the number of foetuses was not determined for some or all of the maiden or multiparous ewes, and/or (b) maiden ewes were mixed with multiparous ewes during lambing and thus lamb survival to marking could not be determined separately for maiden and multiparous ewes.

3.2.3 Questionnaire and measurements

The questionnaire could be completed by the producer either as a hard copy or electronically, or was completed by the research team via a telephone interview with the producer. Follow-up contact with producers was made via telephone, text message or e-mail, where only partial data had been returned. Pre-testing of the questionnaire was done initially with research staff at Murdoch University and then with farmers participating in other sheep production projects to ensure that the questionnaire layout and questions were clear and unambiguous. Modifications to the question design were made in response to feedback during the pre-testing phase. Further refinements to questions were made throughout the study in response to feedback from respondents and following preliminary analysis of the data that informed the statistical methods.

The questionnaire included four questions about general farm details, including location, farm size and the number of Merino and non-Merino ewes on the farm. The remainder of the questionnaire was divided into three sections that included reproductive data for (a) maiden ewes at scanning, (b) maiden ewes at lamb marking and (c) multiparous ewes at scanning and lamb marking. Sheep data were collected for each mob of maiden ewes and for the total population of multiparous ewes for each farm. Data collected for maiden ewes at pregnancy scanning included the number of ewes mated, ewe age, ewe breed, breed of ram used, month of joining period, body condition score at joining, number of ewes scanned, number of foetuses identified at scanning and number of non-pregnant (dry) ewes. Maiden ewe data collected at lamb marking included mob or paddock name, number of ewes, pregnancy status (single, twin, triplet or mixed), month lambing commenced, condition score at lambing, number of lambs marked and number of ewe deaths (if known). Data for multiparous ewes included the number of ewes, ewe breed, breed of rams used, number of ewes scanned, number of foetuses identified at scanning, number of non-pregnant ewes, number of lambs marked and number of ewe deaths (if known).

Respondents had the option of providing additional data outlining the management of maiden ewes, including the length of the joining period, average condition score at joining and lambing, predominant pasture types and feed-on-offer and supplementary feeding strategies. Condition score was reported on a scale of 1 (very thin) to 5 (very fat), as previously described (Kenyon et al., 2014a). For feed-on-offer and condition score, there was a question asking if respondents had measured or

estimated these values and whether they had previously received training in performing these measurements.

Validity of the sample size for respondents was assessed using an assumption of 10% expected difference in mean for reproductive parameters (traits), expected standard deviation of 20 and power of test 80. Based on this, 90% confidence intervals could be determined with 36 responses and 95% confidence intervals determined with 49 responses.

3.2.4 Quantitative variables

Reproductive rate (%) for maiden and multiparous ewes were determined based on the number of foetuses identified at pregnancy scanning expressed relative to the number of ewes scanned. Lamb survival (%) was determined based on the number of live lambs present at lamb marking expressed relative to the number of foetuses identified at scanning. Marking rate (%) was determined based on the number of live lambs present at marking expressed relative to the number of ewes joined. Ewe mortality (%) was determined based on the number of ewe deaths between pregnancy scanning and lamb marking expressed relative to the number of ewes that were pregnant at scanning (i.e., excluded ewes not pregnant at scanning).

3.2.5 Statistical analysis

Farm location was coded into categories based on the Australian Bureau of Agricultural and Resource Economics (ABARE) region that are based on agricultural profile (Australian Bureau of Agricultural and Resource Economics, 2022). Statistical analyses were performed using GENSTAT (VSN International 2017) and IBM SPSS Statistics (version 24) (IBM 2021). Reproductive traits (reproductive rate, lamb survival and marking rate) for maiden and multiparous ewes were compared initially using a paired sample *t*-test (two-tailed), including a 95% confidence interval of the difference. The assumption of normality of the difference between maiden and multiparous ewes for reproductive traits were tested using a Shapiro–Wilk test. This indicated that the assumption of normality for the difference was not met for reproductive rate and ewe mortality *($p <$* 0.001). Subsequently, comparisons of reproductive traits between maiden ewes and multiparous counterparts on the same farm were compared using a two-tailed non-parametric related-samples Wilcoxon signed-rank test. Correlations between reproductive traits for maiden and multiparous ewes on the same farm were determined using bivariate Pearson correlation (two-tailed) and linear regression.

Association between farm, sheep and management factors with reproductive performance traits were analysed with linear mixed-effects models at farm level and mob/paddock level. Models were constructed separately for each of the three reproductive traits (marking rate, reproductive rate and lamb survival) for two maiden ewe age categories (maiden ewe lambs and hogget ewes) and two levels (pooled farm data and mob/paddock data), equating to 12 separate models. Ewe management during lambing (separate or combined management of maiden and multiparous ewes), ewe age, ewe breed, ram breed, month of joining, condition score at joining, feed-on-offer at joining, pasture type at joining, duration of supplementary feeding between joining and marking (at day 0–50, 50–100, 100–150 and lactation), supplementary feeding method (trail feeding or self-feeder), type of supplementary feed, condition score at lambing, average feed-on-offer and management category at lambing (mixed or separate management of single- and multiple-bearing ewes) were fitted as fixed effects. Mob pregnancy status (mob scanned as single, twin, triplet or mixed litter size) was fitted as a fixed effect at mob level. Year of lambing and farm were fitted as

random effects. ABARE region was fitted as a random effect but was not significant and was subsequently removed from the final models. For all analyses, main effects and interactions were only included if they were statistically significant ($p < 0.05$).

3.3 Results

3.3.1 Characteristics of survey participants

A total of 79 respondents provided complete data for maiden and multiparous ewes and were eligible for inclusion in the study. Of these, 16 producers contributed data for two years, and three producers contributed data for three years to give a total of 103 survey responses that represented 111,117 maiden ewes managed in 307 mobs from lambing to marking (Table 1). A total of 302,585 multiparous ewes were included in eligible survey responses (Table 2).

Respondents were located across five Australian states (Table 2). The mean farm size was 3750 hectares (range: 230–115,000 hectares), and the mean number of breeding ewes per farm was 4762 (range: 477–25,000 ewes).

Table 2. Distribution of eligible survey responses by state based on number of respondents (farms) and total number of maiden and multiparous ewes.

			Ewes (*n*)				
	Respondents (*n*)		**Maiden**		**Multiparous**		
State	Non-Merino ewe lambs	Merino hogget ewes	Non-Merino ewe lambs	Merino hogget ewes	Non-Merino	Merino	**Total**
WA	3	11	2945	14,904	9580	37,305	**64,734**
SA	8	4	8974	4645	27,338	19,520	**60,477**
NSW	8	13	15,712	8818	38,216	29,226	**91,972**
VIC	19	11	34,387	13,728	80,448	43,931	**172,494**
TAS	2	0	7004	0	17,021	0	**24,025**
Total	**40**	**39**	**69,022**	**42,095**	**172,603**	**129,982**	**413,702**

Characteristics for the management of sheep are shown in Table 3. Maiden Merino ewes were joined to Merino rams as hogget ewes to lamb at approximately two years-of-age with mean recorded age at joining of 18.5 months. The non-Merino ewe lambs included a range of different breeds, such as composite, Suffolk and Dorper. These were joined with maternal or terminal non-Merino rams to lamb at approximately one year of age with a mean age at joining of 8 months. Differential management during lambing based on ewe litter size was used by 47% of respondents (66% ewes) for maiden ewe lambs and 57% of respondents (61% ewes) for maiden Merino hogget ewes (Table 3).

Table 3. Maiden ewe management characteristics based on eligible survey responses showing number of responses and number of ewes managed by respondents.

	Ewe lambs		Merino hogget ewes	
	Responses (*n*)	Ewes (*n*)	Responses (*n*)	Ewes (*n*)
Age at joining (months)				
6	1	2545		
7	21	33,301		
8	25	28,379		
9	11	5003		
10	1	1361		
16			1	460
17			3	492
18			28	29,066
19			5	4898
20			3	2939
≥21			4	4213
Month of joining				
November			5	1770
December			7	4898
January	1	245	9	12,123
February	9	6188	11	8703
March	39	43,871	9	10,770
April	9	18,924	2	3614
May	1	1361	-	-
June			1	190
Management during lambing				
Mixed	30	23,574	19	16,575
Differential management [A]	27	46,605	25	25,493
Rams used for joining				
Maternal	35	37,944		
Terminal	22	32,235		
Merino			44	42,068
Supplementary feeding during lambing				
Trail feeding	0	0	6	5656
Self-feeder	2	2753	3	1861
No supplementary feeding	30	50,027	17	16,576
Not indicated	25	17,399	17	17,289

[A] Ewes scanned with single and multiple fetuses were managed separately during lambing.

The mean length of the joining period for maiden ewes was 39 days (Table 4). The condition score prior to lambing was reported by 75 respondents (Table 3), of which 31 (41%) were based on direct measurement, 35 (47%) were based on estimation and 9 (12%) did not specify the method used. The mean reported condition score prior to lambing for all maiden ewes was 3.1 (Table 4). Feed-on-offer during lambing was reported by only eight respondents, of which three were based on measurement, four were estimated and one did not specify the method used. Eighty-six per cent (*n* = 68) of respondents reported having been trained in the assessment of feed-on-offer and condition scoring.

Table 4. Characteristics for management of maiden ewes based on eligible survey responses.

	Ewe lamb flocks			Merino hogget flocks		
	n	**Mean ± s.e**	**Range**	***n***	**Mean ± s.e**	**Range**
Joining period length (days)	43	40 ± 1	28–60	36	38 ± 1	28–56
Condition score						
Joining	47	3.15 ± 0.04	2.5–4.0	30	2.90 ± 0.06	2.0–3.7
Lambing	47	3.19 ± 0.05	2.7–4.0	28	3.02 ± 0.07	2.5–4.0
Feed-on-offer during lambing (kg DM/Ha)	6	1700 ± 93	1500–2000	2	750 ± 250	500–1000

s.e: standard error
DM/Ha: dry matter per hectare.

3.3.2 Reproductive performance in maiden and multiparous ewes

Marking rate, reproductive rate and lamb survival for maiden and multiparous ewes are shown in

Maiden non-Merino ewe lambs Multiparous non-Merino ewes

Maiden Merino hogget ewes Multiparous Merino ewes.

Figure 1. A key difference between maiden ewe lambs and maiden Merino hogget ewes and both multiparous ewes was the wider variation in lamb survival and, to a lesser extent, reproductive rate and marking rate between flocks.

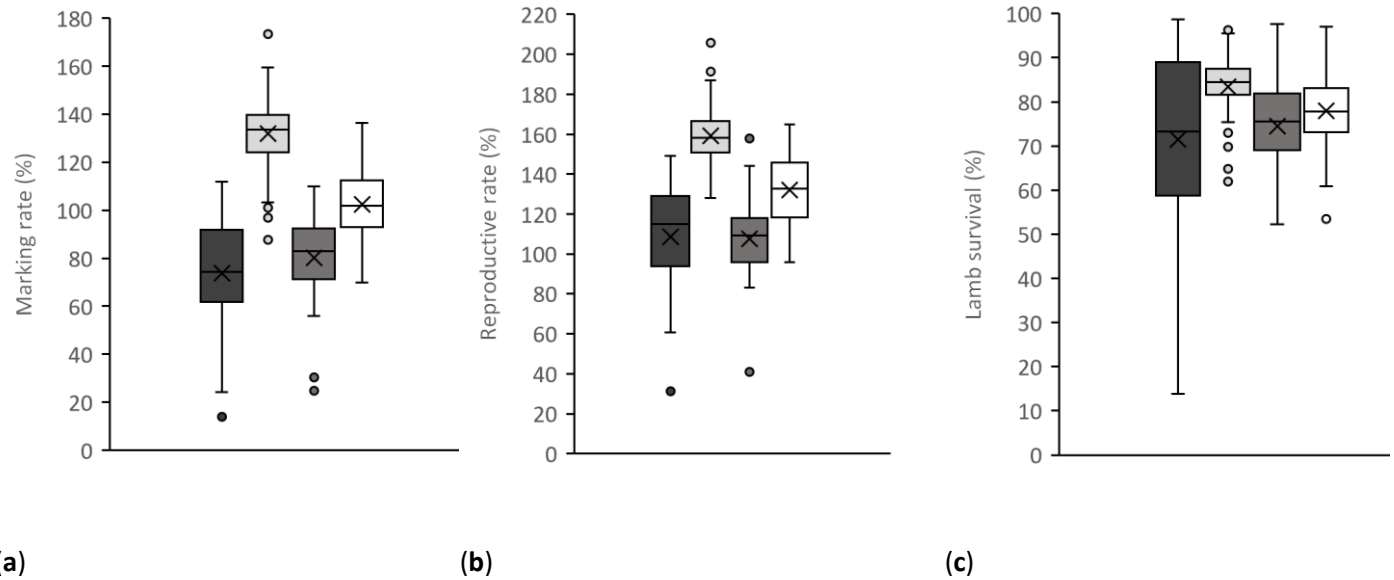

Figure 1. Box and whisker plot for (**a**) marking rate, (**b**) reproductive rate and (**c**) lamb survival in maiden ewe lambs, Merino hogget ewes and equivalent multiparous ewes.

Maiden ewes had a lower marking rate, reproductive rate and lamb survival compared to multiparous ewes on the same farm (Table 5). The average difference in marking rate between maiden and multiparous ewes was 58% for non-Merino ewe lambs and 22% for maiden Merino hogget ewes (Table 5). Lower marking rate in ewe lambs was attributable to differences of 51% for reproductive rate and 16% for lamb survival (Table 5). The poorer marking rates of maiden Merino hogget ewes compared with their multiparous counterparts was largely attributable to a 22% difference in reproductive rate, whilst the difference for lamb survival was only 3% (Table 5).

Ewe mortality of ewe lambs was not different to that of pregnant multiparous ewes (Table 5). However, the difference in mortality of Merino hogget ewes compared to their multiparous counterparts was 0.7% (Table 5).

Table 5. Comparisons between maiden and mature multiparous ewes for reproductive rate, marking rate and lamb survival with mean ± standard error, 95% confidence interval (95% CI) for the difference and non-parametric related samples Wilcoxon signed-rank test.

	Ewe Age Group Mean ± Standard Error		**Difference**	
	Maidens	**Multiparous**	**Mean (95% CI)**	***p*-Value**
Non-Merino ewe lambs				
Marking rate (%) [A]	73.8 ± 2.8	131.9 ± 2.0	−58.1 (−64.3, −51.9)	< 0.001
Reproductive rate (%) [B]	108.6 ± 3.7	159.1 ± 1.9	−50.5 (−59.0, −42.1)	< 0.001
Lamb survival (%) [C]	67.3 ± 1.4	83.4 ± 0.9	−16.0 (−18.8. −13.2)	< 0.001
Ewe mortality (%) [D]	2.6 ± 0.2	2.8 ± 0.2	−0.2 (−0.8, 0.5)	0.378
Merino hogget ewes				
Marking rate (%) [A]	80.1 ± 2.6	102.3 ± 2.2	−22.3 (−26.9, −17.7)	< 0.001
Reproductive rate (%) [B]	107.6 ± 3.3	131.9 ± 2.7	−24.4 (−29.5, −19.2)	< 0.001
Lamb survival (%) [C]	74.5 ± 1.6	77.7 ± 1.3	−3.1 (−5.8, −0.5)	0.026
Ewe mortality (%) [D]	1.7 ± 0.2	2.4 ± 0.3	−0.7 (−1.2, −0.2)	0.006

[A] Marking rate = lambs marked/ewes mated x100.
[B] Reproductive rate = fetuses scanned/ewe joined x100.
[C] Lamb survival = lambs marked (live)/fetuses scanned x 100.
[D] Ewe mortality = ewe deaths between scanning and lamb marking (scanned pregnant)/ewes pregnant x 100.

There were moderate positive correlations between maiden Merino hogget ewes and their multiparous counterparts for marking rate, reproductive rate and lamb survival, marking rate and ewe survival (Table 6). In contrast, there was a very weak positive correlation between ewe lambs and their multiparous counterparts for lamb survival and no correlation for reproductive rate, marking rate or ewe survival (Table 6).

Table 6. Linear regression and bivariate Pearson correlation (two-tailed) between reproductive traits in maiden ewes and corresponding measure for multiparous counterparts.

	Regression			Pearson Correlation	
	Intercept	Slope	R^2	Correlation Co-efficient	*p*-Value
Multiparous ewes vs. non-Merino ewe lambs					
Marking rate (%) [A]	32.40	0.314	0.050	0.223	0.096
Reproductive rate (%) [B]	118.29	−0.061	0.001	−0.032	0.814
Lamb survival (%) [C]	29.55	0.460	0.079	0.280	0.035
Pregnant ewe mortality (%) [D]	2.107	0.180	0.032	0.180	0.222
Multiparous ewes vs. Merino hogget ewes					
Marking rate (%) [A]	14.85	0.637	0.292	0.541	< 0.001
Reproductive rate (%) [B]	4.21	0.783	0.426	0.652	< 0.001
Lamb survival (%) [C]	11.02	0.823	0.389	0.635	< 0.001
Pregnant ewe mortality (%) [D]	0.822	0.368	0.434	0.659	< 0.001

[A] Marking rate = lambs marked/ewes mated × 100

[B] Reproductive rate = fetuses scanned/ewe joined × 100

[C] Lamb survival = lambs marked (live)/fetuses scanned × 100

[D] Pregnant ewe mortality = ewe deaths between scanning and lamb marking (scanned pregnant)/ewes pregnant × 100.

3.3.3 Factors affecting reproductive performance of maiden ewes

Higher marking rates were observed for twin- and triplet-bearing ewes compared with single-bearing ewes (Table 7). This was due to the greater number of foetuses in these mobs and not due to a higher lamb survival with the difference in survival for twin lambs compared to single lambs of 11.4% for ewe lambs and 22.2% for Merino hogget ewes (Table 7 and Figure 2). Reproductive traits were independent of ABARE region, ewe age, ewe breed, ram breed, month of joining, condition score at joining, feed-on-offer at joining, pasture type at joining, condition score at lambing, duration of supplementary feeding, supplementary feeding method, type of supplementary feed or average feed-on-offer ($p < 0.05$).

Table 7. Predicted means ± standard error for the marking (%) and lamb survival (%) according to ewe management group during lambing for non-Merino ewe lambs and maiden Merino hogget ewes.

Management group during lambing	Marking %		Lamb survival %	
	Ewe Lambs	Hogget ewes	Ewe lambs	Hoggets
Mixed	97.3 ± 2.9	91.4 ± 5.0	NA	NA
Single	75.2 ± 2.3	83.3 ± 3.3	76.0 ±1.6	82.7 ± 1.8
Twin	129.8 ± 2.3	122.0 ± 3.6	64.6 ± 1.6	60.5 ±2.0
Triplet	146.0 ± 7.9	NA	43.7 ± 3.9	NA

NA: not available (not measured or no eligible responses).

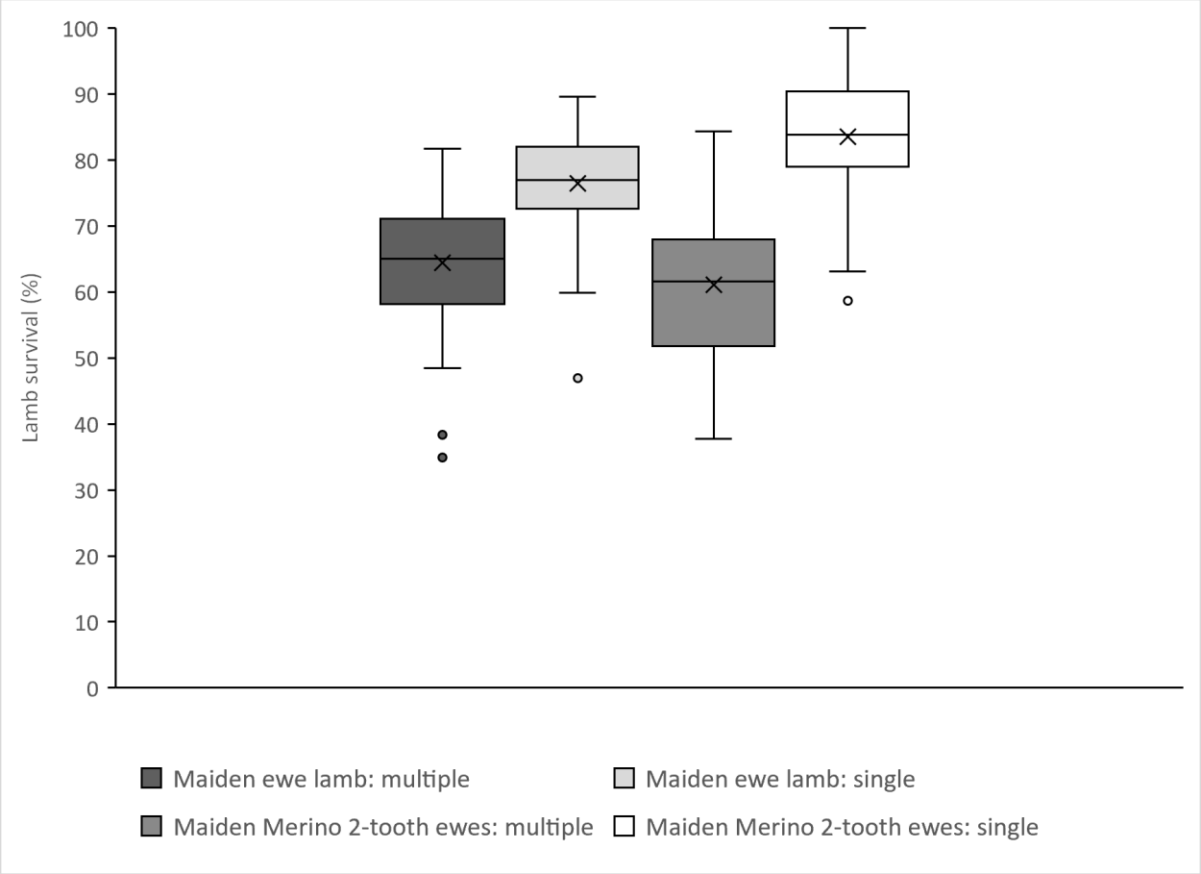

Figure 2. Box and whisker plot of for lamb survival in single- and multiple-born progeny of maiden ewe lambs and maiden Merino hogget ewes managed according to litter size

3.4 Discussion

Maiden ewes mated as non-Merino ewe lambs or Merino hogget ewes produced 44 and 22 fewer lambs to marking per 100 ewes mated than their multiparous counterparts on the same farm. Lower marking rates for maiden compared to multiparous ewes was attributable, albeit to varying degrees, to lower reproductive rate and lower lamb survival. These results support our hypotheses. To our knowledge, this is the first study to compare the marking rate of maiden and multiparous ewes and their components on commercial farms across Australia. Development and adoption of management strategies to improve marking rate for non-Merino ewe lambs should focus on improving both reproductive rate and lamb survival as they contributed nearly equally to the differences in marking rate compared to multiparous ewes. By contrast, the poorer marking rate of Merino hogget ewes compared with their multiparous counterparts were largely due to differences in reproductive rate. Nevertheless, the development and adoption of strategies to improve marking rates for Merino hogget ewes should also focus on improving both reproductive rate and lamb survival. In this study, lamb survival was relatively low for both hogget and multiparous Merino ewes, suggesting that improved lamb survival for Merino ewes across all age groups remains an issue for the Australian sheep industry. Further, the economic value of improving reproductive rate is greater when lamb survival is higher (Young et al., 2014), and improving lamb survival has implications for improving animal welfare (Ferguson et al., 2014).

The 58% difference in marking rate for non-Merino ewe lambs compared with multiparous ewes across 40 farms in our study was similar to the difference recently reported from nine commercial farms representing more than 300,000 records in New Zealand (72 vs. 142%) (Shorten et al., 2021). Likewise, marking rate was more variable between flocks for non-Merino ewe lambs compared with multiparous non-Merino ewes, with the overall average and variation in marking rate between flocks of non-Merino ewe lambs being similar to results reported by both Shorten et al. (2021) and Clune et al (Chapter 5)Clune et al. (2022c). The 22% difference in marking rate for Merino hogget ewes compared to multiparous ewes across 39 farms was greater than the differences measured by Kleemann and Walker (2005) on 14 farms in South Australia (70 vs. 86%) and a recent survey of 1200 Merino producers across Australia (79 vs. 93%) (Sloane, 2018). Over the last decade, more than 5000 sheep producers in Australia have participated in the extension and adoption programs to improve ewe management and reproductive performance, such as Lifetime Ewe Management (Thompson et al., 2020, Trompf et al., 2011) and Bred Well Fed Well (Hancock et al., 2018), but these programs have only focused on the management of multiparous ewes. Similar extension and adoption programs to improve the reproductive performance of maiden ewes could have a significant impact on national marking rates and lamb supply as non-Merino ewe lambs and Merino hogget ewes represent approximately 10 million breeding ewes in Australia (Trompf et al., 2018).

A lower marking rate for non-Merino ewe lambs compared to multiparous ewes on the same farm was attributed to a 51% difference in reproductive rate and a 16% difference in lamb survival. The difference in reproductive rate between these age groups was less than other studies, which have ranged from 75 to 124% (Shorten et al., 2021, Kenyon et al., 2014b), whereas the difference in lamb survival between these age groups tended to be greater than most other studies where the difference was less than 10% (Shorten et al., 2021, Corner et al., 2013, Morel et al., 2010, Mulvaney, 2011). The wider variation in marking rate, reproductive rate and lamb survival between individual flocks of non-Merino ewe lambs was similar to these and other studies, including Clune et al (Chapter 5) and Thompson *et al* (unpublished data). Improved understanding of the degree of variation and causes of poorer reproductive rate and lamb survival in maiden ewes will inform extension and adoption programs to improve maiden ewe reproductive performance.

The weak and generally non-significant correlation between the reproductive performance of ewe lambs and their multiparous counterparts in the current study hinged on the more variable performance of ewe lambs, whereas the performance of maiden hogget ewes was more consistent when compared with their multiparous counterparts on the same farm. Comparisons between studies suggest that the responses in reproductive rate to improved live weight at the start of the joining period (Rosales Nieto et al., 2013b, Rosales Nieto et al., 2013a, Thompson et al., 2021, Shorten et al., 2021), and live weight gain during the joining period (Adalsteinsson, 1979, Viñoles et al., 2012, Thompson et al., 2019), are much greater for ewe lambs than in multiparous ewes regardless of breed. Recently Paganoni et al. (Submitted 2021) also reported that the effects of live weight and condition score at joining on reproductive rate were greater in ewe lambs compared to hogget non-Merino ewes within the same flocks, and the difference between these age groups was greater than the difference between hogget and multiparous Merino ewes. Age of joining also influences the reproductive rate of ewe lambs (Thompson et al., 2021), even though this was not apparent in the current survey data where almost 90% of the ewe lambs were 7 or 8 months of age at joining. Collectively, this implies that the reproductive rate and potential marking rate of ewe lambs are more sensitive to management prior to and during joining than is the case for multiparous ewes. Bunter and Brown (2013) reported that expression of reproduction at hogget (yearling) and adult age is a genetically different trait in maternal (non-Merino) ewes. However, heritabilities for reproductive traits typically ranged 5–15%, which was consistent with low heritabilities for

reproduction traits reported in other studies (Hatcher et al., 2010, Everett-Hincks et al., 2014, Brien et al., 2014). This indicates that whilst progress in reproductive traits can be made with selection, reproductive performance is driven mostly by management. Management strategies to improve the reproductive performance of ewe lambs are more complex and less adopted, which contributes to the poorer and more variable performance of maiden ewes compared to multiparous ewes on commercial farms.

The precise reasons for poorer and more variable survival of lambs born to maiden ewes, especially ewe lambs, compared with their multiparous counterparts were not able to be determined in this study. Maiden ewes were managed separately to multiparous ewes during pregnancy and lambing, and differences in the lambing environment, such as time of lambing, shelter and mob size, could have impacted lamb survival. Whilst the effects of the varying condition score at lambing on the survival of lambs born to hogget ewes is likely to be similar to that that observed in multiparous ewes (Paganoni et al., 2014b), the slightly lower survival of lambs born to hogget ewes may reflect their average condition score at lambing, which was reported to be lower than recommended for mature ewes (Young et al., 2016). By contrast, whilst the level of nutrition and live weight change from pregnancy scanning to lambing is likely to influence the survival of lambs born to ewe lambs (Griffiths et al., 2016), the average condition score of ewe lambs in the current study was reported to be 3.2 at lambing, which is likely to be close to the optimum. It may, therefore, be that factors other than nutritional management during pregnancy and lambing contributed to the much greater difference in survival of lambs between ewe lambs and multiparous ewes compared to maiden hogget ewes and multiparous ewes.

A number of factors may contribute to the survival of lambs between scanning and marking. Important causes of perinatal lamb mortality include dystocia, stillbirths and starvation-mismothering, but these conditions are usually multifactorial and the role of dam parity are not fully understood (Horton et al., 2018, Hinch and Brien, 2014b, Jacobson et al., 2020, Refshauge et al., 2016). Clune et al reported that lamb mortality between birth and marking was the major contributor to lamb loss between scanning and marking for Australian maiden ewe flocks, but that mid-pregnancy abortion caused significant in utero foetal loss in some flocks of ewe lambs (Chapter 5). A review of veterinary investigations for abortions and stillbirths submitted to Australian veterinary diagnostic laboratories reported that 81% of investigations with a diagnosis involved infectious aetiology, with the most common infectious agents implicated being *Listeria* spp., *Campylobacter* spp. and *Toxoplasma gondii* (Chapter 4). More recently, *Chlamydia pecorum* has been associated with abortions and stillbirth for maiden ewe flocks in Australia (Chapter 6)(Westermann et al., 2021), and Chapters 7, 8 and 9 report *T. gondii, Neospora caninum* and *Coxiella burnetii* were not important contributors to foetal and lamb mortality maiden ewe flocks on farms in southern Australia. Immunological naïvety is considered a risk factor for infectious reproductive diseases, and therefore maiden ewes have a higher risk for infectious causes of abortion because they have had less time to be exposed to infection and develop immunity before pregnancy (Buxton, 1990, Katzer et al., 2011, Jensen et al., 1957, Berri et al., 2001). Consequently, sporadic impacts of abortion and perinatal lamb mortality due to infectious disease could explain some of the variability in lamb survival reported in this study for maiden ewe flocks, and especially ewe lambs. Sporadic impacts of infectious disease on lamb survival could also explain the lack of correlation between lamb survival for ewe lambs and multiparous ewes on the same farm. The impacts of infectious disease on lamb survival for maiden ewes warrants further investigation.

We did not observe any effect of condition score or feed-on-offer on reproductive rate or lamb survival. However, there were very few responses that included data for condition score or feed-on-

offer, and of these, a number were estimated rather than measured. Furthermore, condition score assessment is often subject to operator bias, which could result in between-operator variability in condition score assessment (van Burgel et al., 2011). Other studies have reported associations between condition and reproductive rate and lamb survival, and between feed-on-offer at lambing and lamb survival. Therefore, the absence of effects of condition and feed-on-offer on reproductive traits in our study likely reflects the response numbers and reporting bias rather than the absence of a biological association. Further investigation to validate current recommendations for the condition at joining and during pregnancy for maiden ewes are warranted.

There were several limitations to this study. Surveys were distributed widely using electronic communications via a range of agricultural networks. As such, it was not possible to determine the survey response rate to provide an indication of non-responder bias. The most common differences between respondents and non-respondents cited in feedback were that non-respondents and responses not eligible for inclusion did not utilise pregnancy scanning to count foetuses, nor did they manage maiden and multiparous ewes separately at lambing to distinguish lamb survival and marking rates for both categories. The inclusion criteria generated bias in the sample population because only producers that utilised pregnancy scanning for litter size were eligible for inclusion. Subsequently, the sampled population likely included a higher proportion of ewes that were differentially managed according to litter size compared to the general population. Differential management of single- and multi-bearing ewes was used for 66% maiden non-Merino ewe lambs and 61% maiden Merino hogget ewes, which was consistent with the adoption rate for producers that had undertaken Lifetime Ewe Management training (Thompson et al., 2020). It is also possible that producers that have adopted pregnancy scanning were more likely to adopt other management strategies that could impact reproductive performance compared to the broader population. As such, the findings of this study should only be generalised to Australian sheep producers that have adopted pregnancy scanning and should not be extrapolated across the national sheep flock.

3.5 Conclusion

To our knowledge, this is the first study to compare the marking rate for maiden and multiparous ewes and their components on commercial farms across southern Australia. Marking rates for non-Merino ewe lambs and maiden Merino hogget ewes were lower than their multiparous counterparts, and this was attributable to a combination of lower reproductive rate and lower lamb survival. Wide variability in both reproductive rate and lamb survival indicates an opportunity for improvement in reproductive performance for maiden ewes through the adoption of strategies that increase the number of foetuses conceived and the number of lambs surviving between pregnancy scanning and lamb marking. Strategies specific to ewe lambs may be required because their reproductive performance was not correlated with multiparous ewes on the same farm.

4. Sheep abortion and stillbirth investigations in Australia

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4.1 Introduction

Foetal loss and lamb mortality between mid-pregnancy and weaning is an important economic and welfare problem for the Australian sheep industry (Sackett et al., 2006, Lane et al., 2015). A number of diseases endemic in Australia can cause abortion and birth of lambs with poor viability. *Campylobacter* spp., *Listeria* spp. and *Toxoplasma gondii* are reported as leading causes of ovine abortion (Gorrie, 1962, Munday et al., 1966, Plant et al., 1972, Dennis, 1974a, Dennis, 1974b, Broadbent, 1975, Munday et al., 1987, Nilon, 2011). However, most historical studies only report on localised geographical regions or specific putative causes and are considerably dated. To our knowledge, no systematic comprehensive surveys of the causes of ovine abortion across major Australian sheep production areas have been reported.

Identifying the aetiological agent involved in an abortion outbreak has important implications for disease management and control, managing public health risks with zoonotic infections, and preventing further outbreaks. Abortion investigations also play an important role in surveillance for exotic diseases, including *Chlamydia abortus, Brucella melitensis* and *Salmonella enterica* serotype Abortusovis that are associated with abortion in other countries (Ilhan and Yener, 2008, Mearns, 2007a, Habrun et al., 2006). Exclusion of these exotic diseases in abortion investigations has implications for trade through demonstrating freedom from these diseases.

Reaching an aetiological diagnosis for ovine abortion investigations can be challenging. This relies heavily on laboratory methods for demonstration of presence or absence of infectious agents which can be hindered by the type and quality of samples submitted, the availability of diagnostic tests, and difficulty in identifying non-infectious causes of lamb mortality. Most Australian studies reporting causes of ovine abortion pre-date the widespread availability of molecular diagnostic tests (Gorrie, 1962, Munday et al., 1966, Plant et al., 1972, Dennis, 1974a, Dennis, 1974b, Broadbent, 1975, Munday et al., 1987). It is not clear if the introduction of more sensitive tests such as PCR and quantitative PCR have changed the range of diseases diagnosed in abortion or neonatal mortality investigations, or the proportion of investigations where an aetiological diagnosis is made.

A sound understanding of the most common causes of abortion and neonatal mortality would allow veterinary practitioners to develop appropriate protocols for field investigations that ensure appropriate samples are collected. Understanding factors that impact ability of pathologists to make an aetiological diagnosis in these investigations will inform these protocols and allow practitioners to counsel clients on the likelihood of the investigation yielding an aetiological diagnosis.

The aims of this study were to identify the most common causes of ovine abortion and stillbirths reported in Australia based on submissions to state veterinary laboratories and determine factors that influenced whether aetiological diagnoses could be determined for investigations.

4.2 Methodology

4.2.1 Data retrieval

Data were collated for ovine abortion and stillbirth investigations conducted by state government or associated veterinary diagnostic laboratories in Western Australia (Department of Primary Industry and Regional Development Diagnostic Laboratory Services formerly Department of Agriculture and Food Western Australia Animal Health Laboratory), South Australia (VETLAB, with testing performed by Gribbles Veterinary Pathology), Victoria (Agriculture Victoria, with testing performed by AgriBio Laboratories or Gribbles Veterinary Pathology) and Tasmania (Animal Health Laboratory). These laboratories are NATA (National Association of Testing Authorities) accredited under ISO 17025 for veterinary testing, although it was not determined if all the specific tests performed were covered by NATA accreditation in each laboratory. Submissions to veterinary laboratories were made by government and private veterinarians. Diagnostic tests performed by each laboratory included gross pathology, histopathology, microbial culture, molecular diagnostic tests and serology. Specific testing procedures varied between submissions and different database software was used by each laboratory. Retrieval of data for investigations was performed by staff from each laboratory using search terms, ‘abortion’, ‘stillbirth/born’ or ‘infertility’. ‘Infertility’ cases were predominately associated with ram infertility investigations and subsequently omitted from analyses. The time period included for datasets varied between laboratories due to constraints in ability to retrieve results from the databases (Table 8).

Datasets were provided as spreadsheets with summary information for each investigation, including submission identification code, date of submission, farm location (post-code or geographical co-ordinates), summary of case history, sample (specimen) type, diagnostic tests performed, and diagnosis reported for each investigation. The level of detail available for samples varied between laboratories. In some cases, the number of foetuses or stillborn lambs submitted for each investigation was not clear, and in some cases, it was not possible to determine if more than one submission was made for the same farm within the same year. Detailed pathology reports for each investigation were not available. The approximate number of ovine abortion investigations as a proportion of all ovine disease investigations (not including faecal egg counts) was provided for laboratories in Victoria and Tasmania.

4.2.2 Statistical analyses

Datasets were consolidated and cases that included submission of specimens from more than one foetus/lamb from the same farm in the same lambing period were considered as a single investigation. Each investigation was evaluated to determine if a diagnosis was made, if infectious agents were identified by diagnostic testing, and where possible, submission characteristics such as

type and quality of tissue submitted and outcomes for specific tests. The mean annual number of investigations was compared to the number of farms in each state based on the average of the annual number of sheep businesses reported by Australian Bureau of Statistics during the time period from which the datasets were derived (Australian Bureau of Statistics, 2000-2018).

Statistical analyses were performed using IBM SPSS Statistics (version 24). Proportions (e.g. investigations performed, diagnoses made or specific diagnoses) were compared using Chi-square analyses with two-tailed Pearson test for significance. Association between submission of placenta (yes/no) and aetiological diagnosis (yes/no) were compared using Chi-square analyses with two-tailed Pearson test for significance, plus odds ratio and relative risk with 95% confidence interval.

4.3 Results

4.3.1 Abortion and stillbirth investigations

A total of 529 investigations were analysed for the period 2000-2018, although the period of reporting by laboratories varied over this time (Table 8). Most reports were received from Victoria (*n*=248, years 2010-2018) and Tasmania (*n*=144, 2000-2018); and abortion was investigated more often from sheep flocks in these two states. Abortion investigations represented approximately 5-10% of total annual sheep disease investigations (excluding worm egg counts) performed at state government veterinary laboratories for Victoria and Tasmania.

Table 8: Abortion and stillbirth investigations conducted by state government laboratories in Western Australia, South Australia, Victoria and Tasmania between 2000 and 2018

State	Investigations (*n*)	Timeframe		Investigations per year	Farms *(n)*[*]	Mean farms with investigation per year (% farms)
		Years	Duration (years)			
WA	65	2008-2018[#]	11	5.9	5596	0.11[a]
SA	72	2006-2017	12	6	6414	0.09[a]
VIC	248	2010-2018	9	27.6	9855	0.28[b]
TAS	144	2000-2018	19	7.6	1503	0.50[b]
Total	529	-	-	-	-	-

SA - South Australia; TAS – Tasmania; VIC – Victoria; WA – Western Australia

[*] mean number of sheep farms per state over specified timeframe derived from Australian Bureau of Statistics

[ab] Values (proportion) in column with different superscript are significantly different (*P*<0.05)

[#] 2018 data for WA incudes January-June 2018 only

A diagnosis was made for 57% investigations *(n*=300), ranging 49-63% across the four states (Table 9). The proportion of investigations with diagnosis was higher in Victoria compared to the three other states (Table 9).

Datasets from Tasmania and Victoria included detail of diagnostic tests used for each investigation. Histopathology results were available for 139 investigations from Tasmania and 133 investigations from Victoria. Six investigations included only ewe serology or biochemistry, with 1 – 16 ewes sampled per investigation. A diagnosis was made for two of the investigations that used only dam serology or biochemistry, specifically a diagnosis of leptospirosis and copper deficiency. Seroconversion for *T. gondii* was demonstrated in 75-100% serology samples submitted per investigation, however aetiological diagnosis of toxoplasmosis was not made without supportive

histopathological evidence, paired serology or isolation of organism in foetal tissues. Another two investigations included only vaginal swabs without serology or foetal tissue, no diagnosis was made for these investigations.

Table 9: Abortion and stillbirth investigations with aetiological diagnosis between 2000 and 2018

		Investigations with diagnosis	
State	**Total investigations** (*n*)	(*n*)	(%)
WA	65	32	49.2[a]
SA	72	36	50.0[a]
VIC	248	156	62.9[b]
TAS	144	76	52.8[a]
Total	529	300	56.7

[ab] Values (proportion) in column with different superscript are different ($P<0.05$)
SA - South Australia; TAS – Tasmania; VIC – Victoria; WA – Western Australia

4.3.2 Aetiological diagnoses made for abortion and stillbirth investigations

An infectious aetiology was determined in 81.3% of cases where an aetiological diagnosis was determined (Table 10). *Campylobacter* spp. (32.3%), *Listeria* spp. (25.7%) and *Toxoplasma gondii* (9.3%) were the most commonly diagnosed infectious agents; with other infectious agents making up 14% of cases with aetiological diagnosis (Table 11). Most investigations had a single diagnosis, except for one farm in Tasmania with *C. coli* isolated concurrently with *C. jejuni* from one aborted foetus and one farm in Victoria with *C. fetus* and *L. ivanovii* isolated from two separate aborted foetuses during the same lambing period.

Table 10: Diagnoses made for abortion and stillborn lamb disease investigations in WA (2008-2018), SA (2006-2017), VIC (2010-2018) and TAS (2000-2018)

	Diagnoses by state (*n*)				**Overall**	
	WA	SA	VIC	TAS	*n*	% investigations with diagnosis
Campylobacter (not speciated)	1	0	22	5	32	10.7
C. fetus	4	5	21[#]	16	42	14.0
C. jejuni	0	1	4	18	23	7.7
C. coli	0	0	0	1*	1	0.3
TOTAL *Campylobacter* spp.	5	6	47	39	97	32.3[a]
Listeria (not speciated)	0	11	26	0	42	14.0
L. ivanovii	2	0	25	7	32	10.7
L. monocytogenes	3	0	2	1	3	1.0
L innocula	0	0	1[#]	0	1	0.3
TOTAL *Listeria* spp.	5	11	54	8	77	25.7[a]
Toxoplasma gondii	1	4	8	16	28	9.3[b]
Other infectious	6	7	23	6	42	14.0[b]
TOTAL infectious diagnoses	17	28	131^	69^	244	81.3
TOTAL non-infectious diagnoses	15	8	26	7	56	18.7

* *C. coli* isolated concurrently with *C. jejuni* from an aborted foetus
[#]*L. monocytogenes* and *C. fetus* isolated from two separate foetuses from one farm in the same lambing period
^ Total number diagnoses accounts for mixed infections (i.e. more than one infectious aetiology for investigation).
SA - South Australia; TAS – Tasmania; VIC – Victoria; WA – Western Australia
[ab] Values for diagnoses (Campylobacter, Listeria, Toxoplasma, other) with different superscripts are significantly different (P<0.05)

Table 11: Less frequently reported aetiological diagnoses made in ovine abortion investigations between 2000 and 2018

Infectious aetiology (n)[a]	**Non-infectious aetiology** (n)[b]
Leptospira spp. (12)	Maternal illness (17)
Yersinia spp. (8)	Dystocia (13)
Salmonella spp. (5)	Congenital abnormality/anomaly (7)
Chlamydia pecorum (3)	Goitre (7)
Escherichia coli (3)	Nutritional (7)[c]
Arcanobacterium pyogenes (2)	Toxicity (4)[d]
Histophilous somni (2)	Starvation-mismothering-exposure (1)
Aeromonas hydrophila (1)	
Erysipelas rhusiopathie (1)	
Staphylococcus aureus (1)	
Streptococcus parauberis (1)	
Other infectious agent - not specified (3)	

[a] Uncommon infectious agents combined represent 14% investigations with diagnosis
[b] Non-infectious causes combined represent 19% investigations with diagnosis
[c] Includes cases of vitamin E, copper, selenium or cobalt deficiencies
[d] Toxicities included predominantly plant poisonings; phalaris, romulosis and toxic algae

Leptospirosis was diagnosed in 4% of investigations with a diagnosis, with 83% of these cases diagnosed in Victorian flocks in 2018. *Leptospira interrogans* serovar *Hardjo* was the only serovar reported (*n*=2), although serovar was not reported for all cases. Leptospirosis was not diagnosed in Western Australia. Yersinosis was diagnosed for 3% investigations with a diagnosis, with both *Y. enterocolitica* and *Y. pseudotuberculosis* identified as the aetiological agent either together or separately. Other infectious agents each represented less than 2% of diagnosed investigations. 'Maternal illness' and dystocia were the most common non-infectious cause identified. However, 'maternal illness' was non-specific diagnosis and could have involved infectious diseases in some cases. Exotic disease agents were not identified in any of the investigations.

Data were available from all four states for the period 2010-2017 (Table 12), with some variation year-on-year in the frequency of diagnoses for the major infectious causes of abortion. The most frequently reported aetiological diagnoses over this period were listeriosis (30.6% investigations with aetiological diagnosis) and campylobacteriosis (27.6% investigations with diagnosis), and toxoplasmosis (10.3% investigations with diagnosis).

Table 12: Number of diagnoses made for abortion and stillborn lamb disease investigations in Western Australia, South Australia, Victoria and Tasmania between 2010 and 2017

	Year of study								**Overall**	
	2010	2011	2012	2013	2014	2015	2016	2017	n	%
Investigations (*n*)	51	40	45	28	50	55	94	46	409	-
Diagnoses (*n*)										
Listeria	9	7	11	5	8	9	16	6	71	17.4[a]
Campylobacter	10	0	6	7	14	12	10	5	64	15.6[a]
Toxoplasma	4	4	3	0	3	0	10	0	24	5.9[b]
Other infectious causes	2	4	1	4	1	4	9	3	28	6.8[b]
Non-infectious causes	5	5	9	5	3	4	8	6	45	11.0[a]
No diagnosis made	21	20	15	7	21	26	41	26	177	43.3[c]

[abc] Values in column with different superscript are significantly different (*P*<0.05)

Fifty six percent of the investigations where histopathological findings were provided (*n*=272) reported inflammatory lesions. Aetiological diagnoses were made in 76% of this sample, and 97% of these were of an infectious cause. Thirty percent of the investigations where no aetiological diagnosis was made also showed inflammatory changes. The most common lesions described, together or independently, were pneumonia (*n*=42), placentitis (*n*=38) and hepatitis (*n*=27), although detailed information of inflammatory lesions were not available for all cases.

4.3.3 Factors associated with determination of aetiological diagnosis

Of 139 investigations where information on types of samples submitted was available, 55% included placenta. Investigations that included submission of placenta were 2.3 (95% confidence interval (CI) 1.1, 4.5) times more likely to have diagnosis made compared to those investigations without placenta available (*P*=0.017; Table 13).

Table 13: Association between submission of placenta samples and diagnostic success (% investigations with aetiological diagnosis) in ovine abortion investigations.

	Submissions *(n)*	**Aetiological diagnosis made**	**No aetiological diagnosis**
Placenta available	77	48 (62%)	29 (38%)
Placenta not available	62	26 (42%)	36 (58%)

Autolytic changes were reported for 50% of investigations, yet an aetiological diagnosis was still reached in 57% of these, and autolytic changes was not associated with likelihood of aetiological diagnosis being made (*P*=0.578).

4.4 Discussion

This study examined causes of abortion and stillbirths for 529 investigations submitted to state veterinary diagnostic laboratories between 2000 and 2018. Pathologists reported a diagnosis for 57% investigations, with a diagnosis more likely to be made for investigations that included submission of placenta samples. Campylobacteriosis, listeriosis and toxoplasmosis were the most common diagnoses reported. There was no evidence of infection with *Chlamydia abortus*, *Brucella melitensis* or *Salmonella enterica* serotype Abortusovis which are considered exotic to Australia and are important causes of abortion in sheep and public health risks overseas (Habrun et al., 2006, Ilhan and Yener, 2008, Mearns, 2007a).

An infectious aetiology was determined in 46% of investigations. In an additional 30% of cases without a diagnosis, morphological diagnoses suggestive of an infectious aetiology including placentitis, foetal hepatitis and/or pneumonia were reported, suggesting that infectious abortion could be underreported in this sample. This is consistent with previous studies where inflammatory lesions suggestive of infectious causes were found in 7-15% of cases without isolation of an aetiological agent (Kirkbride, 1993, Van Den Brom et al., 2012, van Engelen et al., 2014, Chanton-Greutmann et al., 2002, Oporto et al., 2006).

Abortion associated with *Listeria spp*., *Campylobacter spp*. and *T. gondii* accounted for more than two thirds of investigations with a diagnosis reported. This is consistent with previous studies reporting causes of infectious abortion in Australian sheep (Broadbent, 1975, Plant et al., 1972, Dennis, 1974b, Dennis, 1975, Sergeant et al., 1991). These pathogens are also associated with sheep reproductive losses in many other countries (Dubey, 2009, Buxton and Henderson, 1999, Fenwick et al., 2000, Menzies, 2011). Despite these diseases being endemic in Australia, the incidence of infectious abortion associated with these infections is poorly described.

Campylobacter fetus fetus has been recognised as a cause of ovine abortion in Australia (Broadbent, 1975), which is consistent with the observations of this study. However, in Tasmania, *C. jejuni* was reported more commonly in abortion investigations than *C. fetus. Campylobacter jejuni* is a commensal organism that is commonly shed in faeces by asymptomatic sheep (Yang et al., 2014b). Under some circumstances, *C. jejuni* can be associated with abortion in sheep and goats (Hedstrom et al., 1987, Sanad et al., 2014, Delong et al., 1996, Sahin et al., 2012), but specific risk factors for *C. jejuni*-associated abortion are not well described. A clonal form of *C. jejuni* has become the predominant cause of ovine abortion in some parts of North America (Sahin et al., 2008, Dubey and Kirkbride, 1990, Moeller, 2001). Abortigenic *C. jejuni* strains in Australia have not been studied with molecular characterisation and it is unclear why *C. jejuni*-associated abortion was reported more frequently in Tasmania compared to other states given the widespread distribution of the organism

in Western Australia, South Australia and Victoria (Yang et al., 2014b). A vaccine for *Campylobacter* has been commercially available in Australia since 2013. Given the limitations of the data available, it is not possible to determine if the availability of vaccination has impacted incidence of campylobacter-associated abortion in Australia.

Retrospective evaluation of laboratory data is useful for determining the number of investigations being performed and what aetiological agents were diagnosed, which can subsequently identify changing disease trends and the introduction of novel pathogens (Murray et al., 2019, Vellema and van den Brom, 2014, van Engelen et al., 2014). However, disease investigations reported in this study were derived from submissions by veterinarians to animal health laboratories, which rely on farmers identifying abortion in the flock, reporting this to veterinarians, suitable samples being available for submission to the laboratory and veterinarians making the decision to submit samples for testing. This is associated with potential for bias and the risk of over- or under-reporting specific diseases (Murray et al., 2019). The incidence of abortion in Australian sheep or true incidence of specific diseases therefore cannot be derived from data included in this study. Diseases associated with mid-pregnancy abortion are likely to be underrepresented as foetal tissue is less likely to be recovered.

In this study, aetiological diagnosis was made for 57% investigations. This was comparable with previous Australian studies conducted between 1966 and 1987 where aetiological diagnosis was reported for 25-76% cases (Broadbent, 1975, Dennis, 1974a, Munday et al., 1987, Munday et al., 1966, Plant et al., 1972). Similar findings have been reported in other countries (Mearns, 2007a, Murray et al., 2019, Kirkbride, 1993, Van Den Brom et al., 2012). Differences in number of submissions, predominant diseases, method of sample collection and diagnostic methodology including the availability of molecular diagnostic tools, will contribute to variation in proportion of investigations with aetiological diagnosis, and the types of diagnoses made. Overall, determining aetiological diagnosis in abortion investigations for ruminants remains a challenge in many countries, despite availability of molecular diagnostics.

The type and quality of aborted material submitted for laboratory analysis impacts the ability of pathologists to make an aetiological diagnosis for disease investigations. Inclusion of placental tissue in submissions increased the probability of making an aetiological diagnosis 2.3 times, and this was consistent with previous studies (van Engelen et al., 2014, Holler, 2012, Plant et al., 1972, Van Den Brom et al., 2012). It was not possible to determine the impact of the number of animals included per submission, but it has been previously reported that the likelihood of detecting an infectious agent is improved where samples from more aborting ewes are available (Broadbent, 1975, Haughey et al., 1967).

Autolysis of tissues was commonly reported for samples submitted for histopathology, and this was consistent with other abortion studies (Van Den Brom et al., 2012, van Engelen et al., 2014, Hazlett et al., 2013). Autolysis of specimens is often unavoidable and a consequence of the disease process or maceration prior to expulsion from the uterus. Tissue autolysis may hinder diagnostic success by reducing the viability of causative agents for culture and affecting interpretation of histopathology. Despite this, an aetiological diagnosis was achieved in over 57% of cases where autolysis was described, largely due to use of molecular techniques and/or culture for isolation of infectious agents. Submission of suitable tissue samples for histopathology is especially important for aetiological diagnosis where it is suspected that pathogens could be involved that are both abortigenic and common gut inhabitants of asymptomatic sheep (e.g. *C. jejuni*, *Listeria spp* or *Yersinia spp*.) and where tissue contamination with soil or faeces is evident (Bailey et al., 2003). Veterinary practitioners can improve the likelihood of obtaining an aetiological diagnosis by

encouraging farmers to collect any aborted or perinatal dead lambs and placenta (if available) even if there is evidence of predation, and store these between 4-10^{O} Celsius prior to submission to the practitioner or veterinary diagnostic laboratory.

The inclusion of cultures and molecular diagnostic techniques is likely to improve the ability to make a diagnosis in cases with autolysis, but it is important to note that detection of a pathogen may not be sufficient to determine causation for abortion, and in some cases detection of infectious agents may have been incidental findings. Detection of infectious agents in multiple cases from the same property and supportive histological findings increases confidence in the diagnosis. However, recovery of appropriate samples is difficult on extensive livestock farms and autolysis of samples is common. Further, the use of ewe serology for cases where foetal tissue is not available is unreliable unless paired samples collected close to time of foetal loss are available to demonstrate active infection evidenced as rising titres. Consequently, determining an aetiological diagnosis can be challenging, particularly under conditions where abortion is not readily observed.

Abortion and stillbirth investigations were conducted for a low proportion of farms (up to 0.5% farms per year). It is not clear if low rates of submission are due to low levels of abortion being observed on Australian farms, low rates of voluntarily reporting of abortions to veterinarians by farmers, or veterinarians choosing not to submit specimens for laboratory testing. A range of factors can influence the likelihood of disease investigation, including cost of investigation, access to veterinary services and willingness of farmers to seek veterinary services, veterinary investigative capacity and severity of disease outbreak (Robinson et al., 2012, Sawford et al., 2013, Palmer et al., 2009). The ability of producers to identify an abortion problem in the first instance can be challenging on sheep farms with extensive management systems where the likelihood of observing abortion or locating aborted foetuses in the paddock is low. Further, producers have variable sensitivity thresholds for livestock morbidity and mortality before seeking veterinary advice, and the sporadic nature of low-level abortion events and lack of obvious clinical signs of disease in ewes with many abortive disease syndromes in Australia likely contribute to under-reporting of abortions to veterinarians (Pfeiffer, 2018, Pfeiffer et al., 2017).

Infections with zoonotic potential were identified in this study, including *T.* gondii (Schlüter et al., 2014), *Campylobacter* spp. (Gölz et al., 2014), *Leptospira* spp. (Fang et al., 2015) and *Salmonella* spp. (Allos et al., 2004). Human disease following exposure to ovine aborted material and lambing ewes has previously been reported (Roberts et al., 1967, Kampinga et al., 2000, Baker et al., 2007). This reinforces the importance of appropriate personal protection and hygiene measures for farmers, veterinarians and laboratory workers when handling pregnant and lambing ewes, and aborted material. Recommendations include limiting contact between pregnant or immunocompromised people and lambing ewes or aborted material. General hygiene precautions should be used to avoid ingesting infectious agents (e.g. bacteria, parasites), and if contact with lambing ewes and foetal tissues is unavoidable, open wounds should be covered with waterproof dressings, and the importance of effective hand washing after handling animals or tissues should be emphasised (Elsheikha, 2008).

4.5 Conclusion

Infectious diseases are the most frequent cause of abortion for investigations submitted to state veterinary diagnostic laboratories in southern Australia. The most common diagnoses made are campylobacteriosis, listeriosis and toxoplasmosis, but a wide variety of other infectious and non-infectious causes of abortion are diagnosed in Australian sheep. Whilst sometimes an aetiological

diagnosis in abortion and stillbirth investigations is not reached, veterinary practitioners can improve probability of an aetiological diagnosis by emphasising to farmers the importance collecting any aborted tissues, and especially placenta, submitting multiple foetuses and appropriate storage of the tissues until they can be submitted to the laboratory. Submission of appropriate tissue samples aids diagnosis, even when autolysis is evident. Many of the diseases that cause abortion and stillborn lambs in Australian sheep have zoonotic potential and therefore veterinary practitioners play an important role in educating clients about appropriate hygiene when handling pregnant and lambing ewes and any aborted material.

5. Abortion and lamb mortality between pregnancy scanning and lamb marking for maiden ewes in southern Australia

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5.1 Introduction

The reproductive performance of primiparous (maiden) ewes is an important component of overall flock performance in Australia given maidens represent 20-30% of breeding ewes (Sloane, 2018). Maiden ewes are joined either as ewe lambs at 7 to 10 months of age, or hogget ewes, at 18 to 20 months of age. Economic modelling has shown that improving the reproductive performance of ewe lambs and maiden hogget ewes are key priorities for improving the reproductive performance of the Australian sheep flock (Young et al., 2014). Lamb mortalities in the perinatal period are a major source of reproductive inefficiency for mature ewe flocks (Hinch and Brien, 2014b). However, the nature, timing, and magnitude of foetal and lamb mortality between pregnancy scanning and marking is not well studied for maiden ewes.

Some overseas studies have shown that *in utero* losses, including abortions, during mid- to late-pregnancy may be an important contributor to the reproductive inefficiencies of maiden ewes (Howe et al., 2008, Lafi et al., 2009, Ridler, 2015, West et al., 2006, Kenyon et al., 2014b). Variable levels of pregnancy loss and abortion are reported for maiden ewes. Some studies in New Zealand have reported abortion in less than 5% of maiden ewes (Edwards et al., 2016, Mulvaney, 2011), whilst others have reported abortions ranging from 5-59% for maiden ewes (Howe et al., 2008, Ridler et al., 2015, West et al., 2006, Kenyon et al., 2014b, Hilson et al., 2015, Ridler et al., 2017). In Australia, abortion events are generally sporadic and considered an insignificant contributor to overall lamb mortality, based on studies conducted using mature or mixed-age ewes (Viñoles et al., 2012, Geenty et al., 2014, Allworth et al., 2017, Kleemann and Walker, 2005, Robertson et al., 2017). An incidence of abortion of 2% is considered 'normal', but detection of abortion in sheep managed in extensive production systems is challenging. Furthermore, most Australian studies investigating lamb mortalities between scanning and lamb marking do not distinguish between losses occurring *in utero* and those in the perinatal period. Anecdotal reports from Australian sheep producers suggest that abortions post-scanning may be contributing to poor reproductive outcomes for maiden ewes, but the relative contribution of abortion to overall lamb mortality for maiden ewes in Australia is unclear.

There are several possible causes of pregnancy loss and abortion between scanning and birth in maiden ewes. Younger ewes appear to be more susceptible to endemic infectious causes of abortion such as campylobacteriosis and toxoplasmosis (Chapter 4), as they have had less time to develop immunity prior to pregnancy (West, 2002). Lower ewe liveweight at joining and during early pregnancy has also been associated with increased rates of abortion during mid- to late-pregnancy in ewe lamb flocks in New Zealand (Ridler et al., 2015). Although, this association was not observed in other studies (Ridler et al., 2017). Multiple factors may be contributing to *in utero* losses and lamb mortality concurrently in a single flock, making it challenging to identify specific risk factors.

The aims of this study were to (i) quantify abortion and lamb mortality from maiden ewes joined either as ewe lambs or hogget ewes, (ii) investigate the timing of abortion and lamb mortalities between pregnancy scanning in mid-pregnancy and lamb marking, and (iii) determine whether abortion was associated with ewe live weight or body condition during pregnancy. We hypothesised that abortion will be a significant contributor to overall mortality between scanning and lamb marking for maiden ewes on commercial farms in Australia.

5.2 Methodology

All procedures were conducted according to guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes and were approved by the Murdoch University Animal Ethics Committee (R3004/17). The owners of sheep included in this study provided consent to participate.

5.2.1 Research sites, animals, and experimental design

This observational cohort study was conducted using 30 maiden ewe flocks on 28 farms across Western Australia (*n* = 11 flocks), South Australia (*n* = 9 flocks) and Victoria (*n* = 10 flocks) between 2018 and 2020. Farms were located in medium (400-600mm rainfall annually) to high (600-1000mm rainfall annually) rainfall zones (Figure 3). The study was performed in consecutive years using different study flocks on two farms; one from Western Australia (2018 and 2019; Flocks 3 and 14) and one from Victoria (2019 and 2020; Flocks 19 and 27). All other farms were sampled in a single year.

Farms were selected on convenience sampling with criteria for inclusion based on; having at least 200 maiden ewes available for the study, a capacity to monitor ewes and their progeny over the study period, and with a sheep genotype and management system that were generally representative of commercial sheep farms in the region. Some farms included in the study managed flocks of stud sheep, but management and stocking density were comparable to commercial sheep flocks in these regions.

Flocks of approximately 200 ewe lambs (*n* = 19 flocks; 7-10 months of age at joining) or Merino hogget ewes (*n* = 11 flocks; 18-20 months of age at joining) were monitored from joining to lamb marking on each farm. For farms with more than 200 maiden ewes, a subset of approximately 200 ewes were randomly selected from the larger cohort for inclusion in the study. Ewe lambs were of non-Merino breeds except for one research site (Flock 8) in Western Australia which joined Merino ewe lambs to Merino rams. Most sires were the same breed as the ewes in the study flock, however White Suffolk rams were used in two flocks joining maiden Merino hogget ewes in South Australia (Flock 9 and 12). All rams were confirmed to be negative for *Brucella ovis* via serology prior to joining. Most ewes were naturally joined with a ratio of 50 ewes per ram and joined for an average

of 38 days. Some flocks were separated into smaller mobs at joining to facilitate single-sire joining. All or some of the ewes in five flocks were artificially inseminated followed by a period of natural joining.

Each farm ran self-replacing flocks and sheep were managed as per standard farm practice including monitoring of condition score to guide nutrition and grazing management. Seven of the 30 flocks had been vaccinated against *Campylobacter* spp. (Coopers Ovilis Campyvax®, MSD Animal Health) according to the manufacturer's instructions (Appendix 2 Table A2.1). On most farms, the study flock was kept separate from other maiden and mature sheep. Some farms managed their single- and multiple-bearing maiden ewes together in a single mob whilst others separated their single- and multiple-bearing ewes prior to the start of lambing.

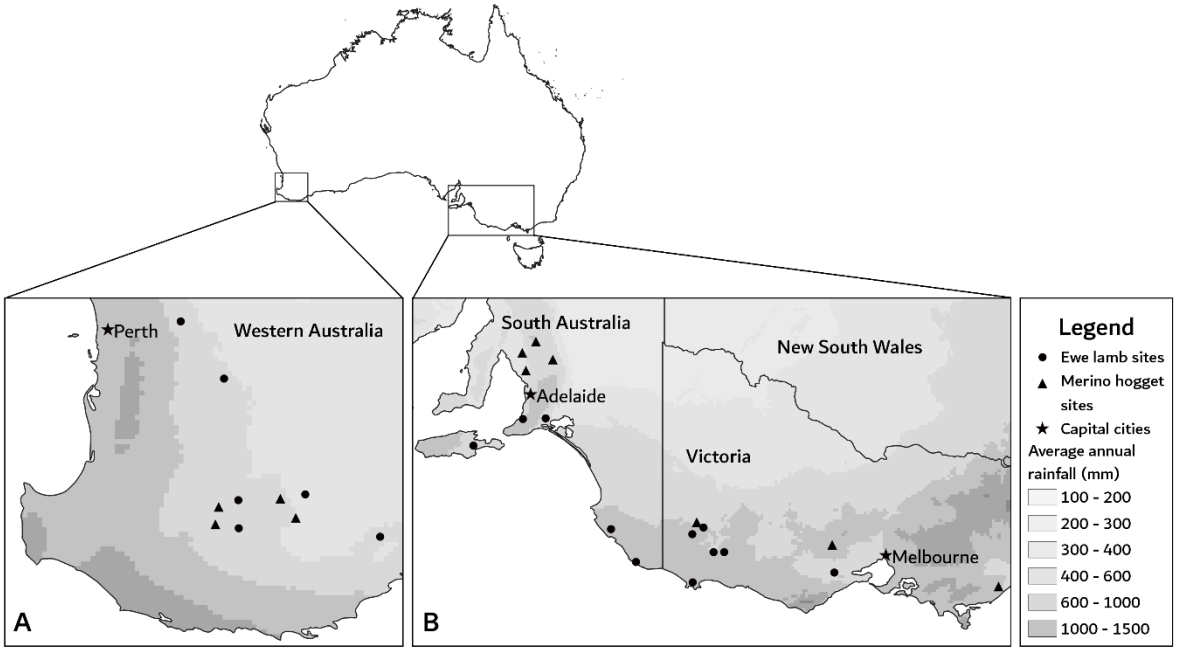

Figure 3: Approximate location of each farm where maiden ewes were monitored in (A) Western Australia and (B) South Australia and Victoria. Data for average annual rainfall was sourced from Australian Government Bureau of Meteorology (2021).

5.2.2 Animal measurements and sample collection

The study involved observations and measurements at five timepoints between joining and lamb marking, plus lambing rounds performed by farm staff. Lamb marking was performed approximately 6 weeks from the start of lambing (Table 14).

Table 14: Summary of timepoints, corresponding stage of the reproductive cycle and measurements performed throughout the study period for each flock.

Timepoint	Stage of reproductive cycle	Measurements
1. Joining	Approx. 0-14 days prior to joining	Ewes: CS and LW
2. First pregnancy scan (Scan 1)	Average of 85 days from the start of joining	Ewes: CS, LW and pregnancy ultrasound for foetal number

3. Second pregnancy scan (Scan 2)	Average of 118 days from the start of joining	Ewes: CS, LW and pregnancy ultrasound for foetal viability
4. Pre-lambing	Average of 138 days from the start of joining	Ewes: CS and LW
5. Lambing	Lambing period	Lambs: number of lambs born to each ewe, birth status of lambs (dead/alive) and total number of lambs born^
6. Lamb marking	Approx. 6 weeks from the start of lambing	Ewes: CS and LW Lambs: Number of lambs marked to each ewe and total number of lambs marked^

CS: condition score, LW: liveweight

^ Lambs were not tagged at birth for some flocks. In these flocks, number of lambs born per ewe was determined by distance observation and ewe lactation status was used to determine if the ewe reared a lamb.

5.2.3 Liveweight and condition score

Liveweight and condition score were recorded for ewes at the five timepoints as outlined in Table 14. Condition score was determined by palpation on a scale of 1 (very thin) to 5 (very fat) as described by Jefferies (1961) and van Burgel et al. (2011). All investigators had received training in body condition score assessment and where possible, condition score was assessed by the same person across all time points for each flock.

5.2.4 Assessment of pregnancy status and abortion

Ewes were pregnancy scanned using transabdominal ultrasonography twice during pregnancy (Table 14). Pregnancy scanning for foetal number and viability were performed by experienced researchers, veterinarians or private contractors. On average, the first pregnancy scan (Scan 1) was performed at 85 days from the introduction of rams (range 62-101). The second pregnancy scan (Scan 2) was performed at, on average, 118 days from the introduction of rams (range 107-136) and on average, 33 days (range 21-44) after the first scan. Where possible, scanning was performed by the same operator at Scan 1 and Scan 2 at a single site.

Ewes that were not pregnant, that is, no visible foetus or placentomes detected, were removed from the study after sampling at Scan 1. Pregnancy viability at Scan 2 was confirmed by detection of foetal heartbeats and/or vigorous foetal movements. Loss of pregnancy (*i.e.,* no foetus detected) or foetal mortality (*i.e.,* no evidence of foetal viability) between Scan 1 and Scan 2 was validated using lambing records and udder inspection at marking and will be defined as 'mid-pregnancy abortion' hereafter. Partial loss of foetuses (*e.g.* detection of twin pregnancy at Scan 1 and single pregnancy at Scan 2) was not detected as foetal number was not reliably determined at Scan 2. Therefore, any ewes that may have had partial loss of foetuses were not categorised as mid-pregnancy abortion.

Ewes were checked by farm staff at least twice weekly during pregnancy by observing ewes in their paddocks. For flocks where mid-pregnancy abortion was detected at Scan 2, ewes were subsequently checked at least every second day and farm staff were alerted to possibility of detecting aborted foetuses.

5.2.5 Measurements during the lambing period

Farm staff checked the lambing ewes once or twice daily throughout the lambing period. For flocks that tagged lambs at birth (*n*=19; Table 15), pregnant ewes were assigned a unique paint brand on their sides or fitted with a unique neck tag before lambing to enable the lamb birth type (single, twin

or triplet), birth status (dead or alive) and identification to be recorded against the dam identification within 24 hours of birth.

Maternal parentage was determined via DNA testing alone in one flock (Flock 10) and in combination with tagging at birth for two flocks (Flock 19 and 27). A combination of tagging at birth and proximity sensors at lamb marking were used to determine maternal parentage in one flock (Flock 1) (Sohi et al., 2017, Paganoni et al., 2021).

Maternal parentage of lambs was not determined for eight flocks (Table 15). In seven of these flocks, pregnant ewes were assigned a unique paint brand on their sides or fitted with a unique neck tag before lambing. Lamb birth type and birth status were recorded against the dam by observation and ewe lactation status was assessed at marking to determine if the ewe was rearing a lamb. For these flocks, the total number of lambs born per flock was estimated based on the number of lambs present at marking plus the number of dead lambs collected or observed during the lambing period. The number of lambs born may be underestimated because it is unlikely that all lambs that died were observed (*e.g.,* lost to predation). For Flock 24, lambing rounds were not performed, but a third pregnancy scan was performed at the pre-lambing visit (134 days from the start of joining) to determine if pregnancy losses had occurred since Scan 2 and these results were used as a proxy for the number of lambs born.

Table 15: The number of flocks which determined birth type and rear type of lambs born to ewe lambs and maiden hogget ewes by tagging at birth, DNA testing at marking, tagging at birth and using sensors for maternal pedigree at marking, and those using other methods to assess lamb birth type, lamb birth status and whether ewes were rearing a lamb.

Method of determining birth type, rear type, and maternal parentage of individual lambs	Ewe lambs (*n*)	Hoggets (*n*)
Tagging at birth	13	5
DNA testing at marking	2	1
Tagging at birth and sensors for maternal pedigree at marking	0	1
Other^	4	4

^ Individual lamb birth type, rear type and maternal parentage not determined. The total number of lambs born was estimated using a count of live lambs at marking plus the number of dead lambs recovered, or via a pregnancy scan at the pre-lambing timepoint. The number of lambs born per ewe was determined by distance observation. Ewe lactation status was used to determine whether ewes were rearing a lamb.

Lambs that were dead at lambing rounds (*i.e.*, died between birth and tagging) were categorised as 'born' and therefore were included in lamb mortalities between birth and marking. For flocks where lambs were tagged at birth, identification of live lambs was recorded at lamb marking to determine lamb survival to marking for individual ewes. Some dead lambs could not be allocated to a ewe but were included in the total count of number of lambs born at the flock-level. It is possible that some lambs that died were not recovered during lambing rounds and therefore the number of lambs born may be underestimated for some flocks.

Ewe udders were assessed at lamb marking to determine lactation status using the categories lactating (wet) or non-lactating (dry). Ewes that failed to lamb were identified using lambing records (no lamb/s allocated to ewe) and ewe lactation status at marking (dry). Rear type, or number of lambs reared to marking per ewe, was determined by recording ear tag identification of lambs

present at marking and validated using ewe udder assessment. Ewe mortalities during the lambing period were recorded by the farm staff during daily inspections.

5.2.6 Lamb cause of death and detection of infectious disease

Lambs that died in the first three days following birth were retained for necropsy to determine cause of death as described in detail and reported elsewhere by (Chapter 6). Tissue samples from aborted (*n* = 2) or stillborn (*n* = 33) lambs recovered from a subset of eight flocks (Flocks 1, 2, 3, 7, 11, 14, 16 and 19) were tested for evidence of infectious disease using microbial culture and/or molecular diagnostics as reported in Chapter 6. Ewe serology were performed for *Toxoplasma gondii* (Chapter 7), *Campylobacter* spp. (Chapter 10), *Neospora caninum* (Chapter 8) and *Coxiella burnetii* (Chapter 9) and reported elsewhere.

5.2.7 Quantitative variables

Conception rate (%) was calculated for each flock as the number of ewes pregnant at Scan 1 divided by the number of ewes present at joining. Scanning rate (%) was calculated for each flock as the number of foetuses identified at Scan 1 divided by the number of ewes present at joining.

Mid-pregnancy abortion frequency (%) was calculated using either number of foetuses that were lost or not viable at Scan 2 expressed as proportion of foetuses detected at Scan 1, or the number of ewes with evidence of abortion at Scan 2 expressed as proportion of ewes determined to be pregnant at Scan 1.

Foetal and lamb mortality between Scan 1 and marking were calculated using the number of foetuses identified at Scan 1, viability of pregnancy at Scan 2, number of lambs born and marked per ewe, and lactation status.

5.2.8 Statistical analyses

Data from Flock 6 and 9 (maiden Merino hogget ewe flocks) and Flock 25 (ewe lamb flock) were incomplete (Appendix 2 Table A2.1 and Appendix 2 Table A2.2). Data from these flocks were included in descriptive statistics, where appropriate, but excluded from analyses using General Linear Mixed Models (GLMM) where data for the outcome variable (mid-pregnancy abortion or overall lamb mortality) were incomplete.

Data were analysed by the following methods using GENSTAT (VSN International 2017). Estimates of mid-pregnancy abortion between Scan 1 and 2 were assessed by fitting GLMM. The approach used a logit transformation and binomial distribution. Using additive models, logits were predicted as a function of birth type (scanned single or multiple), liveweight and condition score at joining and interactions thereof as fixed effects while flock was fitted as a random effect. Estimates of overall mortality from Scan 1 to marking were assessed by fitting GLMM. The approach used a logit transformation and binomial distribution. Using additive models, logits were predicted as a function of birth type, liveweight and condition score at joining, liveweight and condition score change from joining to Scan 1 and interactions thereof as fixed effects while flock was fitted as a random effect.

Lamb mortality (%) between Scan 1 and Scan 2, Scan 2 and birth and birth and marking within flocks were compared using a 2-tailed Chi-square test. Statistical significance was accepted where $P \leq 0.05$.

5.3 Results

5.3.1 Mid-pregnancy abortion – ewe lambs

A total of 2968 pregnant maiden ewe lambs from 19 flocks were included in this study. Mid-pregnancy abortion was observed in 14/19 (73.7%) flocks (Appendix 2 Table A2.1). The frequency of mid-pregnancy abortion was ≥2% of ewes in 6/19 (31.6%) flocks (Appendix 2 Table A2.1). The frequency of mid-pregnancy abortion ranged from 2.1-50% for ewes in these flocks, representing 3.4-48.4% of foetuses identified at Scan 1 (Table 16).

Mid-pregnancy abortion accounted for 12.4% of overall lamb mortality between Scan 1 and marking. However, there was considerable between-flock variation (Appendix 2 Table A2.1). The majority (63.6%) of mid-pregnancy abortions were observed on four farms (Flock 3, 14, 19 and 24) in which mid-pregnancy abortion accounted for 16.4- 68.1% of total foetal and lamb mortalities observed between Scan 1 and marking (Table 16 and Appendix 2 Table A2.1).

Visual evidence of abortion such as aborted foetuses, retained foetal membranes, vaginal discharge or staining of the perineal region or hindlegs before the expected lambing date were noted for five flocks. Aborted foetuses were recovered from only three flocks, all of which were between Scan 2 and the start of lambing (Appendix 2 Table A2.1).

Mid-pregnancy abortion was observed in both Flock 3 (6.2% foetuses; 2018) and Flock 14 (21.7% foetuses; 2019) which were located on the same farm (Appendix 2 Table A2.1). For the other farm that was sampled over consecutive years, mid-pregnancy abortion was observed in Flock 19 (8.6% foetuses, 2019), but not Flock 27 (2020). Campylobacteriosis was diagnosed in Flock 19 by microbial culture, and vaccination for *Campylobacter* spp. was implemented by the farmer for Flock 27 in 2020.

Table 16: Overall reproductive performance and timing of foetal and lamb mortalities for 19 flocks of ewe lambs and 11 flocks of maiden Merino hogget ewes across southern Australia between 2018 and 2020.

	Ewe lambs		Merino hoggets	
	Mean	**Range**	**Mean**	**Range**
Conception rate (%)[1]	73.4	45.4 – 92.4	87.1	58.5 – 97.1
Scanning rate (%)[2]	112.7	57.7 – 155.6	104.7	59.6 – 135.2
Mid-pregnancy abortion				
% foetuses [3]	5.5	0 - 48.4	0.8	0 - 3.7
% ewes [4]	5.7	0 – 50.0	0.9	0 - 4.4
Foetal loss between scan 2 & birth (% foetuses) [5]	10.5	0 - 27.5	10.3	0 - 40.2
Lamb mortality between birth & marking (% lambs) [6]	18.0	8.7 - 28.1	19.0	10.6 – 26.0
Overall foetal/lamb mortality between Scan 1 & marking (% foetuses)	35.8	14.3 – 71.1	29.4	19.7 – 52.7

[1] Number of ewes pregnant at Scan 1/number of ewes joined (%)
[2] Number of foetuses identified at Scan 1/number of ewes joined (%)
[3] Number of foetuses lost between Scan 1 and Scan 2/number of foetuses identified at scan 1 (%)
[4] Number of ewes with foetal loss between Scan 1 and Scan 2/number of ewes joined (%)
[5] Number of foetuses present at Scan 2 but not accounted for at lambing/number of foetuses identified at Scan 1 (%). Includes lambs that were born and not recovered at lambing rounds (*i.e.,* lost to predation).
[6] Number of lambs that died between birth and marking/number of foetuses identified at Scan 1 (%). Includes lambs dead at birth (full-term).

5.3.2 Timing of foetal and lamb mortality – ewe lambs

Timing for abortion and lamb mortality in ewe lamb flocks are shown in Table 16. On average, 35.8% of foetuses identified at Scan 1 failed to survive to lamb marking (Table 16). The relative contribution of foetal or lamb mortality within each time period varied between flocks (Appendix 2 Table A2.1). Lamb mortality between birth and marking was the largest contributor to lamb mortality for most farms (Appendix 2 Table A2.1). Ewe mortalities (*n*=28) during the lambing period resulted in mortality for 0.83% foetuses (*n*=38) identified at Scan 1.

5.3.3 Factors associated with foetal and lamb mortality - ewe lambs

Liveweight and condition score for single- and multiple-bearing ewe lambs are shown in Table 17. Average liveweight and body condition score at joining for single-bearing ewes were 48.0kg and 3.3 respectively, and for multiple-bearing ewes were 49.8kg and 3.4 respectively (Table 17). Liveweight and condition score at joining, liveweight change from joining to Scan 1, and had no effect on mid-pregnancy abortion ($P>0.05$), nor overall lamb mortality between scanning and marking ($P>0.05$).

Lamb birth type (litter size) had no effect on mid-pregnancy abortion (Table 17). Overall lamb mortality between scanning and marking was lower for lambs scanned as singles (24.5%) compared to lambs scanned as multiples (twins or triplets; 31.2%) ($P<0.001$).

Table 17: Mean of flock means for liveweight and body condition score for single- and multiple-bearing ewes from ewe lamb and maiden Merino hogget ewe flocks

	Mean liveweight and body condition score (range)			
	Ewe lamb		Merino hogget ewes	
	Single	Multiple	Single	Multiple
Liveweight (kg)				
Joining [1]	48.0 (39.7 - 65.6)	49.8 (40.4 - 67.4)	48.6 (37.5 - 67.5)	49.2 (42.3 - 67.8)
Scan 1 [1]	50.5 (41.0 - 65.6)	53.5 (41.9 - 67.6)	50.8 (37.9 – 73.0)	53.8 (49.3 - 75.1)
Scan 2 [2]	51.0 (34.2 - 68.5)	56.3 (36.3 - 72.6)	51.9 (38.4 - 68.3)	56.1 (48.0 - 69.3)
Pre-lambing [2]	55.4 (39.7 - 77.6)	61.9 (41.9 - 82.9)	55.9 (41.0 - 76.5)	61.9 (52.1 – 78.0)
Body condition score				
Joining [1]	3.3 (2.4 – 4.0)	3.4 (2.4 - 4.0)	2.9 (2.6 - 3.6)	3.0 (2.6 - 3.5)
Scan 1 [1]	3.4 (2.8 – 4.0)	3.4 (2.7 – 4.0)	2.9 (2.6 - 3.6)	3.0 (2.6 - 3.4)
Scan 2 [2]	3.3 (2.6 - 3.9)	3.3 (2.7 – 4.0)	2.8 (2.0 - 3.6)	2.9 (2.5 - 3.7)
Pre-lambing [2]	3.3 (2.7 - 4.1)	3.4 (2.6 - 4.1)	2.8 (2.5 - 3.5)	2.8 (2.5 - 3.5)

[1] Flocks 6 and 9 excluded from analyses
[2] Flocks 6, 9 and 25 excluded from analyses.

5.3.4 Mid-pregnancy abortion – Merino hogget ewes

A total of 1886 pregnant maiden Merino ewe hogget ewes from 11 flocks were included in this study. Mid-pregnancy abortions were detected in 6/11 (54.5%) flocks (Appendix 2 Table A2.2) and accounted for mortality of 0.8% foetuses (Table 16). Mid-pregnancy abortion contributed 2.8% of overall lamb mortality between Scan 1 and marking.

The frequency of mid-pregnancy abortion was ≥2% of ewes in 2/11 (18.2%) flocks where abortion frequency ranged 2.2-4.4% pregnant ewes and 1.7-3.7% of foetuses identified at Scan 1 (Appendix A2.2 Table 2). No aborted foetuses were recovered from hogget ewe flocks. Blood staining on the hindlegs of two ewes was observed in Flock 13 where 0.9% of ewes had mid-pregnancy abortion, but no visual evidence of abortion was reported in other flocks.

5.3.5 Timing of foetal and lamb mortality – Merino hogget ewes

On average, 29.4% of foetuses identified at Scan 1 failed to survive to lamb marking for Merino hogget ewes (Table 16). As with the ewe lambs, the relative contribution of foetal or lamb mortality within each time period varied between flocks (Appendix 2 Table A2.2). Lamb mortality between birth and marking was the greatest contributor to foetal and lamb mortality (Appendix 2 Table A2.2). Ewe mortalities (*n*=7) during the lambing period resulted in mortality of 0.35% foetuses (*n*=8) identified at Scan 1.

5.3.6 Factors associated with foetal and lamb mortality – Merino hogget ewes

Liveweight and condition score for single- and multiple-bearing maiden Merino hogget ewes are shown in Table 17. Average liveweight and body condition score at joining for single-bearing ewes were 48.6kg and 2.9 respectively, and for multiple-bearing ewes were 49.2 and 3.0 respectively (Table 17). Liveweight and condition score at joining, and liveweight change from joining to Scan 1 had no effect on mid-pregnancy abortion (*P*>0.3), nor overall foetal and lamb mortality between scanning and marking.

Mid-pregnancy abortion was 1.63% greater for single foetuses compared to multiple foetuses (*P*=0.022; Table 18). However, overall mortality between Scan 1 and marking for lambs scanned as multiples was greater than lambs scanned as singles (47.4% vs. 30.3%; *P*<0.001).

Table 18: Transformed and back-transformed means for frequency of mid-pregnancy abortion in maiden ewe lambs and Merino hogget ewes determined using General Linear Mixed Model

	Mid-pregnancy abortion frequency Transformed data (back transformed mean)[1]	
Litter size	Ewe lambs	Hoggets
Single	-4.21 (1.46%)	-3.91 (1.98%)
Multiples	-4.37 (1.25%)	-5.67 (0.34%)
Standard error of the difference	0.256	0.771

[1] Number of foetuses aborted between Scan 1 and Scan 2 / number of foetuses detected at Scan 1 (%)

5.4 Discussion

Pregnancy scanning rates for maiden ewes and mortality of their lambs between pregnancy scanning and lamb marking varied widely between flocks. Lamb mortality was greatest between birth and marking for maiden Merino hogget ewe flocks and most flocks of ewe lambs. However, abortion during mid- and late-pregnancy were significant contributors to overall lamb mortality for some ewe lamb flocks. Therefore, our hypothesis that abortion will be a significant contributor to lamb mortality between scanning and lamb marking for maiden ewes is partially accepted. Over 50% of foetuses identified at pregnancy scanning subsequently aborted or died in some flocks, representing substantial production and economic losses for these enterprises. However, evidence from other flocks in our study shows that mortality below 20% is achievable for lambs born to ewe lambs and maiden Merino hogget ewes in southern Australia. Our observations indicate that strategies which reduce perinatal lamb mortality should be prioritised for improving lamb survival for maiden ewes. However, identifying and addressing mid-pregnancy abortions may improve reproductive performance in some ewe lamb flocks. The causes of mid-pregnancy abortion are poorly understood and further investigation of the pathophysiology and economic losses associated with mid- and late-pregnancy abortion are warranted. This will inform cost-benefit analyses for interventions to address these losses.

The frequency of mid-pregnancy abortion was greater than the widely accepted ‘normal’ level of abortion of 2% for one third of ewe lamb flocks. The mean frequency of mid-pregnancy abortion of 5.7% across ewe lamb flocks was consistent with the apparent 9% foetal mortality between scanning and birth for non-Merino ewes up to 18 months of age at lambing recorded on the Maternal Sheep Genetics Australia database (Daniel Brown, pers. comms). It is also consistent with studies from New Zealand which reported that the frequency of abortion ranged from 3.7-8.1% in commercially managed ewe lamb flocks based on sequential scanning performed at similar timepoints to our study (Ridler et al., 2017, Ridler et al., 2015). In contrast to ewe lambs, the mean frequency of mid-pregnancy abortion was less than 1% for Merino hogget ewe flocks, making mid-pregnancy abortion a minor contributor to overall lamb mortality in these flocks. This was consistent with an Australian study reporting an apparent 3% foetal mortality between scanning and birth in mixed age ewes, although this may have included lambs that died soon after birth but were not identified at lambing rounds (Geenty et al., 2014).

Mid- to late-pregnancy abortion was largely inconspicuous, even in flocks with a high frequency detected by scanning. Evidence of abortion such as an aborted foetus, retained foetal membranes or bloodstaining of the hind legs was only observed in five ewe lamb flocks. In most cases this was detected by research staff because of additional monitoring of sheep occurring as part of the study. It is likely that some foetal mortality during mid-pregnancy resulted in resorption of the foetus. Otherwise, paddock topography, predation, and the length of pasture in good seasons make it challenging for farmers to detect aborted foetuses or foetal membranes during inspection of flocks, particularly given the small size of the foetus with mid-pregnancy abortion. Disease investigation of abortion cases is more likely to result in aetiological diagnosis where placental tissue is available (Chapter 4). For flocks where mid-pregnancy abortion is suspected, farmers should be advised to be extra vigilant to identify aborting ewes, and where possible, collect material to rule out infectious causes.

Errors in assessing foetal number at scanning may contribute to error in estimation of abortion and lamb mortality between scanning and marking. In this study, the use of sequential pregnancy scans was an effective strategy for determining mid-pregnancy abortion given that complete pregnancy loss between Scan 1 and Scan 2 was the principle measure used to identify ewes with mid-pregnancy abortion. However, the frequency of abortion, including late pregnancy abortion, may be underestimated using only two scans at the timepoints used in this study. Further, distinguishing late-pregnancy abortion from perinatal lamb deaths is challenging on extensively managed sheep farms due to issues recovering dead lambs related to predation and paddock characteristics. A third pregnancy scan (after day 130 of pregnancy) identified late-pregnancy abortion (pregnancy loss between Scan 2 and pre-lambing) in some ewes that had viable pregnancies at Scan 2 for Flock 24. Therefore, a later second scan or third scan may help to quantify *in utero* losses and distinguish these from perinatal losses in flocks with evidence of mid-pregnancy abortion. The decision to use additional scans to identify flocks with mid-pregnancy abortions should consider the costs associated with an additional scan and risk of handling multiple-bearing ewes in late gestation.

Mid-pregnancy abortion was not associated with either liveweight or condition score at joining or liveweight change in early pregnancy. This is in agreeance with studies in ewe lambs in New Zealand under pastoral conditions (Kenyon et al., 2008, Morris et al., 2005, Mulvaney et al., 2010). By contrast, Mulvaney et al. (2008) and Ridler et al. (2015) reported that a higher frequency of abortion was associated with lower liveweight at joining and lower liveweight gain in early pregnancy. High liveweight gains during early pregnancy were also associated with foetal mortality in the study by Mulvaney et al. (2008). However, the overall average pre-joining liveweight in this study (48kg for single-bearing ewes and 49.8kg for multiple-bearing ewes) was higher than the pre-joining liveweight of ewe lambs in the study by Mulvaney et al. (2008) (36kg) which may explain why we observed no association between liveweight at joining and mid-pregnancy abortion. Whilst standardized methods were used for measurement of body condition score, assessment is subject to operator bias (van Burgel et al., 2011). Calibration of body condition score measurement between assessors could have reduced between-operator variability in body condition score assessment.

Overall lamb mortality in ewe lamb flocks (36%) was within the range of 19-43% observed for the progeny of ewe lambs on commercial farms in New Zealand (Kenyon et al., 2014b, Thompson et al., 2021). There was marked variation between farms and even between years on the same farms joining ewe lambs as per Thompson et al. (2021). However, this variation was not explained by liveweight at joining, liveweight profile during early pregnancy or lamb birth type. Inconsistent reproductive performance in ewe lambs has been identified as a barrier to adoption of joining ewe lambs (Kenyon et al., 2004). Whilst ewe lamb reproductive performance can be improved by

optimising ovulation and reproductive rate by liveweight at joining (Rosales Nieto et al., 2013a, Paganoni et al., 2014a, Rosales Nieto et al., 2015), observations from this study indicate that efforts to reduce foetal and lamb mortality between scanning and marking will also be valuable in improving the reproductive output of ewe lamb flocks.

Average overall lamb mortality between scanning and marking in maiden Merino hogget ewe flocks in this study (29%) was comparable to other studies reporting 33% lamb mortality in Merino hogget ewe flocks (Kilgour, 1992), and within the lamb mortality range of 25-30% reported for mixed age Merino ewes (Allworth et al., 2017). As with ewe lambs, the reproductive performance of maiden Merino hogget ewes can be improved with interventions to optimise fertility and fecundity (Kleemann and Walker, 2005). However, findings from our study suggest that there is scope to improve maiden hogget ewe reproductive performance with interventions that improve lamb survival, and especially in the period between birth and marking.

Most lamb deaths occurred at or after birth. Lamb necropsies were conducted on a subset of farms and are reported elsewhere (Chapter 6). Dystocia, stillbirths, and starvation-mismothering accounted for most mortalities which is consistent with other Australian studies (Bruce et al., 2021, Hinch and Brien, 2014b). Higher rates of mortality were observed for multiple-born lambs which is consistent with previous observations for predominantly mature ewe flocks (Kleemann and Walker, 2005, Lockwood et al., 2020, Lockwood et al., 2019a, Paganoni et al., 2014b, Oldham et al., 2011). However, there was considerable variation in lamb mortality between birth and marking between flocks in this study. This may be explained by differences in genetics, environmental factors and other management factors (*e.g.* mob size and supplementary feeding) on lamb survival (Refshauge et al., 2016, Lockwood et al., 2020, Jacobson et al., 2020, Hinch and Brien, 2014b). The use of lamb necropsies to identify cause of death can be used to inform targeted strategies for improving perinatal lamb survival. Conducting necropsies also provides opportunity for detection of infectious diseases that may be associated with abortion or poorer lamb viability. Identification of factors impacting lamb survival in the perinatal period informs targeted strategies that can improve reproductive performance of maiden flocks.

We did not identify any relationship between ewe condition score or liveweight at joining or liveweight profile of and overall foetal and lamb mortality in ewe lamb flocks. This was consistent with other recent work reporting that liveweight at joining and liveweight profile may have relatively little impact on the mortality of lambs born to ewe lambs (Thompson et al., 2021). A range of other factors could have contributed to abortion and perinatal mortality. Stillbirths, dystocia and starvation-mismothering accounted for the majority of perinatal mortalities identified by gross pathology for the subset of flocks for which lamb necropsies were performed (Chapter 6). These causes of perinatal mortality are often multifactorial and related to genetic, environmental and management factors (Hinch and Brien, 2014b, Jacobson et al., 2020).

Sporadic impacts of endemic diseases could also explain between-flock variation in lamb mortality and absence of relationship with ewe liveweight or liveweight profile. There was evidence that endemic diseases were contributing to abortions and stillbirths in some flocks in this study. For example, abortions, stillbirths and polyarthritis associated with *Chlamydia pecorum* were identified in a subset of flocks in this study (Chapter 6)(Ostfeld et al., 2020), and campylobacteriosis (*Campylobacter fetus* fetus) was identified in one flock (Chapter 10). There was no evidence that infection with *Toxoplasma gondii* (Chapter 7), *Neospora caninum* (Chapter 8) or *Coxiella burnetii* (Chapter 9) were important contributors to foetal and lamb wastage in these flocks. Younger ewes are more susceptible to some endemic diseases because they are less likely to have developed immunity through previous exposure to infection. Disease investigations are warranted for maiden

ewe lamb flocks with disappointing or inconsistent lamb survival to inform targeted strategies addressing lamb survival. Further studies to improve understanding of the causes of mid- and late-pregnancy abortion in Australian ewes will inform targeted strategies addressing abortion and lamb survival.

5.5 Conclusion

Lamb mortality between scanning and lamb marking is highly variable for maiden ewe flocks. Perinatal lamb deaths represent the most important source of reproductive loss between scanning and marking in most maiden ewe flocks. However, significant *in utero* losses occur due to mid-pregnancy abortion in some flocks. Liveweight or condition score at joining or liveweight change in early pregnancy had no effect on mid-pregnancy abortion or overall lamb mortality in these flocks. Sequential pregnancy scanning can be used to identify mid-pregnancy abortion and differentiate *in utero* losses from perinatal mortality. Disease investigations are warranted for maiden ewe flocks with evidence of abortion or disappointing lamb survival. Differentiating losses associated with mid- and late-pregnancy abortion from perinatal losses and determination of aetiological diagnosis where infectious disease is implicated will inform strategies to improve reproductive performance and lamb survival in maiden ewe flocks.

6. Causes of abortion and perinatal lamb deaths: necropsies and detection of infectious disease

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6.1 Introduction

Improving lamb survival is an important economic and welfare issue for sheep industries worldwide. Approximately 10% of single-born lambs and 30% of twin-born lambs die prior to weaning under extensive grazing conditions across Australia, with most losses occurring in the first 48 hours of life (Hinch and Brien, 2014b, Refshauge et al., 2016, Oldham et al., 2011). The starvation-mismothering-exposure complex, stillbirths, and dystocia are the most common causes of lamb mortality during the perinatal period (Hinch and Brien, 2014b, Refshauge et al., 2016, Jacobson et al., 2020). Lower lamb survival has been reported for maiden ewes compared to adult flocks in Australia (Kleemann and Walker, 2005, Knight et al., 1975, Kilgour, 1992) and overseas (Kenyon et al., 2014b, Knight et al., 1975, Young et al., 2010, McMillan, 1983, Pettigrew et al., 2018). However, causes of mortality for lambs born to maiden ewes are not well described and it is not clear if the main factors contributing to lamb mortality in maiden ewes are similar to those for multiparous ewes.

Infectious diseases may contribute to lamb mortality through abortion, stillbirths and birth of weak lambs that are more likely to die soon after birth. Campylobacteriosis, listeriosis and toxoplasmosis were the most common aetiological agents identified in sheep abortion investigations submitted to Australian veterinary diagnostic laboratories between 2006 and 2019 (Chapter 4)(Refshauge et al., 2020b). This is consistent with older reports describing these as the most common infectious causes of abortion and perinatal mortality in Australian sheep (Broadbent, 1975, Dennis, 1974b, Plant et al., 1972, Munday et al., 1966). Sporadic abortion associated with *Chlamydia pecorum* has been reported in sheep from Australia (Chapter 6)(Westermann et al., 2021) and overseas (Clemente et al., 2011, Williams, 2019), but the epidemiology of *C. pecorum*-associated abortion in sheep remains poorly understood.

Maiden ewes may be more susceptible to infectious diseases, as younger ewes are less likely to have developed immunocompetency to infection prior to pregnancy (Quinlivan and Joppt, 1982, Dempster et al., 2011). Most recent Australian studies that included lamb necropsies were conducted with multiparous ewes, and cause of death was assigned based on gross post-mortem findings without adjunct laboratory investigation (Refshauge et al., 2016, Lockwood et al., 2019a,

Lockwood et al., 2019b, Holst et al., 2002, Behrendt et al., 2019, Robertson et al., 2020). In general, relatively few investigations for abortion and perinatal lamb death are submitted to veterinary diagnostic laboratories for exclusion of infectious diseases (Chapter 4). Consequently, the role of infectious diseases as a contributor to mortality of lambs born to maiden ewes are not well described and it is possible that the contribution of infectious disease to perinatal mortality may be underrecognized.

The aim of this study was to determine the common causes of perinatal death for lambs born to maiden ewes, and whether infectious disease was implicated. In doing so, we identified *C. pecorum* in a surprisingly high proportion of aborted and stillborn lambs from multiple farms, and subsequently expanded the study to determine molecular characteristics for *C. pecorum* strains detected in aborted and stillborn lambs.

6.2 Methodology

All procedures for monitoring ewes and sample collection were conducted according to guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes, and were approved by the Murdoch University Animal Ethics Committee (R3004/17).

6.2.1 Animals and study sites

Eleven flocks of maiden ewes from ten farms were monitored between the start of joining and lamb marking at approximately 6 weeks from the start of lambing (Table 19). All farms were located in southern Western Australia in a region with Mediterranean climate characterised by hot dry summer and cool wet winter.

On each farm, Merino or non-Merino ewes were mated as either ewe lambs (7-10 months, *n* = 7 flocks) or maiden hogget ewes (18-20 months, *n* = 4 flocks; Table 19). All rams were of the same breed as the ewes to which they were joined. Rams were confirmed seronegative for *Brucella ovis* prior to joining using a modified complement fixation test (Corner, 1987) where three local *B. ovis* isolates were used as the antigen (DDLS freeze-dried culture collection numbers 0735, 1655, 1794). On two farms, ewes were artificially inseminated followed by a period of natural joining. All other flocks were mated naturally with an average joining period of 38 days (range 32-46 days). All ewes were managed as per standard farm practice including use of body condition monitoring to guide nutrition and grazing management, with no experimental interventions imposed by this study other than monitoring of ewes and lambs as described (Table 19).

Table 19: Characteristics of maiden ewe flocks from Western Australia

Flock code	Year	Location	Ewe breed	Ewe age at joining (months)	Pregnant ewes (*n*)[A]	Pre-lambing body condition score[B]
Hoggets						
1	2018	Kojonup	Merino	18-20	186	2.7
2	2018	Kojonup	Merino	18-20	178	2.7
10	2019	Broomehill	Merino	18-20	169	2.5
15	2019	Katanning	Merino	18-20	204	2.8
Ewe lambs						
3[D]	2018	Narrogin	Composite[C]	7-9	148	3.1
14[D]	2019	Narrogin	Composite[C]	7-9	168	3.1
4	2018	York	Composite[C] & White Suffolk	7-9	130	3.1
7	2019	Kojonup	Composite[C]	7-9	151	3
8	2019	Katanning	Merino	7-9	86	2.7
11	2019	Kojonup	Dorper	8-10	146	3
16	2019	Ongerup	White Suffolk	7-10	103	3.1

[A] Determined by transabdominal ultrasound conducted 62-87 days from the start of joining or artificial insemination
[B] Average body condition score of the flock assessed approximately 140 days from the start of joining
[C] Composite: mixed (non-Merino) breed ewes
[D] Flock 3 and 14 located on same farm, sampled different years

6.2.2 Measurements

Ewes were pregnancy scanned via transabdominal ultrasonography at 62-87 days from the start of joining to determine litter size and foetal viability. Ewe body condition score was recorded at approximately 140 days from the start of joining using a scale of 1 (very thin) to 5 (very fat) as previously described (Jefferies, 1961).

Farm staff checked the lambing flocks once or twice daily throughout the lambing period. Lambs were identified with an ear tag and their birth type and dam pedigree were recorded within 24 hours of birth for most (8/11) flocks. The total number of lambs born for each flock was calculated using records of the number of lambs tagged at birth plus the number of dead lambs collected. On farms where tagging at birth was not performed, the number of lambs born was calculated based on number of lambs present at marking plus the number of dead lambs collected. Number of lambs born may have been underestimated at these sites because it is unlikely that all lambs that died were recovered for necropsy.

6.2.3 Lamb necropsies and sample collection

Lambs that died in the first three days following birth were retained for necropsy to determine cause of death. Dead lambs were either refrigerated (4^{o}C) or frozen (-20^{o}C) for up to five days before necropsies were performed. One aborted foetus and one foetal membrane were also recovered from Flock 3 prior to the start of lambing and submitted for necropsy and diagnostic testing for infectious agents.

Lamb necropsies were performed by a single person using methods described by Everett-Hincks and Duncan (2008). Briefly, post-mortem examination included recording the weight, sex and details of the external appearance of the lamb along with gross examination of thoracic and abdominal organs. Brain tissues were assessed for lesions only in lambs that had not been frozen. Cause of death was classified according to methods previously described (Everett-Hincks and Duncan, 2008), and described in more detail in Table 20.

Table 20: Cause of death classifications modified from Everett-Hincks and Duncan [30]

Classification	Post-mortem observations
Dystocia	Evidence of oedema to the head or neck
Stillborn	Full-term appearance; not walked or breathed
Abortion/prematurity	Pre-term appearance (size, wool covering); not walked or breathed
Starvation-mismothering-exposure complex	Evidence that had walked and breathed Empty stomach contents Mobilisation of peri-renal and peri-cardial fat
Other	Cause of death determined based on the gross appearance of affected organ systems

Tissue samples from aborted or stillborn lambs from flocks with at least two lambs classified as abortion or stillbirth were submitted to the Department of Primary Industry and Regional Development Diagnostic Laboratory Service (South Perth, Western Australia). The type of tissues submitted varied between cases (Appendix 3 Table A3.1), with liver and placenta submitted for all cases except where these were not available due to predation.

6.2.4 Laboratory investigation

Histology, bacteriology and molecular diagnostics for endemic and exotic abortigenic agents were performed by the Department of Primary Industry and Regional Development's Diagnostics and Laboratory Services. Bacteriology comprised culture on blood agar plus selective culture for *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp.. Molecular testing included polymerase chain reactions (PCRs) for *Brucella* spp., *Campylobacter* spp., *Leptospira* spp., *Toxoplasma gondii*, *Coxiella burnetii*, pestiviruses and *Chlamydia* spp. that are described in more detail below.

All aborted tissue samples underwent routine bacteriological culture on Columbia agar (Oxoid) with 5% equine blood and MacConkey agar (Oxoid). Additionally, foetal liver, cotyledon and placenta samples were subject to selective isolation for *Listeria, Salmonella* and *Campylobacter* (PathWest Media, Western Australia). All cultures were incubated at 37°C with 5% CO_2 except for the *Campylobacter* cultures which were incubated under microaerophilic conditions.

Histopathology was performed on formalin fixed tissues processed to haematoxylin and eosin (H&E) slides; a subset of cases was also subjected to immunohistochemistry. Representative specimens were processed from 10% buffered formalin solution to paraffin embedded tissue in a Logos Milestone histological processer and blocked using standard histological techniques. Sections were trimmed at 4µm thickness and stained to H&E in a Leica autostainer XL with Leica CV5030 coverslipper. Selected sections were subject to immunohistochemistry with an anti-*Chlamydia* polyclonal antibody (B47829R, Progen) and an anti-*Toxoplasma gondii* polyclonal antibody (B65201R, Biodesign). Both antibodies were visualised using the Dako Envision Dual-link system and Dakocytomation DAB+ (both Dako, Agilent) according to the manufacturers' instructions.

6.2.5 Molecular testing – Nucleic acid extraction

DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen) and run on the automated Qiacube platform (Qiagen) following the Purification of DNA from tissues protocol. RNA was extracted using the MagMAX-96 Viral RNA Isolation Kit (Thermo Fisher Scientific) on the MagMAX Express-96 (Thermo Fisher Scientific) magnetic bead processor.

6.2.6 Molecular testing – *Chlamydia* spp.

Chlamydia quantitative polymerase chain reactions (qPCRs), targeting the outer membrane protein A (*omp*A) gene, were performed on all foetal liver, cotyledon and placenta samples using species-specific assays for the detection of *C. pecorum* (Pantchev et al., 2010), *C. abortus* and *C. psittaci* (Pantchev et al., 2009). Positive *C. pecorum* detections were confirmed via 298 bp and 806 bp *Chlamydiales* 16S rRNA gene fragments PCRs (Everett et al., 1999) followed by Sanger sequencing.

Chlamydia qPCR assays were performed in 25 µL reaction volumes containing 0.5 µM of each primer, 0.2 µM of probe, 12.5 µL of Rotor-Gene Multiplex Master Mix (Qiagen) and 5 µL of extracted DNA. The qPCR reactions were run on a Rotor-Gene Q (Qiagen) real-time PCR cycler under the following conditions: initial denaturation at 95^{o}C for 5 min followed by 45 cycles of 95^{o}C for 15 sec and 60^{o}C for 15 sec with fluorescent probe acquisition occurring during the 60^{o}C annealing/extension step. A 25 µL reaction volume was also used for the *Chlamydiales* PCRs and contained 0.4 µM of each primer, 12.5 µL of HotStarTaq Master Mix (Qiagen) and 5 µL of extracted DNA. Conventional PCR was performed on a DNA Engine (Bio-Rad) thermal cycler under the following conditions: initial denaturation at 95^{o}C for 5 min followed by 40 cycles of 94^{o}C for 30 sec, 55^{o}C for 45 sec and 72^{o}C for 45 sec with a final elongation step of 72^{o}C for 7 min. To minimise contamination risk synthetic positive control gBlocks Gene Fragments (Integrated DNA technologies) were designed for all PCRs.

Conventional PCR amplicons were purified using the Qiaquick PCR purification kit (Qiagen) and forward and reverse sequencing reactions were prepared in 12 µl volumes containing approximately 12–18 ng of PCR product and 9.6 pmol of primer. All amplicons were sequenced at the Australian Genome Research Facility (AGRF Perth Node) and sequence and BLAST analysis was performed using Geneious R11 (Geneious).

6.2.7 Molecular testing – other abortifacents

Screening for *Brucella* spp. (Ouahrani-Bettache et al., 1996), *Campylobacter* spp. (Linton et al., 1996), *Coxiella burnetii* (Banazis et al., 2010, Lockhart et al., 2011), pathogenic *Leptospira* spp. (Smythe et al., 2002), *Toxoplasma gondii* (VetMAX *T. gondii* Kit - Thermo Fisher Scientific) and Pestivirus (Hyndman et al., 1998, Hoffmann et al., 2006) was undertaken via PCR. Further screening for *Brucella* spp. (López-Goñi et al., 2008) was performed at The Australian Centre for Disease Preparedness, Geelong, Victoria. All primer sequences, probes, final concentrations, and cycling conditions for diagnostic PCRs and molecular testing performed in this study are outlined in Appendix 3 Table A3.2.

6.2.8 *C. pecorum* genotyping by Multi Locus Sequence Typing (MLST) and ompA

Prior to genotyping, the *C. pecorum* positive DNA samples from six liver and two cotyledon samples taken from eight aborted and/or stillborn lambs from four different farms (Appendix 3 Table A3.1) were quantified for *C. pecorum* genome copy number (tested in duplicate) using standard curve calibrated and High Resolution Melt (HRM) *C. pecorum* qPCR assay (Jelocnik et al., 2019a). The genome copy number/µl in samples ranged from 3.41×10^{2} – 2.47×10^{5} copies/µl DNA template, with geometric mean of 7.26×10^{3} copies/µl (Table 21). The *C. pecorum*-specific MLST (Jelocnik et al., 2014a, Jelocnik et al., 2019b) and *ompA* gene sequence analyses (Mohamad et al., 2014) are the most commonly used molecular typing targets for *C. pecorum*, due to their recognised congruence with whole-genome phylogeny. Full-length *omp*A genotyping (Islam et al., 2019) and *C. pecorum*

MLST (Jelocnik et al., 2019b) were applied as previously described to these eight *C. pecorum* positive DNA samples.

The resultant MLST sequences were confirmed for sequence type (ST) by using the online *Chlamydiales* PubMLST database (Jolley et al., 2018). Both concatenated *C. pecorum* MLST, and *omp*A sequence and phylogenetic analyses were performed in GeneiousPrime 2020 (Geneious). The concatenated MLST sequences of eight samples from this study were aligned using ClustalOmega (as implemented in Geneious) to other 36 publicly available livestock *C. pecorum* MLST sequences retrieved from the *Chlamydiales* PubMLST database(Jolley et al., 2018). Using the concatenated MLST sequences 3095 bp alignment for the 44 *C. pecorum* global and Australian livestock strains, we have constructed a mid-point rooted approximately-maximum-likelihood phylogenetic tree, using FastTree 2.1.11 (Price et al., 2010).

The *omp*A sequences from this study were analysed by BLASTn (BLASTn) to evaluate their % sequence similarity to Top BLAST hits, and aligned using ClustalOmega (as implemented in Geneious) to other publicly available *C. pecorum omp*A sequences retrieved from GenBank (GenBank). Using the 980 bp *omp*A alignment for the eight *C. pecorum* strains described in this study and additional 20 previously described strains, we constructed a mid-point rooted Bayesian phylogenetic tree, using MrBayes (Huelsenbeck and Ronquist, 2001) as implemented in GeneiousPrime (Figure 4). The tree parameters included: GTR + I+ G nucleotide substitution model, with four Markov Chain Monte Carlo chains of million generations, subsampled every 10 000 runs, and 100 000 trees discarded. The *omp*A sequences from this study were deposited in Genbank under accession numbers MW273771-MW273778. The MLST sequences were deposited in the *Chlamydiales* PubMLST database (Jolley et al., 2018).

Table 21: Mean *C. pecorum* loads detected by qPCR, and sequence type and sequence identity identified using MLST and ompA

Flock code	Strain name	Tissue	Mean Ct	Mean qPCR Loads (copies/ul)	MLST	ompA % sequence identity
3	FarmF1_Foetus1	Cotyledon	21.53	10,552	ST 23	100% E58
3	FarmF1_StillbornLamb2	Liver	21.24	12,900	ST 23	100% E58
3	FarmF1_Foetus3	Liver	18.46	88,353	ST 23	100% E58
3	FarmF1_StillbornLamb4	Liver	24.69	1176	ST 23	100% E58
1	FarmA_StillbornLamb1	Liver	26.47	341	ST 23	100% E58
7	FarmH_StillbornLamb1	Liver	22.09	7110	ST 23	100% E58
7	FarmH_StillbornLamb2	Cotyledon	16.97	247,296	ST 23	100% E58
16	FarmJ_StillbornLamb	Liver	25.06	911	ST 23	100% E58

6.2.9 Statistical analyses

Lamb mortality (%) between birth and marking for single-born lambs and multiple-born lambs (twins and triplets) were compared using two-tailed z-test (Sergeant, 2021). Only farms where lambs were tagged at birth (Flocks 1, 2, 15, 8, 4, 7, 11, 16) were included in calculation of mortality for single- and multiple-born lambs. The proportion of cases with *C. pecorum* detected for each ewe age category (ewe lambs and hogget ewes) were compared using two-tailed z-test (Sergeant, 2021).

6.3 Results

6.3.1 Lamb mortality in study flocks

From a total of 1963 lambs born, 1395 individual lamb records (including birth type, dam pedigree and survival) were available. Lamb mortality for study flocks are outlined in Table 22. Overall, lamb mortality from birth to marking ranged 12.6 - 27.1% for Merino hogget flocks and 9.3 - 40.7% for non-Merino ewe lamb flocks. Mortality rate for multiple-born lambs (twins or triplets) was 12% higher than for single-born lambs (P = <0.001). One aborted foetus was recovered from Flock 3, and sequential pregnancy ultrasounds identified 7% of ewes with evidence of pregnancy loss occurring between day 80 and 117 from the start of joining in that flock. No overt evidence of outbreak of abortion ('abortion storm') or ewe illness was observed by the farmers in any of the flocks during the study.

Table 22: Lamb mortality (birth to marking) for lambs born to maiden ewes in Western Australia

		Mortality (%)[A]		
Flock code	**Lambs born (*n*)**	**All birth types**	**Singles**	**Multiple**
Hoggets				
1	249	19.7	9.8	29.4
2	210	27.1	17.2	42.7
10	151[B]	12.6[B]	Unknown	Unknown
15	277	23.5	16.0	29.1
Ewe lambs				
3[E]	169[B]	29.0[B]	Unknown	Unknown
14[E]	150[B]	40.7[B]	Unknown	Unknown
4	197	16.8	15.4	17.2
7	196[C]	10.7[C]	15.8[C]	14.5[C]
8	89	18.0	7.0	25.0
11	145	20.0	15.7	24.2
16	130	24.6	13.0	29.2
TOTAL	**1963**	**22.0**	**14.0[D]**	**26.4[D]**

[A] Birth to marking
[B] Lambs not tagged at birth (number lambs born may be underestimated)
[C] Not all lambs tagged and assigned to birth type and/or dam
[D] Mortality calculated based on data only from flocks that had individual lamb survival data (birth type, rear type, dam pedigree)
[E] Flock 3 and 14 located on same farm, sampled different years

6.3.2 Necropsies and cause of death

A total of 298 lamb necropsies were performed, which represented 69.1% of lambs that died between birth and marking. Remaining cases without necropsy either were not recovered by the farmers or died after 72 hours of age.

The cause of death assigned at necropsy are shown in Table 23. Cause of death was established for 76% (227/298) of cases. The starvation-mismothering- exposure complex, dystocia, and stillbirths accounted for 96% (218/227) of cases where cause of death was identified. Predation and decomposition were reported for 26% of necropsies. Overall, abortion, prematurity and stillbirth represented 21% necropsies where a cause of death category was assigned. Ewe death during the

lambing period (*n*= 16) and subsequent death of their progeny was associated with 5% (23/431) lamb mortalities from birth to marking.

Table 23: Cause of lamb death identified at necropsy

		Cause of death category (*n*)					
Flock code	**Necropsies** *n* (%)[A]	Abortion & premature	Stillbirth	Dystocia	SME[B]	Infection	Undetermined
Hoggets							
1	34 (69.4)	0	11	8	7	0	8
2	39 (68.4)	0	3	5	16	0	15
10	19 (100)[C]	0	1	0	13	1	4
15	32 (49.2)	0	3	3	16	3	7
Ewe lambs							
3[D]	49 (100) [C]	2	11	13	10	0	13
14[D]	48 (78.7)[C]	0	3	14	23	1	7
4	12 (36.4)	0	1	7	2	0	2
7	21 (100) [C]	0	4	3	7	0	7
8	7 (43.8)	0	2	3	0	0	2
11	23 (79.3)	0	3	15	2	0	3
16	14 (43.8)	2	2	2	5	0	3
Total							
n	298	4	44	73	101	5	71
% necropsy	-	1	15	24	34	2	24
% necropsy with diagnosis	-	1.8	19.4	32.1	44.5	2.2	-
% lambs born	15.2	-	-	-	-	-	-

[A] Lamb necropsies expressed as % of all lamb mortalities birth to marking
[B] SME: Starvation-mismothering-exposure complex
[C] Lambs not tagged at birth (necropsy proportion may be overestimated)
[D] Flock 3 and 14 located on same farm, sampled different years

6.3.3 Laboratory investigation for abortion and stillbirth cases – pathogen detection

Specimens for 35 cases classified as abortion or stillbirth from six farms were tested for evidence of infectious disease (Table 24 and Appendix 3 Table A3.1). *Chlamydia pecorum* DNA was detected by qPCR in 39% (13/33) of stillborn or premature cases and 100% (2/2) of abortion cases, with *C. pecorum* detected at five of the six farms (Table 24). *Chlamydia pecorum* DNA detection in aborted or stillborn progeny was higher for cases born to ewe lambs (64%, 14/22) compared with Merino hogget ewes (8%, 1/13; $P = 0.001$).

The only abortigenic bacteria isolated via culture was *Trueperella pyogenes* (*n* = 2), including in one case where *C. pecorum* was concurrently detected using qPCR. *Toxoplasma gondii, Listeria* spp., *Campylobacter fetus*, *Campylobacter jejuni* and exotic abortigenic agents (*C. abortus, B. melitensis, S. enterica* serovar Abortusovis) were not detected by culture or molecular diagnostics (Table 24).

Table 24: Detection of infectious agents from aborted or stillborn lambs in Western Australia

	Hoggets		Ewe lambs					
Flock	1	2	3[A]	14[A]	7	11	16	TOTAL
Cases submitted (*n*)								
Total	10	3	10	4	4	2	2	35
Aborted foetus & membranes	0	0	1	0	0	0	0	1
Aborted membranes only	0	0	1	0	0	0	0	1
Stillborn lamb	10	3	8	4	4	2	2[B]	33
***Chlamydia* spp.**								
C. pecorum								
qPCR positive	1	0	9	0	3	1	1	15
Sequencing – *C. pecorum*	1	0	4	0	3	1	1	10
Insufficient amplification	0	0	5	0	0	0	0	5
C. abortus	0	0	0	0	0	0	0	0
C. psittaci	0	0	0	0	0	0	0	0
Other								
Listeria (culture)	0	0	0	0	0	0	0	0
Salmonella (culture)	0	0	0	0	0	0	0	0
Trueperella pyogenes (culture)	-	-	1	-	1	-	-	2
Campylobacter (culture)	0	0	0	0	0	0	0	0
Campylobacter (PCR)	0	0	2[C]	0	0	0	0	2[C]
Leptospira (PCR)	0	0	0	0	0	0	0	0
Toxoplasma (qPCR)	0	0	0	0	0	0	0	0
Coxiella (qPCR)	0	0	0	0	0	0	0	0
Brucella (PCR)	0	0	0	0	0	0	0	0
Pan-pestivirus (qPCR)	0	0	0	0	0	0	0	0

[A] Flock 3 and 14 located on same farm, sampled different years

[b] Premature twins

[C] *C. sputorum* and *C. mucosalis* by sequencing (suspected contaminant)

6.3.4 Laboratory investigation for abortion and stillbirth cases – histopathology

The majority of tissues submitted had significant autolysis and/or had been frozen which negatively impacted histopathological assessment (Appendix 3 Table A3.1). In addition, due to the opportunistic nature of some of the sample collection, the range of tissues available for examination was variable, which prevented standardised evaluation of every individual. Cases where fixed tissues were available for histological assessment (*n* = 17) are summarised in Appendix 3 Table A3.3. For cases in which *C. pecorum* was detected by qPCR and fixed tissue were available for histopathology (*n* = 9), lesions observed included placentitis (*n* = 4) epicarditis (*n* = 3), meningitis and encephalitis (*n* = 2), portal hepatitis and renal pyelitis (*n* = 2).

Necrotising placentitis with neutrophilic vasculitis was present for three lambs, with variable placental mineralisation. Another lamb displayed histiocytic infiltration of the allantoic mesenchyme without necrosis or vasculitis. Three of the five placental samples were subjected to *Chlamydia* IHC and all three were positive, with cytoplasmic staining of trophoblasts and macrophages.

Epicarditis was noted in three lambs, with mild, multifocal histiocytic and lymphocytic infiltrates present in each. Two lambs displayed a multifocal, histiocytic meningitis, moderate in intensity in one and mild in the second. The more severe case also had multifocal neutrophilic encephalitis and

multifocal glial nodule formation. These sections were both *Chlamydia* spp. and *Toxoplasma gondii* IHC negative. Two lambs had mild, multifocal infiltrates of histiocytes and lymphocytes in the portal triads of the liver. Two lambs had histiocytic and variably neutrophilic infiltrates in the submucosa of the renal pelvis. The more severe case also displayed several renal epithelial cells distended by small, round, basophilic intracytoplasmic bodies compatible with chlamydial inclusions. The epithelium in the second case had sloughed and was unavailable for examination.

In addition, five lambs displayed meconium or squames in small pulmonary airways and five had a mild macrophage or neutrophil infiltrate in the alveoli. Of the five with inflammatory infiltrates in alveoli, two displayed positive staining of macrophage cytoplasm by *Chlamydia* IHC.

6.3.5 Molecular characterisation of C. pecorum using MLST and ompA genotyping

The *C. pecorum* MLST and *omp*A genotyping was applied to samples from aborted (*n* = 2) or stillborn lambs (*n* = 6) from four farms, and compared to previously reported *C. pecorum* MLST and *omp*A sequences. The *C. pecorum* strain sequence types (STs) detected in the aborted and stillborn lambs were denoted as ST23. This genotype was identical to, and clustering with strains previously associated with pathogenicity including recently described ovine abortion from NSW (e.g. NSW_F1, NSW_F2, NSW_F3), ovine polyarthritis and/or conjunctivitis (e.g. Australian Mer_Ovi1_Jnt and Nar_S24_LE), and sporadic bovine encephalomyelitis (US E58, and Australian NSW/Bov/SBE, WA_Bov65_Brain) (Figure 4). The remaining *C. pecorum* STs that have been previously described are mainly sheep and cattle rectal strains, and strains from pig and goat hosts. These other ST clustered in three distinct and diverse larger clades.

Similarly, the *omp*A sequences detected in aborted and stillborn lambs in this study were genetically identical to previously reported isolates and strains associated with pathogenicity (e.g. Australian Mer/Ovi1/Jnt), US E58, and Australian NSW/Bov/SBE) clustering together in a well-supported clade (Appendix 3 Figure A3.1). The remaining the *omp*A sequences from sheep, goat and/or bovine rectal and other strains clustered in several genetically diverse clades (Appendix 3 Figure A3.1).

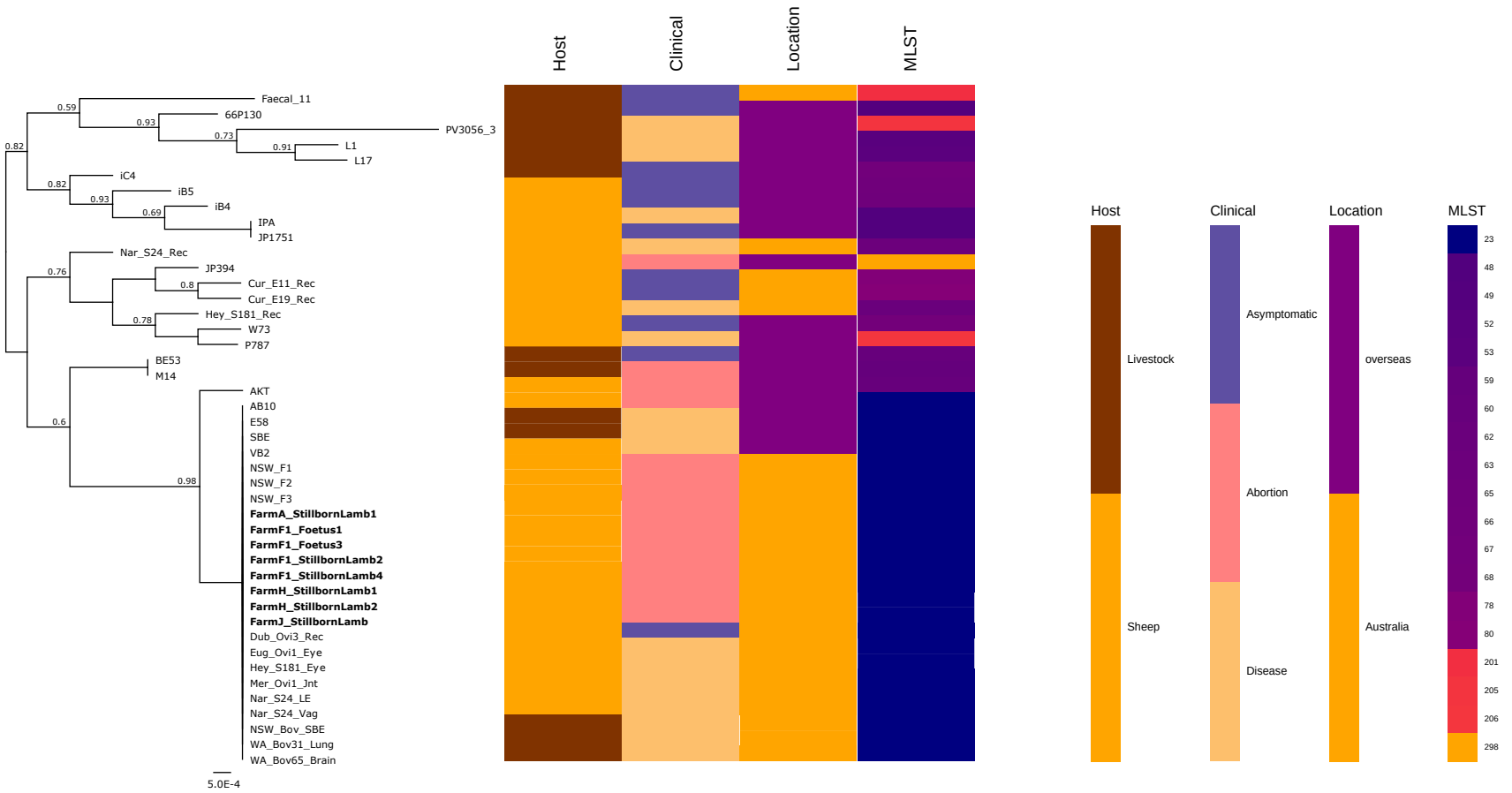

Figure 4: Phylogenetic relationships of the *C. pecorum* strains from sheep and other livestock hosts.[A]

[A] The mid-point rooted tree was constructed using a 3095 bp concatenated MLST sequences alignment from the 44 *C. pecorum* strains, including the abortigenic *C. pecorum* strains described in this study (outlined in bold). Support values are displayed on the tree nodes. As displayed on the legend, metadata for each strain denotes: hosts including sheep and other livestock (goat, cattle and pig); clinical manifestations such as asymptomatic, abortion, and disease including Sporadic Bovine Encephalomyelitis (SBE), polyarthritis, pneumonia, orchitis, conjunctivitis, and metritis; geographical location (overseas and/or Australia) and STs. The figure displaying metadata was created with Phandango (Hadfield et al., 2017).

6.4 Discussion

The detection of *C. pecorum* in aborted and stillborn lambs from maiden ewes from multiple farms was the most striking observation in this study. *Chlamydia pecorum* has predominantly been associated with polyarthritis (Lloyd et al., 2017, Walker et al., 2016, Ostfeld et al., 2020), keratoconjunctivitis (Jelocnik et al., 2019a) and asymptomatic gastrointestinal carriage and faecal shedding in Australian sheep (Yang et al., 2014a). Abortion due to *C. pecorum* is sporadic and not commonly reported (Chapter 6)(Westermann et al., 2021, Williams, 2019), and the role of *C. pecorum* as an abortigenic agent is not well defined. Thereby, detection of this organism in aborted and stillborn lambs from multiple farms with no epidemiological or geographical relationship is notable, and *C. pecorum* should be considered as a differential diagnosis for abortion and perinatal mortality in Australian sheep.

Determining the aetiology in abortion and perinatal lamb death investigations is inherently challenging (Chapter 4), and conclusive diagnosis of disease cannot be made based only on detection of a pathogen in tissue samples. Nevertheless, several observations from this study suggest *C. pecorum* was a likely aetiological agent associated with abortion and stillbirth on these farms. Firstly, other endemic and exotic abortigenic agents were not detected. Secondly, histopathological changes for cases where *C. pecorum* was detected were consistent with those reported for *C. abortus* and previously described *C. pecorum* abortion in small ruminants (Giannitti et al., 2016, Sammin et al., 2009, Westermann et al., 2021). The high loads of *C. pecorum* detected in placenta and foetal liver from aborted and stillborn lambs (Table 21) was consistent with observations for other clinical diseases associated with *C. pecorum* (Jelocnik et al., 2014a, Walker et al., 2016, Ostfeld et al., 2020). Finally, MLST and *ompA* characterisation of high load *C. pecorum* DNA from aborted and stillborn lambs identified ST23 type strains that were identical to other globally distributed ST23 strains associated with pathology in sheep and cattle, including abortion (Westermann et al., 2021, Struthers et al., 2021), arthritis (Jelocnik et al., 2013, Jelocnik et al., 2014b) and conjunctivitis (Jelocnik et al., 2013, Jelocnik et al., 2014b) in sheep, and sporadic bovine encephalopathy in cattle (Jelocnik et al., 2014a, Jelocnik et al., 2013). Emerging evidence of abortigenic potential of *C. pecorum* is perhaps not surprising given the closely related *C. abortus* is an important cause of abortion in sheep in other countries, and *C. psittaci* is a cause of abortion in horses (Jenkins et al., 2018, Longbottom and Coulter, 2003).

Chlamydia pecorum was detected in aborted and stillborn lambs from five out of six farms. However, the degree to which *C. pecorum* ST23 contributed overall lamb mortalities could not be determined. Infectious disease screening was not conducted for lambs that died from causes other than abortion or stillbirth, including those classified as starvation-mismothering. However, similar to other bacterial infections of the pregnant uterus, it is likely that *C. pecorum*-associated placentitis results in a spectrum of outcomes, including abortion, stillbirths, lambs that are born alive, but weak and with low birth weights and poor survival, congenital infections or even normal offspring, depending on the severity of placental pathology and colonisation (Westermann et al., 2021, Philips and Clarkson, 1998). Future investigations should determine whether infection contributes to reduced lamb viability, as well as abortion or stillbirth, and factors that impact outcome for infection.

Chlamydia pecorum detection was higher for aborted and stillborn progeny of younger ewes (ewe lambs) compared to hogget ewes. This was consistent with a recent case report from New South Wales, Australia where *C. pecorum* abortion was reported in maiden ewe lambs, with no evidence of abortion storm in multiparous ewes on the same property (Westermann et al., 2021). Ewes mated as ewe lambs (under 12 months of age) may be more susceptible to *C. pecorum* ST23 infection and

pathology. The reproductive performance of ewe lambs is highly variable. Improved understanding about the impact of *C. pecorum* ST23 on reproductive performance of ewe lambs and opportunities to mitigate impacts could inform management recommendations to improve their reproductive performance.

Chlamydia pecorum is endemic in Australian livestock (Walker et al., 2016, Yang et al., 2014a, Jelocnik et al., 2013), including sheep and cattle, and ubiquitous in livestock worldwide (Longbottom and Coulter, 2003). The route by which ewes became infected was not tested in our study. Faecal-oral transmission has been hypothesised, however mucosal shedding has been reported and transmission routes such as oculo- oral- or nasal contact, sexual transmission or inhalation are plausible (Longbottom and Coulter, 2003).

Asymptomatic *C. pecorum* infections are commonly detected in sheep, with faecal carriage detected 30% faecal samples over three time points and flock point prevalence ranging 0 – 94% in Australian sheep (Yang et al., 2014a). However, MLST and *omp*A genotyping has demonstrated ST23 detected in cases of abortion, arthritis and conjunctivitis are distinct from gastrointestinal strains detected in rectal swabs (Figure 4). Therefore, studies that do not characterise *C. pecorum* ST cannot assess prevalence for the pathogenic ST23 genotype and the epidemiology for *C. pecorum* ST23 in Australian sheep remains poorly understood. Frequency of abortion, conjunctivitis and lameness are typically not reported for flocks included epidemiological studies because these conditions are challenging to detect in extensively managed sheep and may not be evident at the time of sampling. The frequency of conjunctivitis and arthritis was not able to be determined for flocks in our study, however polyarthritis associated with *C. pecorum* was detected in sheep from Farm F (Ostfeld et al., 2020).

Overall lamb mortality for flocks in this study was comparable with ranges reported in other Australian studies (Hinch and Brien, 2014b). Stillbirths accounted for 19% of necropsies where cause of death was determined, and despite the detection of *C. pecorum,* was not markedly different to stillbirths as proportion of total losses reported in other Australian studies (Refshauge et al., 2016). Notably, stillbirths and abortion associated with *C. pecorum* ST23 were detected in flocks without overt evidence of abortion storm (*i.e.* observation of abortions by the farmer) or illness in ewes that would have normally triggered a veterinary investigation. This suggests *C. pecorum* may be associated with subclinical losses that go undetected on Australian farms, and explains why *C. pecorum* abortion is not more widely reported.

Listeriosis, campylobacteriosis and toxoplasmosis are the most common infectious causes of abortion and perinatal death in Australian sheep (Chapter 4)(Refshauge et al., 2020b). These diseases are sporadic and were not detected in any of the aborted or stillborn lambs in this study. There was no evidence of exotic infectious diseases. *Trueperella pyogenes* was cultured in two cases, but the significance of this finding was not clear. Although *T. pyogenes* has been reported as a primary abortigenic agent (Chapter 4)(Kirkbride, 1993), the commensal nature of the organism on the mucosal surfaces predisposes aborted and birth material to secondary contamination (Rzewuska et al., 2019). Regarding the case where both *T. pyogenes* and *C. pecorum* were detected in the same lamb, co-infections with other pathogens has been reported for both species (Pantchev et al., 2010, Rzewuska et al., 2019). However, the role of a synergistic interaction between *C. pecorum* and *T. pyogenes*, precipitating in disease, has not been established. This observation also serves as a reminder to consider mixed and co-infections during abortion investigations.

Starvation-mismothering-exposure complex and dystocia accounted for most lamb mortalities that occurred in the perinatal period and mortality was higher for multiple-born lambs compared to

single-born lambs. This was consistent with studies reported for multiparous ewes in Australia (Hinch and Brien, 2014b, Refshauge et al., 2016, Kleemann and Walker, 2005, Dennis, 1974a), and maiden ewes in New Zealand (Young et al., 2010, McMillan, 1983). Strategies to reduce dystocia and starvation-mismothering-exposure, including provision of adequate shelter for lambing ewes and managing ewe nutrition during pregnancy to optimise lamb birthweights may help optimise survival for progeny of maiden ewes (Jacobson et al., 2020).

The ability to assess brain and neurological tissue at necropsy was impacted by freezing of some carcasses and the extent of time lapsed prior to necropsy. This impacted comparison of proportion of mortalities attributable to dystocia relative to other studies that use brain and spinal cord lesion scores to determine cases as dystocia B (stillbirth) or dystocia C (birth injury) where obvious subcutaneous oedema of the head or neck is not present (Refshauge et al., 2016, Holst et al., 2002). Additionally, predation and decomposition were evident in approximately one quarter of necropsies, contributing to number of cases where cause of death could not be determined.

6.5 Conclusion

Chlamydia pecorum was detected in abortions and stillborn progeny of maiden ewes from multiple farms and should be considered as a differential diagnosis for abortion and perinatal mortality in Australian sheep. The *C. pecorum* strains detected from abortions and stillborn lambs belong to the ST23 clade that has previously been associated with abortions in sheep and cattle, and other diseases including polyarthritis, conjunctivitis and sporadic bovine encephalitis. Further investigation to quantify impact of *C. pecorum* as a cause of abortion, stillbirth or poor lamb viability in sheep, and determine factors that impact infection outcome are warranted. Starvation-mismothering-exposure complex, dystocia and stillbirths accounted for most lamb mortalities for lambs born to maiden ewes, which is consistent with that reported for adult ewes.

7. *Toxoplasma gondii* and reproductive performance of maiden ewes

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7.1 Introduction

The number of lambs weaned from primiparous (maiden) ewes is typically lower and more variable compared to multiparous ewes (Kleemann and Walker, 2005, Fowler, 2007)(Chapter 3). However, little research has investigated the causes of foetal and lamb mortality occurring between pregnancy diagnosis in mid-pregnancy and weaning for maiden ewes. Toxoplasmosis is a globally important disease of sheep that is caused by infection with *Toxoplasma gondii* and can cause early embryonic deaths, abortions, stillbirths, premature lambs and the birth of weak lambs that have poor survival rates. Zoonotic transmission of *T. gondii* via ingestion of viable cysts in undercooked sheepmeat is also an important public health issue (Dubey et al., 2020).

Sheep can be infected with *T. gondii* via ingestion of feed or water contaminated with oocysts that have been shed by a feline definitive host (Innes et al., 2009). Vertical transmission after pregnancy-induced recrudescence of persistent infections has also been reported (Costa et al., 2021, Williams et al., 2005). Reproductive disease is generally observed only following a primary infection in a naïve pregnant ewe, and *T. gondii* infection usually confers long-lasting protective immunity (Buxton and Innes, 1995). Hence, the likelihood of infection and thus immunity increases with age (Dubey and Kirkbride, 1989). Young ewes are therefore more likely to be immunologically naïve and susceptible to reproductive disease if exposed to infective oocysts during gestation.

Toxoplasmosis is one of the most commonly diagnosed causes of abortion in ewes in southern Australia (Chapter 4)(Clune et al., 2021b). However, the incidence of reproductive disease associated with toxoplasmosis in Australian ewes is not well described. Serological surveys conducted in Australian sheep have demonstrated that *T. gondii* has a broad geographical distribution with reports of seropositivity on 41 – 97% of the studied farms and mean individual animal seropositivity ranging from 7-62% (Table 25). However, most of these studies were restricted to specific regions or do not discriminate between age groups of sheep.

Table 25: Individual animal and flock-level *T. gondii* seroprevalence values from seroprevalence surveys conducted across Australia between 1975 and 2021.

Study	State(s)^	Individual animal seroprevalence *n* (%)	Flock-level seroprevalence *n* (%)
Munday (1975)	TAS	Lambs: 27/160 (16.9%) Adults: 89/144 (61.7%)	
O'Donoghue et al. (1987)	SA	86/ 1159 (7.4%)	27/59 (45.8%)
Plant et al. (1982)	NSW	515/5724 (9%)	219/534 (41%)
Kiermeier et al. (2008)	NSW, VIC, QLD, SA, WA	Lambs: 37/246 (14.9%) Adults: 126/388 (32.5%)	
McGregor and Harvey (2011)	NSW	33/489 (6.7%)	6/10 (60%)
Taggart et al. (2020)	SA	318/560 (57%)	
Lanyon and O'Handley (2020)	SA	209/875 (23.9%)	28/29 (96.6%)
Hamilton et al. (2021)		46/401 (11.5%)	

^NSW: New South Wales; QLD: Queensland; SA: South Australia; TAS: Tasmania; WA: Western Australia; VIC: Victoria.

Whilst *T. gondii* is endemic in Australia, its impact on ewe reproduction, and specifically reproduction for maiden ewes, is not well quantified. A seroprevalence survey in South Australia reported a negative correlation between within-flock *T. gondii* seroprevalence and lamb marking rate (Lanyon and O'Handley, 2020). However, this study did not investigate seroprevalence between ewe age groups or determine the timing of *T. gondii* seroconversion relative to reproductive outcome. Similarly, a study in Uruguay identified lower lambing rates for ewes that seroconverted for *T. gondii* during gestation (Savio and Nieto, 1995). Vaccination against *T. gondii* was associated with increased lamb marking percentages in extensively managed maiden ewe lambs in New Zealand which suggests that toxoplasmosis was impacting reproduction in flocks that were not vaccinated (Kenyon et al., 2004).

The aim of this study was to assess the impact of *T. gondii* infection on the reproductive performance of maiden ewes in southern Australia. This will inform the need for targeted control strategies for *T. gondii* in Australian sheep.

7.2 Methodology

All procedures were conducted according to guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes and were approved by the Murdoch University Animal Ethics Committee (R3004/17). The owners of sheep included in this study provided informed consent to participate.

7.2.1 Study design, animals and research sites

This cohort study was conducted as part of a larger project investigating reproductive performance of maiden ewes using 30 maiden flocks on 28 farms located in Western Australia (*n* = 11), South

Australia (*n* = 9), and Victoria (*n* = 10) (Figure 5) between 2018 and 2020 as previously described (Chapter 5). Briefly, farms were located over a wide geographic area that incorporated different rainfall zones (Figure 5 and Appendix 4 Table A4.1). Farms were selected for inclusion based on; having at least 200 maiden ewes available for the study, capacity to monitor ewes and their progeny over the study period, and with sheep genotype and management that were generally representative of standard commercial sheep farms in the region. Some farms included in the study managed flocks of stud sheep which may increase frequency of monitoring relative to commercial flocks, but the housing (*i.e.* all flocks were managed in paddocks for the duration of the study) and stocking intensity were broadly comparable to commercial sheep flocks in these regions.

Each flock was monitored during gestation and lambing. Merino and non-Merino breeds were included in the study (Appendix 4 Table A4.1). Non-Merino flocks included Border Leicester, Dorper, White Suffolk and composite breeds. Ewes were mated as either ewe lambs (7-10 months, *n* = 19 flocks) or maiden hoggets (18-20 months, *n* = 11 flocks). Most ewes were naturally mated, and all rams used were confirmed to be negative for *Brucella ovis* via serology prior to joining. At each farm, twenty mature multiparous ewes aged three years or older that had been bred on the same farm were randomly selected for blood sampling only (Appendix 4 Table A4.2). All farms ran self-replacing flocks and ewes included in the study were managed according to standard farm practice.

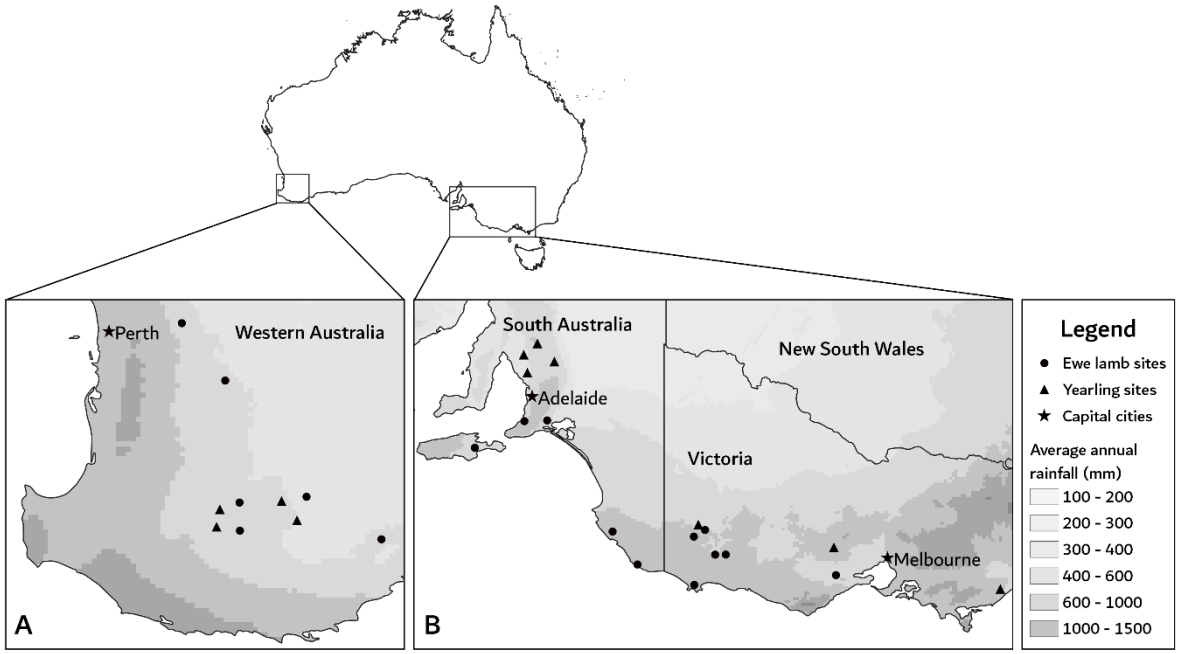

Figure 5: Approximate location of farms sampled in Western Australia (A) and South Australia and Victoria (B) adapted from Clune et al. (2021d).

7.2.2 Animal measurements and sample collection

Monitoring of ewes and determination of pregnancy outcome are described in Chapter 5. Briefly, foetal mortality for maiden ewes was determined based on sequential transabdominal pregnancy ultrasounds (scans). Scan 1 was conducted approximately 85 days (range 62-101) from the start of joining. Scan 2 was conducted at least 30 days after the first scan at approximately 118 days (range

107-136) from the start of joining. The outcome of pregnancy and perinatal lamb mortality were determined based on number (single, twin or triplet) and survival status (lambs dead or alive) for lambs at lambing rounds and at lamb marking (approximately six weeks from the start of lambing). Ewe lactation status (lactating or not lactating) was determined by visual observation and/or palpation of the udder at lamb marking. Mid-pregnancy abortion was determined based on loss of pregnancy (i.e., no foetus/es detected) or foetal mortality (*i.e.,* no evidence of foetal viability) between scan 1 and scan 2 and validated using lambing records (*i.e.* no lamb allocated to ewe at lambing rounds) and udder inspection (*i.e.* no evidence of lactation) at marking as described by Clune et al. (2022c). Ewes that "failed to rear" were determined based on evidence of pregnancy at scan 1 with no live lamb present at lamb marking and no evidence of lactation at lamb marking.

Blood samples for maiden ewes were collected at five timepoints: pre-joining, scan 1, scan 2, pre-lambing (approximately 140 days from start of joining) and lamb marking (approximately 6 weeks from start of lambing). Blood samples for mature ewes were obtained at a single timepoint during the study period, but timing of sampling relative to lambing and their reproductive outcome was not recorded (Appendix 4 Table A4.3). All blood samples were obtained by jugular venepuncture into serum vacutainer tubes with clot activator and stored on ice or at 2^{o}C. Within 72 hours of collection, blood samples were centrifuged at 4000 rpm for 10 minutes and serum was decanted into 2mL low protein-binding polypropylene screw cap micro tubes and stored at -20^{o}C prior to serological testing.

7.2.3 Serology sample selection

The sample size necessary to estimate true prevalence was 239 ewes based on assumed true seroprevalence prevalence of 15% in maiden ewes, assumed test sensitivity 90%, assumed specificity 99% and desired precision 5%. As 30 flocks were included in the study, this sample size was achieved with at least 8 ewes sampled per flock.

All samples from mature ewes and a sub-sample of at least 40 maiden ewes from each flock were selected for *T. gondii* serology. Samples for maiden ewes that were identified as pregnant at the first pregnancy scan but failed to successfully rear a lamb were prioritised for screening. That is, sample selection for maiden ewes was biased towards ewes that were pregnant and failed to rear a lamb. This included maiden ewes that were determined to have aborted and ewes for which lamb mortality occurred in the perinatal period. Samples for maiden ewes that reared lambs were also included for screening where flocks had less than 40 ewes that failed to rear a lamb (Appendix 4 Table A4.1). Blood samples collected at lamb marking were used for serology where available. For maiden ewes where blood samples from marking were not available, blood samples collected at the latest available timepoint were used (*i.e.* blood sample collected at scan 2 or pre-lambing after foetal mortality was detected).

For maiden ewes that returned a 'positive' result using indirect ELISA at the last time-point, serum samples from earlier timepoints (pre-joining, scan 1, scan 2 and pre-lambing) were tested to determine the timing of seroconversion relative to gestation and foetal or lamb mortality.

7.2.4 *Toxoplasma gondii* serology

Anti-*T. gondii* IgG seropositivity was determined using commercial indirect ELISA (ID Screen Toxoplasmosis Indirect Multispecies, ID Vet, France) according to the manufacturer's instructions

(IDvet, 2018). Testing was performed by VETPATH Laboratories (Perth, Western Australia). The results were read at 450 nm using a Multiskan FC, Thermo Scientific spectrophotometer. Positive and negative internal controls were included with each plate. Optical density (OD) values were expressed as the mean percentage of sample/positive (S/P) values, as recommended by the manufacturer:

$S/P\ value = (OD_{sample} - OD_{negative.control})/(OD_{positive\ control} - OD_{negative.control})$

Serum samples were classified as positive (S/P value ≥50), doubtful (S/P value 40 to <50) or seronegative (S/P value <40) according to the manufacturer's recommendation. This assay has a specificity of 100% (95% CI 98.2, 100) and a sensitivity of 100% (95% CI 89.1, 100) using sheep sera validated against latex agglutination test (MAST Group) according to the manufacturer's internal validation report (IDvet, 2018). A subset of sera from Australian sheep previously tested with a validated modified agglutination test (Hamilton et al., 2021) in a separate study were re-tested using the commercial indirect ELISA, with sensitivity 90.5% (95% CI 71.1, 98.3) and specificity 100% (95% CI 64.6, 100) (Appendix 4 Table A4.3).

Samples that returned a 'doubtful' result (Appendix 4 Table A4.4) and a subset of samples that returned a negative result (Appendix 4 Table A4.5) were re-tested using an alternate commercial ELISA test (IDEXX Toxotest, IDEXX Laboratories, Switzerland), according to manufacturer's instructions. Re-testing was performed by Department of Primary Industry and Regional Development Diagnostic Laboratory Service. Each plate included positive and negative controls. The results were read at 450 nm using a Multiskan EX, Thermo Fisher Scientific spectrophotometer. Optical density (OD) values were expressed as the mean percentage of sample/positive (S/P) values, as recommended by the manufacturer:

$S/P\ value = (OD_{sample} - OD_{negative.control})/(OD_{positive\ control} - OD_{negative.control})$

Serum samples were classified as positive (S/P value ≥100), weak positive (S/P value 30 to <100), suspect (S/P value 20≤ 30) or negative (S/P value < 20) according to the manufacturer's recommendation. This assay has reported specificity 97.5% (95% CI 92.5, 99.4) and a sensitivity 90.9% (95% CI 83.4, 95.6) for sheep (Mainar-Jaime and Barberán, 2007).

Samples that were 'doubtful' for the first test (using ID Screen®) but 'positive' or 'weak positive' for the second test (using IDEXX Toxotest) were categorised 'positive'. Results that were 'doubtful' for first test and 'negative' or 'suspect' for second test were considered negative (Appendix 4 Table A4.4).

7.2.5 Statistical methods

Lamb mortality was calculated based on the number of lambs alive at marking expressed as a proportion of the number of foetuses identified at scan 1. Lamb mortality was classified as 'abortion' based on detection of pregnancy loss between scan 1 and scan 2 (and validated with lambing records and ewe lactation status). Abortion was expressed as a proportion (%) of ewes with abortion detected between scan 1 and scan 2 relative to the number of ewes that were confirmed pregnant at scan 1.

Apparent *T. gondii* seropositivity was calculated using the number of ewes categorised as positive expressed a proportion (%) of the ewes tested, with 95% confidence intervals determined using

Jeffreys method (Brown et al., 2001). Proportion *T. gondii* seropositivity were compared for the ewe age categories and states using a two-tailed two-sample proportion z-test.

The true seropositivity and 95% credible intervals (95% CrI) were estimated using Bayesian inference, considering the sensitivity and specificity and their 95% CrI derived from manufacturer's internal validation report (IDvet, 2018) as beta-pert distribution for priors (Speybroeck et al., 2013).

7.3 Results

7.3.1 Maiden reproductive performance

Foetal loss and lamb mortality for progeny of maiden ewes have been reported in more detail in Chapter 5. Briefly, foetal and lamb mortality between scan 1 and lamb marking was 36% (1567/4351 foetuses; range 14 - 71%) for maiden ewe lambs and 28% (582/2103 foetuses; range 20 - 53%) for maiden hoggets. Mid-pregnancy abortion was detected in 14/19 maiden ewe lamb flocks and 6/11 maiden hogget flocks. In maiden ewe lamb flocks, mid-pregnancy abortion was detected in 5.2% (155/2968) ewes, ranging 0 – 50.0% across flocks. In maiden hogget flocks, mid-pregnancy abortion was detected in 0.8% (16/1886) ewes, ranging 0 – 4.4% across flocks.

7.3.2 *Toxoplasma gondii* seropositivity

Apparent and true *T. gondii* seroprevalence for ewe age categories are shown in Table 26. Apparent *T. gondii* seroprevalence for maiden ewes (ewe lambs and hoggets combined) was 1.1% (95% CI 0.6, 1.8). Apparent individual-animal *T. gondii* seroprevalence was higher for mature ewes compared to maiden ewe lambs ($P < 0.001$) and maiden hogget ewes ($P < 0.001$). There was no difference in the apparent seroprevalence between maiden ewes mated as ewe lambs or hoggets ($P = 0.214$).

Toxoplasma gondii seropositivity was detected in at least one ewe for 16/28 (57%) of the farms. *Toxoplasma gondii* seropositivity was identified in 12/30 (40%) of maiden ewe flocks and 11/28 (39%) of mature ewe flocks. For flocks where *T. gondii* seropositivity was detected, within-flock seroprevalence ranged from 1-5% for maiden ewes (Appendix 4 Table A4.1) and 5-50% for mature ewes (Appendix 4 Table A4.2). The majority (82%) of seropositive mature ewes were detected on five farms where within-flock seroprevalence for mature ewes ranged 25-50% (Appendix 4 Table A4.2).

Table 26: Apparent and estimated true seropositivity to *T. gondii* for maiden ewes mated as ewe lambs (approximately one-year-old at sampling) or hoggets (approximately two-years-old at sampling) and mature multiparous ewes (aged three-years-old or older) from 28 Australian farms.

	Ewes sampled				
	Flocks (*n*)	Individual ewes (*n*)	Seropositive samples (*n*)	**Apparent seropositivity** % (95% CI)	**Estimated true seropositivity** % (95% CrI)
Maiden ewes					
Ewe lambs	19	839	7	0.8 (0.4, 1.6)[a]	0.7 (0.1, 1.6)
Hoggets	11	440	7	1.6 (0.7, 3.1)[a]	1.6 (0.4, 3.1)
Mature ewes	28	558	45	8.1 (6.0, 10.5)[b]	8.1 (5.8, 10.6)

95% CI: 95% confidence interval

95% CrI: 95% credible interval

[ab] Apparent seropositivity proportion (%) with different superscripts are significantly different (two sample proportion z-test (2-tailed) $P < 0.05$)

There was no difference in the proportion of maiden ewes seropositive to *T. gondii* between states (Table 27). The proportion of mature ewes that were seropositive to *T. gondii* was greater for South Australia and Victoria compared to Western Australia (Table 27).

Table 27: Apparent seropositivity to *T. gondii* at state-level for maiden ewes mated as ewe lambs (approximately one-year-old at sampling) or hoggets (approximately two-years old at sampling) and mature multiparous ewes (aged 3 years or older) from 28 Australian farms.

State^	**Ewe lambs**		**Hoggets**		**Mature ewes**	
	Sampled (*n*)	Seropositive (*n* (%))	Sampled (*n*)	Seropositive (*n* (%))	Sampled (*n*)	Seropositive (*n* (%))
WA	338	2 (0.6)[a]	160	3 (1.9)[a]	200	5 (2.5)[a]
SA	221	2 (0.9)[a]	160	2 (1.3)[a]	178	17 (9.6)[b]
VIC	280	3 (1.1)[a]	120	2 (1.7)[a]	180	23 (12.8)[b]

^SA: South Australia; WA: Western Australia; VIC: Victoria

[ab] Apparent seropositivity proportions (%) within columns with different superscripts are significantly different (two sample proportion z-test (two-tailed) $P < 0.05$)

7.3.3 Timing of *T. gondii* seroconversion in maiden ewes

A total of 1279 maiden ewes were screened for *T. gondii* seropositivity (Table 26), of which 1097 were pregnant at scan 1 but failed to rear a lamb to marking (Appendix 4 Table 4.1). Of the 1097 ewes that were tested and failed to rear a lamb, mid pregnancy abortion (pregnancy loss between scan 1 and scan 2) was detected in 161 ewes.

Toxoplasma gondii seropositivity was detected for 7 ewes joined as ewe lambs and 7 ewes joined as hoggets (Table 26). Of these, outcome of pregnancy and serial blood samples were available for 12 ewes. The timing of detection of seroconversion in these 12 ewes is shown in **Table 28**. Seropositivity to *T. gondii* at joining was detected in 1/12 ewes that were seropositive to *T. gondii* at lamb marking (or last available sample). Seroconversion to *T. gondii* after joining was detected for

11/1097 (1.0%, 95% CI 0.5, 1.7) of the maiden ewes that failed to raise a lamb based on detection of seropositivity at lamb marking (or last available sample) but not at joining.

For the subset of ewes selected for serology that had mid-pregnancy abortion, 1/161 (0.6%, 95% CI 0.1, 2.9) ewes had evidence of *T. gondii* seroconversion after joining evident as *T. gondii* seropositivity at marking and with no evidence of seropositivity at joining (ewe ID 12114; **Table 28**). Seropositivity was also detected in a second ewe that aborted but did not have serial blood samples available to determine timing of seroconversion. One ewe that was opportunistically identified with late-pregnancy abortion had evidence of *T. gondii* seroconversion after joining (ewe ID 13091; **Table 28**).

Serum samples categorised as negative for *T. gondii* IgG at a sampling timepoint after detection of *T. gondii* seroconversion were detected for only one ewe (Ewe 3479; **Table 28**).

Table 28: Timing of detection for *T. gondii* IgG seroconversion using indirect ELISA for maiden ewes (*n* = 12) sampled across southern Australia between 2018 and 2020. The earliest detection of seroconversion is bolded.

Ewe ID	Farm	Timing of foetal or lamb loss	***T. gondii* seroconversion status**				
			Pre-joining	Scan 1	Scan 2	Pre-lambing	Lamb marking
Seroconversion detected pre-joining							
9463	13	Perinatal death	**Positive**	Positive	Positive	NA	Positive
Seroconversion first detected during pregnancy							
18842	20	Late abortion/ perinatal lamb death	Negative	**Positive**	Positive	NA	Positive
18857	20	Perinatal death	Negative	**Positive**	Positive	NA	Positive
3479	3	Late abortion/ perinatal lamb death	Negative	**Positive**	Negative	Negative	Positive
12114	12	Mid Abortion (scan 1 – scan 2)	Negative	Negative	**Positive**	NA	NA
13091	7	Late abortion * (scan 2 – pre-lambing)	Negative	Negative	Negative	**Positive**	NA
18121	21	Perinatal death	Negative	Negative	Negative	**Positive**	Positive
Seroconversion first detected at marking							
16527	10	Perinatal death	Negative	Negative	Negative	NA	**Positive**
16528	10	Perinatal death	Negative	Negative	Negative	NA	**Positive**
17152	15	Perinatal death	Negative	Negative	Negative	Negative	**Positive**
18190	22	Perinatal death	Negative	Negative	Negative	Negative	**Positive**
23364	29	Raised twins	Negative	NA	NA	Negative	**Positive**

NA – not available for testing as the ewe was not present for sampling or had been removed from the study flock after abortion was confirmed

*confirmed late abortion based on observation of purulent vaginal discharge and opportunistic transabdominal ultrasound at the pre-lambing visit

7.4 Discussion

Toxoplasmosis was not an important contributor to foetal and lamb mortality between pregnancy scanning and marking for the maiden ewe flocks in this study. Whilst there was serological evidence of widespread exposure to *T. gondii* at farm level, seroconversion after joining was evident for only 1% of maiden ewes that were confirmed to be pregnant and subsequently failed to raise a lamb. Low frequency of *T. gondii* seropositivity in maiden ewes was consistent with the absence of detection of *T. gondii* using qPCR on aborted and stillborn lambs from a subset of farms in this study reported in Chapter 6. These findings are in accord with a recent review of submissions to Australian veterinary laboratories that reported *T. gondii* was implicated in 5% of sheep abortion investigations, and suggests that toxoplasmosis is a sporadic cause of abortion in Australian sheep (Chapter 4). Our findings indicate that routine vaccination for toxoplasmosis is unlikely to be economically justified for many Australian sheep producers unless there is evidence demonstrating high risk of exposure to *T. gondii* in the specific region.

This study used serial serology to assess the timing of seroconversion relative to abortion or lamb death. Abortions may occur acutely, or up to 8 weeks post-infection, with the outcome of infection being largely dependent on the stage of gestation when *T. gondii* infection occurred (Castaño et al., 2016, Munday and Dubey, 1986). *Toxoplasma gondii* IgG antibodies are detectable by P30 ELISA between 3 and 10 weeks after infection (Sager et al., 2003) and persist for several years (Dubey and Beattie, 1988). So, it is unlikely that antibodies would have failed to rise to detectable levels or waned to below detectable levels by lamb marking if ewes had become infected with *T. gondii* during pregnancy and foetal or lamb mortality was related to toxoplasmosis. Based on these assumptions, toxoplasmosis was a plausible aetiology for abortions and lamb mortalities in ewes 12114, 13091, 18842 and 18857 (**Table 28**). However, attempting to relate maternal serostatus to the occurrence of abortion or lamb mortality can be unreliable due to the variable timeframe between detection of seroconversion and lamb mortality (Munday and Dubey, 1986). Toxoplasmosis-associated reproductive disease may be confirmed using foetal serology (Munday and Dubey, 1986), molecular diagnostics (Masala et al., 2003), and histopathology (Pereira-Bueno et al., 2004). Additionally, quantitative serology to evaluate changes in antibody titres for ewes with suspected toxoplasmosis would allow more accurate interpretation of serial serological results. Nevertheless, even if foetal and lamb mortality for all ewes that were seropositive at lamb marking in this study were due to toxoplasmosis, this still represented a very small contributor to overall foetal and lamb mortality in these flocks.

The unclear association between timing of *T. gondii* seroconversion and abortion in some ewes was also consistent with observations that other factors were likely to be contributing to abortion and perinatal lamb deaths in these flocks. Abortions, stillbirths and polyarthritis associated with *Chlamydia pecorum* were detected in maiden ewe flocks from Western Australia, and non-infectious causes of death (including dystocia and starvation-mismothering) were important contributors to cause of lamb death identified at necropsy (Chapter 6)(Ostfeld et al., 2020). Previous Australian studies have reported dystocia and starvation-mismothering to be important contributors to perinatal lamb mortality (Bruce et al., 2021, Jacobson et al., 2020, Hinch and Brien, 2014b), and sporadic abortion associated with *C. pecorum* infections in ewe lambs (Westermann et al., 2021). There was no evidence neosporosis or coxiellosis were contributing to foetal or lamb mortality in the same flocks reported in this study (Chapter 8 and Chapter 9). Abortions associated with *Campylobacter fetus* fetus were detected by microbial culture from aborted lambs in one flock included in this study (Chapter 10). A review of abortion investigations submitted to Australian veterinary laboratories reported campylobacteriosis and listeriosis to be the most common diagnoses made for investigations of ovine abortion or stillbirth (Chapter 4).

This study reported seropositivity for ewes that were determined to be pregnant by transabdominal ultrasound and subsequently aborted or failed to raise lambs. However, toxoplasmosis can also have impacts on early pregnancy before pregnancy scanning, including embryonic death, resorption and early foetal mortality (Dubey, 2009). Therefore, it is possible that infections occurring early in pregnancy could result in maiden ewes being not pregnant at scanning. This study did not determine association between *T. gondii* seropositivity and pregnancy status at scan 1. Although, the low seropositivity reported in this study for maiden ewes that were pregnant at scan 1 suggests that toxoplasmosis was unlikely to be an important contributor to early reproductive losses on these farms. Further investigation is required to determine if infection in early pregnancy is an important contributor to early pregnancy loss evident as ewes that are not pregnant at scanning.

Toxoplasma gondii seropositivity was detected for 57% of farms in this study, providing further evidence for the parasite's endemicity and suggesting that exposure to *T. gon*dii occurs on many Australian sheep farms. This was consistent with other Australian studies reporting farm-level seroprevalence ranging 41 – 97% (Appendix 4 Table A4.1). Seropositivity in mature ewes (8.1%) was within the 7% – 57% range reported for other Australian studies in the last 15 years, and similar to national seroprevalence 11.5% (46/401) for mutton (mature sheep) reported by Hamilton *et al* in a recent Australian abattoir survey (Appendix 4 Table A4.1). Individual-animal *T. gondii* seropositivity for maiden ewes in our study (1.1%) was lower than two previous studies reporting seroprevalence of 15% and 17% for Australian slaughter-age lambs in abattoir surveys (Appendix 4 Table A4.1). Our study used a commercial indirect ELISA that has good sensitivity and specificity relative to latex agglutination test for sheep sera reported by the manufacturer (IDvet, 2018), suggesting that differences in testing methodology are unlikely to explain the difference in seroprevalence between the studies. It is therefore more likely that the low seropositivity in maiden ewes reflects the sporadic nature of *T. gondii* infection in Australian sheep rather than differences in serological methods.

Flock and animal seropositivity in mature ewes indicated variable exposure to *T. gondii* on Australian sheep farms. Most seropositive mature ewes were concentrated on five farms where within-farm seropositivity ranged 25-50%. Variable seropositivity in Australian sheep contrasts to the more consistent exposure reported for sheep farms in New Zealand, United States and United Kingdom (Dempster et al., 2011, Dubey and Kirkbride, 1989, Katzer et al., 2011), and suggests sporadic point source exposure to oocysts, likely via contaminated drinking water or feed source, are the major source of *T. gondii* exposure on Australian sheep farms. The higher (50%) seropositivity observed for ewes on a farm on Kangaroo Island (Appendix 4 Table A4.3) was consistent with previous studies reporting high *T. gondii* seroprevalence on Kangaroo Island (Taggart et al., 2020, Lanyon and O'Handley, 2020). Interestingly, there was no evidence of seropositivity in the maiden ewes from the farm on Kangaroo Island, consistent with sporadic exposure to *T. gondii*. The risk of *T. gondii* exposure is associated with a range of factors including abundance of the feline definitive host, access to surface water and rainfall (Shapiro et al., 2019, Stelzer et al., 2019). Further work is warranted to determine regional differences in *T. gondii* exposure and incidence of toxoplasmosis. Improved understanding of regional variation and risk factors for *T. gondii* exposure on Australian sheep farms would inform cost-benefit analyses for interventions to reduce the risk of toxoplasmosis.

Sampling maiden ewes was biased towards ewes that were determined to be pregnant and subsequently failed to rear a lamb. If toxoplasmosis was an important contributor to foetal and lamb mortality on these farms, then this sampling bias could result in overestimation of seroprevalence relative to the general population. Notwithstanding this, the low frequency of seropositivity and

seroconversions during pregnancy in maiden ewes that failed to raise lambs does not support the role of *T. gondii* as an important cause of reproductive losses between scanning and marking on these farms. A case control study would allow for determination of odds ratios for 'fail to rear' for ewes that were seropositive for *T. gondii*. However, due to the very low frequency of seropositivity to *T. gondii* in these flocks we therefore decided to target 'fail to rear' ewes to improve our confidence in the level of seropositivity in this cohort.

One ewe (Ewe 3479; **Table 28**) was seronegative at two timepoints after testing seropositive at scan 1 then subsequently tested seropositive at marking. The reason for fluctuating serostatus was not determined. However, this could be due to false positive results, issues with test reproducibility or failure of the test to detect fluctuating titres below the detection limit. There is a lack of published data validating the test against other 'gold standard' tests such as microagglutination tests or PCR. A commercial modified agglutination test for *T. gondii* was validated for Australian sheep (Hamilton et al., 2021), but this test is no longer available. The indirect ELISA used in this study was found to have good agreement with modified agglutination test on a subset of sheep sera collected from another study (Appendix 4 Table A4.5). Further validation of commercial indirect ELISA for natural *T. gondii* infections in Australian sheep will inform improved estimation of true prevalence for field studies.

Whilst the observations from this study do not support the need for widespread routine vaccination to reduce foetal and lamb mortality for maiden ewes in Australia, *T. gondii* is endemic on Australian farms and associated with sporadic reproductive losses on some farms (Clune et al., 2021b). The low rates of seropositivity in maiden and multiparous ewes suggests a lack of protective immunity in a large proportion of ewes (Buxton and Innes, 1995). These ewes remain susceptible to reproductive disease if a toxoplasmosis outbreak was to occur during gestation. Control of feral and domestic cat populations on sheep farms and measures to prevent contamination of feed and water sources from cat faeces can reduce the risk of toxoplasmosis outbreaks in susceptible sheep. Vaccination could be warranted in some regions where high farm and individual animal seroprevalence is identified (Lanyon and O'Handley, 2020), and high incidence of reproductive losses due to *T. gondii* are confirmed using foetal and lamb necropsy and laboratory investigation. The interpretation of *T. gondii* serology for the purpose of diagnosing toxoplasmosis is challenging, particularly for field investigations of reproductive loss in extensive sheep production systems where abortions or unusually high incidence of perinatal mortality are challenging to detect at the time losses are occurring. Diagnosis of toxoplasmosis should be supported with detection of *T. gondii* in tissues where possible, as well as the exclusion of other endemic pathogens.

7.5 Conclusion

Toxoplasmosis was not a significant contributor to abortion and perinatal lamb mortality for maiden ewes on farms across southern Australia. Seropositivity to *T. gondii* for mature multiparous ewes was detected by indirect ELISA on more than half of the farms included in this study. However, only 1% of maiden ewes that had confirmed pregnancy and subsequently failed to raise a lamb had evidence of *T. gondii* seroconversion after joining. Low frequency of *T. gondii* seropositivity in maiden ewes during gestation was consistent with the absence of detection of *T. gondii* using qPCR on aborted and stillborn lambs from a subset of farms.

8. *Neospora caninum* and reproductive performance of maiden ewes

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8.1 Introduction

The reproductive performance of maiden ewes (primiparous ewes in their first breeding season) is often variable and lower than multiparous ewes with higher levels of lamb loss between pregnancy diagnosis and lamb marking (Kleemann and Walker, 2005, Allworth et al., 2017, Kilgour, 1992, Shorten et al., 2021). A number of endemic diseases may cause abortion and poor viability of lambs in Australia (Chapter 4)(Refshauge et al., 2020b), and younger sheep are more likely to be immunologically naïve and susceptible to clinical disease following exposure.

Neospora caninum is recognised globally as an important cause of infectious abortion and reproductive loss in cattle (Dubey and Schares, 2011, Reichel et al., 2013). Abortion and perinatal deaths due to neosporosis have also been observed in both naturally and experimentally infected sheep (González-Warleta et al., 2014, Hässig et al., 2003, Buxton et al., 2001). Cerebral neosporosis has also been reported in Australian sheep (Bishop et al., 2010). Reproductive losses in sheep associated with natural infection and neosporosis are generally sporadic, although larger flock outbreaks have been described (Hässig et al., 2003, González-Warleta et al., 2014). *Neospora caninum* has been implicated in the reduced reproductive performance of ewe lamb flocks in New Zealand (Howe et al., 2012, Howe et al., 2008, West et al., 2006). Neosporosis is not a frequently diagnosed cause of abortion or stillbirths in Australian sheep (Chapter 4)(Refshauge et al., 2020b). However, relatively few abortion investigations are conducted and the significance of *N. caninum* as a reproductive pathogen for sheep in Australia remains unclear.

Neospora caninum is endemic in Australian cattle with seropositivity detected in 29-95% of beef herds from New South Wales, Queensland and South Australia and overall individual animal seroprevalence ranging 2.5-15% (Moloney et al., 2017, Nasir et al., 2012, Stoessel et al., 2003). There is considerable variation in seroprevalence in Australian cattle with one study reporting seroprevalence for cattle management groups ranging 0-94% (Fordyce et al., 2013). By contrast, *N. caninum* seroprevalence in sheep is not well studied. One survey in New South Wales reported detection of seropositivity in at least one animal for 3 of 5 sheep farms and an overall individual animal seroprevalence of 2.2% (Bishop et al., 2010). Therefore, the incidence of *N. caninum* infection and neosporosis in Australian sheep is largely unknown.

There are several factors that suggest that *N. caninum* could pose a risk for reproductive disease in Australian sheep. There is considerable geographical overlap of sheep and cattle production in southern Australia. Endemicity of *N. caninum* in Australian cattle and common presence of the domestic and wild dogs (definitive host) on sheep farms suggests widespread potential for *N. caninum* exposure for sheep. Sheep have been demonstrated to be susceptible to *N. caninum* infections and cases of ovine neosporosis have been described for sheep managed under extensive farming systems that are consistent with those typical for Australian sheep farms (Bishop et al., 2010, Howe et al., 2012, Howe et al., 2008). Consequently, *N. caninum* may play a role in the suboptimal reproductive performance for Australian maiden ewes.

The aims of this study were to (i) determine the seroprevalence of *N. caninum* in Australian sheep and (ii) examine if natural exposure to *N. caninum* is associated with poor reproductive performance of maiden ewes in southern Australia.

8.2 Methodology

All procedures were conducted according to guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes and were approved by the Murdoch University Animal Ethics Committee (R3004/17).

8.2.1 Sampling and location

Thirty flocks of maiden ewes (approximately 200 ewes per flock) on 28 farms located in Western Australia (*n*=11), South Australia (*n*=9), and Victoria (*n*=10) were monitored from the start of joining to lamb marking (tagging). Farms were selected based on convenience sampling and were located in a range of geographic regions and rainfall zones (Figure 6 and Appendix 5 Table A5.1). Maiden ewes were mated as either ewe lambs (7-10 months, *n* = 19 flocks) or hogget ewes (18-20 months, *n* = 11 flocks). At each farm twenty mature ewes (aged three years or older) that had been bred on the same farm were randomly selected for this study. All farms ran self-replacing flocks and ewes included in the study were managed according to typical farm practice. Information on the presence of dogs and cattle were recorded for each farm.

A sub-sample of least 40 maiden ewes from each flock were selected for *N. caninum* serology based on evidence of abortion or perinatal mortality of their offspring (Appendix 5 Table A5.1). For flocks where less than 40 ewes that failed to rear a lamb were identified, ewes that had reared single or twin lambs were included (Appendix 5 Table A5.1). Reproductive status was not known for maiden ewes from one farm in South Australia (Farm 6).

Mature multiparous ewes aged 3 years or older (n=20 per farm) were randomly selected for *N. caninum* serology (Appendix 5 Table A5.2). Reproductive status of mature ewes was not known.

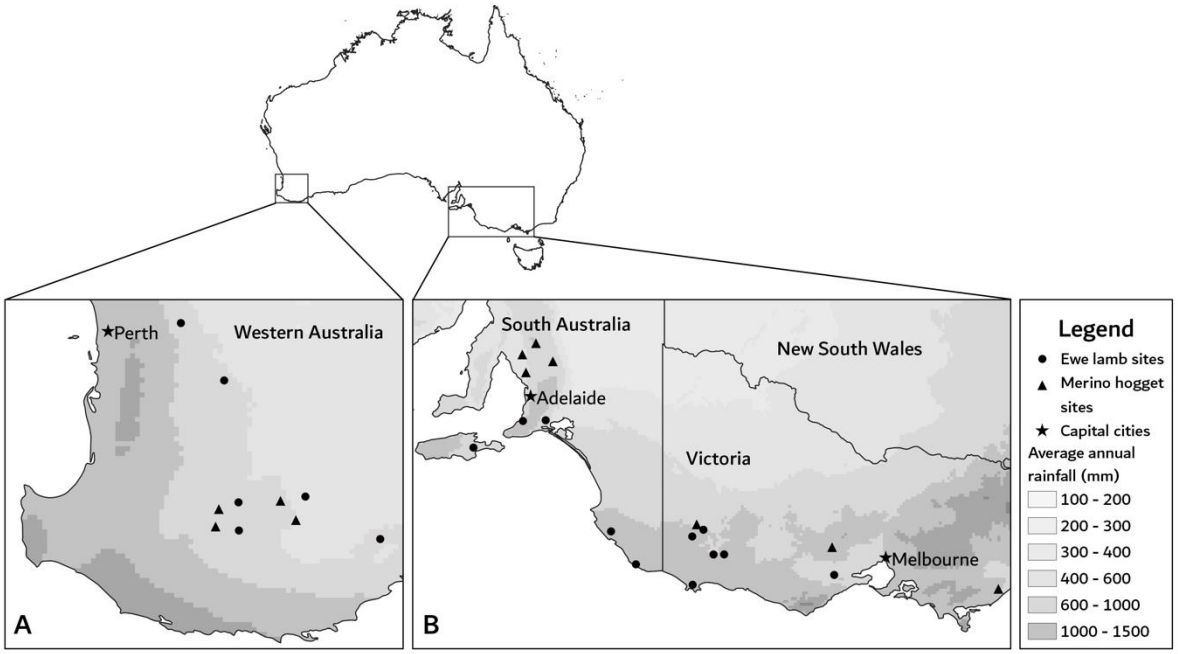

Figure 6: Approximate location of each property where samples were collected in Western Australia (Map A) and in South Australia and Victoria (Map B). Average annual rainfall data sourced from Australian Government Bureau of Meteorology (2021)

8.2.2 Animal measurements and sample collection

Foetal and perinatal lamb mortality for maiden ewes was determined based on sequential transabdominal ultrasonography, lambing inspections and lamb marking (tagging) data. Sequential transabdominal ultrasonography was performed at day 62-101 (scan 1) and day 108-136 (scan 2) from the start of joining. Maiden ewes were inspected at pre-lambing (approximately day 140 from start of joining). Lamb survival status (dead or alive) was recorded at lambing inspections and lamb marking (approximately 6 weeks from the start of lambing), with confirmation using ewe lactation status recorded at lamb marking.

Blood samples from maiden ewes were obtained pre- joining, scan 1, scan 2, pre-lambing and lamb marking. Samples collected at lamb marking were used for serology. Where a blood sample collected at lamb marking was not available (i.e the ewe was removed from the flock by the farmer after abortion was confirmed), then the last blood sample collected at pre-lambing or scan 2 was used for serology. Blood samples from multiparous mature ewes were collected on a single occasion, with the timing of sampling relative to lambing and the reproductive outcome for pregnancy was not recorded. Blood samples were obtained by jugular venepuncture and collected into serum vacutainer tubes with clot activator and stored on ice. Within 72 hours of collection, blood samples were centrifuged at 4000 rpm for 10 minutes and serum was decanted into 2mL low protein-binding polypropylene screw cap micro tubes and stored at -20^{o}C prior to serological testing.

8.2.3 *Neospora caninum* serology

Anti-*N. caninum* IgG serology was determined using indirect ELISA (ID Screen *Neospora caninum* Indirect, ID Vet, France) according to the manufacturer's instructions. The results were read at 450 nm using a Multiskan FC, Thermo Scientific spectrophotometer. Each plate included positive and

negative internal controls. Optical density (OD) values were expressed as the mean percentage of sample/positive (S/P) values, as recommended by the manufacturer:

$$S/P\ value = (OD_{sample} - OD_{negative.control})/(OD_{positive\ control} - OD_{negative.control})$$

Serum samples were classified as positive (S/P value ≥50), doubtful (S/P value 40 to <50) or seronegative (S/P value <40) according to manufacturer's recommendation.

Samples that returned a 'doubtful' result were re-tested using the same methods described above. If the second test was positive, the sample was categorised as positive, if the second test was doubtful or negative, the sample was categorised as negative.

Serology were performed by VETPATH Laboratories (Perth, Western Australia). VETPATH Laboratories are NATA (National Association of Testing Authorities) accredited under ISO 17025 for veterinary testing. However, NATA accreditation does not cover this specific test. The manufacturer reports sensitivity 100% (95% confidence interval (CI) 98.7, 100%) for bovine samples and specificity 100% (95% CI 97.7, 100%) using bovine, ovine and caprine sera for the commercial indirect ELISA kit used in this study. The manufacturer internal validation report states that only 5 positive sheep samples were used validation of sensitivity for ovine sera, therefore we used other published data for estimation of true prevalence. Sensitivity for *N. caninum* indirect ELISA in sheep has been reported ranging 95% – 100% and specificity ranging 96% – 100% (Osawa et al., 1998, Abo-Shehada and Abu-Halaweh, 2010, Reichel et al., 2008).

8.2.4 Statistical analyses

Lamb mortality was calculated based on the number of foetuses identified at the first pregnancy scan (scan 1) and the number of lambs marked. Mortalities were classified as 'abortion' based on evidence of pregnancy loss between scan 1 and scan 2, plus validation with lambing records (no lamb allocated to ewe at lambing inspections) and ewe lactation status (ewe not lactating at lamb marking). Abortion was expressed as a proportion (%) using the number of ewes with pregnancy loss between scan1 and scan 2 as a proportion of the number of ewes that were confirmed pregnant at scan 1.

Apparent seroprevalence was calculated based on the number of positive samples expressed as a proportion (%) of samples tested. Confidence intervals for apparent seroprevalence were determined using Jeffrey's method (Brown et al., 2001). Seroprevalence were compared for the ewe age categories using a two sample z-test to compare sample proportions (2-tailed) with P-value < 0.05 accepted as significant (Sergeant, 2021).

The true prevalence and 95% credible intervals (95% CrI) were estimated using Bayesian inference, considering a uniform prior distribution for sensitivity ranging from 95.0% to 100%, and specificity between 96.0% and 98.8% (Speybroeck et al., 2013). Estimates were obtained using the 'prevalence' and 'rjags' package in R (Devleesschauwer et al., 2015). Prior probabilities for the sensitivity and specificity were derived from Osawa et al. (1998), Abo-Shehada and Abu-Halaweh (2010), Reichel et al. (2008).

8.3 Results

8.3.1 Flock reproduction and farm characteristics

Overall, foetal and lamb mortality between scan 1 and lamb marking was 36% (1567/4351 foetuses identified at scan 1; range 14 - 71%) for maiden ewe lambs and 28% (582/2103 foetuses identified at scan 1; range 20 - 53%) for maiden hoggets. Abortion was detected in 14/19 maiden ewe lamb flocks and 6/11 maiden hogget flocks based on pregnancy loss between scan 1 and scan 2. In maiden ewe lamb flocks, abortion was detected in 5.2% (155/2968) ewes, ranging 0 – 50.0% across flocks. In maiden hogget flocks, abortion was detected in 0.8% (16/1886) ewes, ranging 0 – 4.4% across flocks.

Dogs were present on all farms in this study. Cattle were managed on the same property for 6/28 farms, however cattle were farmed in the same region for all farms.

8.3.2 Seropositivity for *N. caninum*

Apparent and true seroprevalence for ewes are shown in Table 29. *Neospora caninum* seroprevalence was 0.16% (95% CI 0.03, 0.5) in maiden ewes and 0% (95% CI 0; 0.45) in mature ewes (Table 29). There was no difference in apparent seroprevalence between ewe lamb and hogget flocks (P = 0.670), or between maiden and mature ewes (P = 0.334). Estimated true prevalence was less than 0.5% for all three ewe age categories (Table 29).

Seropositivity for *N. caninum* was not detected in any mature ewes (Appendix 5 Table A5.2). Seropositivity was detected in two maiden ewes, one from a Merino hogget flock in Victoria and the other from a Border Leicester ewe lamb flock in South Australia (Appendix 5 Table A5.1). Both of these ewes returned doubtful results at the initial test then positive result with a subsequent test (Appendix 5 Table A5.3). Both ewes categorised as seropositive were determined to be pregnant with a single viable foetus detected at the first and second pregnancy scan. In both cases, the lamb did not survive to marking. Within flock *N. caninum* apparent seroprevalence for both of the maiden ewe flocks with seropositivity detected (i.e. at least one positive ewe) was 2.5% (95% CI 0.3, 11.1).

Table 29: Apparent seroprevalence and estimated true prevalence for *N. caninum* using indirect ELISA for maiden ewes mated as ewe lambs (approximately one-year-old at sampling) or hoggets (approximately two-years old at sampling) and mature multiparous ewes (aged 3 years or older) from 28 Australian farms

	Ewes sampled				
	Flocks (n)	Individual ewes (n)	Seropositive samples (n)	**Apparent Seroprevalence** % (95% CI)	**Estimated True Seroprevalence** % (95% CrI)
Maiden ewes					
Ewe lambs	19	839	1	0.12 (0.01, 0.56)	0.2 (0.0, 0.6)
Hogget	11	440	1	0.23 (0.02, 1.06)	0.4 (0.0, 1.2)
Mature ewes	28	558	0	0 (0, 0.45)	0.2 (0.0, 0.5)

95% CI: 95% confidence interval
95% CrI: 95% credible interval

8.4 Discussion

There was no evidence that *N. caninum* infection was an important contributor to abortions or perinatal lamb deaths in maiden ewe flocks located across a wide geographic region in Australia.

This is consistent with absence of neosporosis diagnoses in ovine abortion and stillbirth investigations submitted to Australian state veterinary laboratories (Chapter 4)(Refshauge et al., 2020b) and limited reports of field infections in Australian sheep (Bishop et al., 2010). Very low seroprevalence in ewes suggests that exposure to *N. caninum* on these farms is very low despite widespread exposure to dogs and proximity to cattle farms.

Seropositivity was detected in two maiden ewes that failed to raise lambs. However, it was not possible to determine whether *N. caninum* infection contributed to the death of their progeny. Seropositivity at a single timepoint indicates evidence of previous exposure, but does not indicate how recently the infection occurred or if infection resulted in clinical disease. An aetiological diagnosis is generally based on demonstration of *N. caninum* in tissue via immunohistochemistry (Lindsay and Dubey, 2020) or molecular diagnostic techniques and concurrent histopathological lesions (Moreno et al., 2012). Notwithstanding, even if both lambs born to seropositive ewes did die due to neosporosis, this still reflects a very low incidence and does not support widespread involvement of *N. caninum* in the reproductive wastage observed in these flocks.

This is the largest *N. caninum* seroprevalence survey conducted in Australian sheep. Very low seroprevalence was consistent with a previous seroprevalence survey for five farms in New South Wales reporting slightly higher overall individual seroprevalence of 2.2% (5/232) (Bishop et al., 2010). These observations suggest that seroprevalence in Australian sheep is considerably lower than reported for cattle. Reasons for lower seroprevalence in sheep compared to Australian cattle were not able to be determined. Seroprevalence could be lower in our study compared to previous cattle studies due to characteristics related to the study population (including age and location of sheep sampled), differences in diagnostic tests used, differences in the incidence of vertical transmission between sheep and cattle, differences in grazing behaviour between sheep and cattle, and different susceptibility to natural infection (Abo-Shehada and Abu-Halaweh, 2010). Higher seroprevalence has been reported in cattle from New South Wales and Queensland compared to cattle from South Australia (Bishop et al., 2010, Fordyce et al., 2013, Moloney et al., 2017, Nasir et al., 2012, Stoessel et al., 2003). Regional differences in the distribution of *N. caninum* infection have also been reported overseas and may be related to climatic factors and management differences (Abo-Shehada and Abu-Halaweh, 2010, Frössling et al., 2008, Liu et al., 2015).

Few studies have investigated the association between individual ewe *N. caninum* serological status and reproductive outcomes. Our observations of low *N. caninum* seroprevalence in maiden ewes that aborted or failed to read lambs were consistent with a study in the United Kingdom reporting low *N. caninum* seroprevalence (0.45%) for 660 aborting ewes (Helmick et al., 2002). Two studies in New Zealand reported *N. caninum* seroprevalence ranging 1.8% to 36% in aborting ewes (Howe et al., 2012, Howe et al., 2008). However, there was no difference in seroprevalence between aborting ewes and those which raised lambs. These studies highlighted challenges interpreting serological data and the need for laboratory testing using aborted material to confirm a diagnosis of neosporosis (Howe et al., 2012, Howe et al., 2008).

Seroprevalence was determined using indirect ELISA. The commercial indirect ELISA has been used for seroprevalence surveys for *N. caninum* in sheep in other countries including Brazil (Cosendey et al., 2018) and Costa Rica (Villagra-Blanco et al., 2019), but the sensitivity and specificity of this indirect ELISA has not been validated for Australian sheep under field conditions. The present study did not include a reference test (e.g. IFAT) and therefore it was not possible to evaluate sensitivity of the commercial indirect ELISA. It is possible that the two sera samples categorised as positive were false positive reactions. The proportion of false positives is expected to increase for infections that have low prevalence. Further evaluation of the indirect ELISA specificity and sensitivity using sera

collected from naturally infected Australian sheep would improve estimates for true prevalence. However, neosporosis cases are sporadic in Australian sheep with a single report in the literature describing cerebral neosporosis detection in Merino sheep on one farm and seropositivity detection on three farms (Bishop et al., 2010). This presents challenges for further evaluating assay sensitivity and specificity for Australian sheep under field conditions.

Selection of farms was based on convenience sampling and sampling of maiden ewes was biased towards ewes that had aborted or failed to rear lambs. This bias could result in overestimation of the seroprevalence in the general population of one- and two-year old ewes if *N. caninum* was an important contributor to abortion and perinatal deaths in Australian sheep. The very low seroprevalence in randomly selected mature ewes from the same farms suggests that sampling bias related to reproductive status for maiden ewes did not affect the conclusion that there was no evidence of widespread exposure to *N. caninum* during pregnancy for maiden ewes on these farms.

Based on the limited data available, *N. caninum* does not appear to be an important reproductive pathogen in Australian sheep. However, relatively few abortion investigations are conducted in Australian sheep, primarily because detecting abortions and recovering aborted lambs and/or foetal membranes for disease investigations is challenging in extensive sheep management system. Furthermore, screening for *N. caninum* is not routine in ovine abortion investigations and therefore neosporosis may be underreported. This study utilised a single diagnostic test (indirect ELISA), and it is possible that exposure to *N. caninum* was underestimated without concurrent alternate methods such as IFAT or molecular diagnostics (Howe et al., 2012). Infections with *N. caninum* can occur in Australian cattle in the absence of detectable antibodies (McInnes et al., 2006), but it is not clear how common this is for sheep. Campylobacteriosis, listeriosis and toxoplasmosis are the most common diagnoses made in abortion investigations in Australia (Chapter 4)(Refshauge et al., 2020b). More recently cases for *Chlamydia pecorum* abortion have been reported for maiden ewes in Australia, including flocks in this study (Chapter 6)(Westermann et al., 2021).

Our observations suggest that suspicion of *N. caninum* abortion should be raised when seropositivity is detected in Australian sheep and other causes of abortion have been ruled out because there is no evidence that background exposure to *N. caninum* is widespread in southern Australia. Sheep may become infected with *N. caninum* by horizontal transmission (ingestion of feed or water contaminated with oocysts shed by canine definitive host) or vertical transmission (transplacental infection of the foetus by the dam during pregnancy) (González-Warleta et al., 2018) On Australian sheep farms where *N. caninum* infection is absent or low, the risk for neosporosis outbreaks can be mitigated through strategies reducing exogenous infection via the canine definitive host including: controlling wild or stray dogs and foxes; restricting access of whelping bitches, pups or young dogs near pregnant sheep; and preventing domestic and wild canids from ingesting fresh ruminant meat and offal (Reichel et al., 2014, King et al., 2010). Management strategies aimed at limiting potential for *N. caninum* transmission from dogs to sheep are likely to be effective in managing transmission for other sheep parasites with canine definitive hosts endemic in Australia that have important economic and/or zoonosis implications including *Taenia ovis* and *Echinococcus granulosus.*

8.5 Conclusion

There was no evidence of widespread exposure to *N. caninum* in breeding ewes on sheep farms in southern Australia using indirect ELISA. Furthermore, there was no evidence that *N. caninum* was an important contributor to abortions or perinatal lamb deaths observed for maiden ewes on the farms in this study.

9. *Coxiella burnetii* and reproductive performance of maiden ewes

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9.1 Introduction

The reproductive performance of maiden (primiparous) ewes is often lower than that observed for mature multiparous ewes (Shorten et al., 2021, Allworth et al., 2017, Kleemann and Walker, 2005). Higher incidence of foetal or lamb mortality from pregnancy diagnosis to lamb marking has been reported for maiden ewes compared to multiparous ewes (Kleemann and Walker, 2005, Allworth et al., 2017, Kilgour, 1992). However, the causes of losses that occur during this period are not well defined. A number of endemic diseases may cause abortion and poor viability of lambs in Australia (Chapter 4)(Refshauge et al., 2020b). It is not clear if infectious diseases are an important contributor to foetal and lamb mortality for maiden ewes in Australia.

Coxiella burnetii is endemic in Australian livestock, including sheep, but prevalence and impact on sheep health is not well studied (Tan, 2018). Infections in sheep can be asymptomatic, but in pregnant ewes can cause abortion, stillbirth and the birth of weak lambs that are less likely to survive (Arricau-Bouvery and Rodolakis, 2005, Martinov et al., 1989, Berri et al., 2001, Brooks et al., 1986, Berri et al., 2005). The outcome of infection in the pregnant ewe may be influenced by strain virulence, severity of placental infection, and maternal and foetal immunity (Agerholm, 2013). Abortions are more likely to occur during gestation following primary infection, with no lasting impacts on reproduction in subsequent pregnancies (Berri et al., 2001, Berri et al., 2007). Therefore, younger ewes that are immunologically naïve are at risk of abortion if infection occurs during pregnancy.

Seroprevalence for *C. burnetii* in Australian sheep has been reported to range from 0 - 18.7% depending on the location, serological assay and cut-off values used (Derrick et al., 1959, Smith, 1962, Rowan and Keast, 1965, Tan, 2018, Banazis et al., 2010). However, most of these studies are either over 50 years old or localised to specific regions or single farms. Consequently, the prevalence of *C. burnetii* for the current Australian sheep population is poorly quantified and the impact of *C. burnetii* on the reproductive performance of Australian sheep has not been assessed.

Apart from impacts for sheep health and production, *C. burnetii* has important zoonotic implications. Australia has one of the highest rates of human Q-fever cases reported globally (Gidding et al., 2009, Eastwood et al., 2018). Livestock are considered to be an important reservoir for infection in humans (Fournier et al., 1998, Angelakis and Raoult, 2010). Improved understanding of the role of sheep as a potential source of *C. burnetii* infections will inform recommendations for managing Q-fever risk in susceptible people including occupational risks for farmers, veterinary staff and abattoir workers (Sloan-Gardner et al., 2016, Bond et al., 2016).

The aims of this study were to (i) determine if natural *C. burnetii* exposure is associated with poor reproductive performance of maiden ewes in southern Australia, (ii) determine if natural exposure to *C. burnetii* is widespread in maiden and multiparous ewes, and (iii) determine if ewes represent an important reservoir for *C. burnetii* infection in humans. We hypothesised that (i) *C. burnetii* infection and seropositivity is associated with foetal and lamb loss in maiden ewes, and (ii) ewes will demonstrate seropositivity for *C. burnetii* indicating that ewes are reservoir for infection.

9.2 Methodology

All procedures were conducted according to guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes and were approved by the Murdoch University Animal Ethics Committee (R3004/17).

9.2.1 Animals and research sites

This cross-sectional study was conducted at 28 farms using 30 study flocks located in Western Australia (*n* = 11), South Australia (*n* = 9), and Victoria (*n* = 10) between 2018 and 2020 (Figure 7; Appendix 5 Table A5.1). Farms were selected based on convenience sampling, with eligibility for inclusion based on the farm having sufficient number of maiden ewes available for the study, capacity to monitor ewes and their progeny over the study period, and sheep genotype and management that were generally representative of standard commercial sheep farms in the region. Approximately two-hundred maiden ewes at each farm were randomly selected at joining. All farms ran self-replacing flocks and ewes included in the study were managed according to standard farm practice.

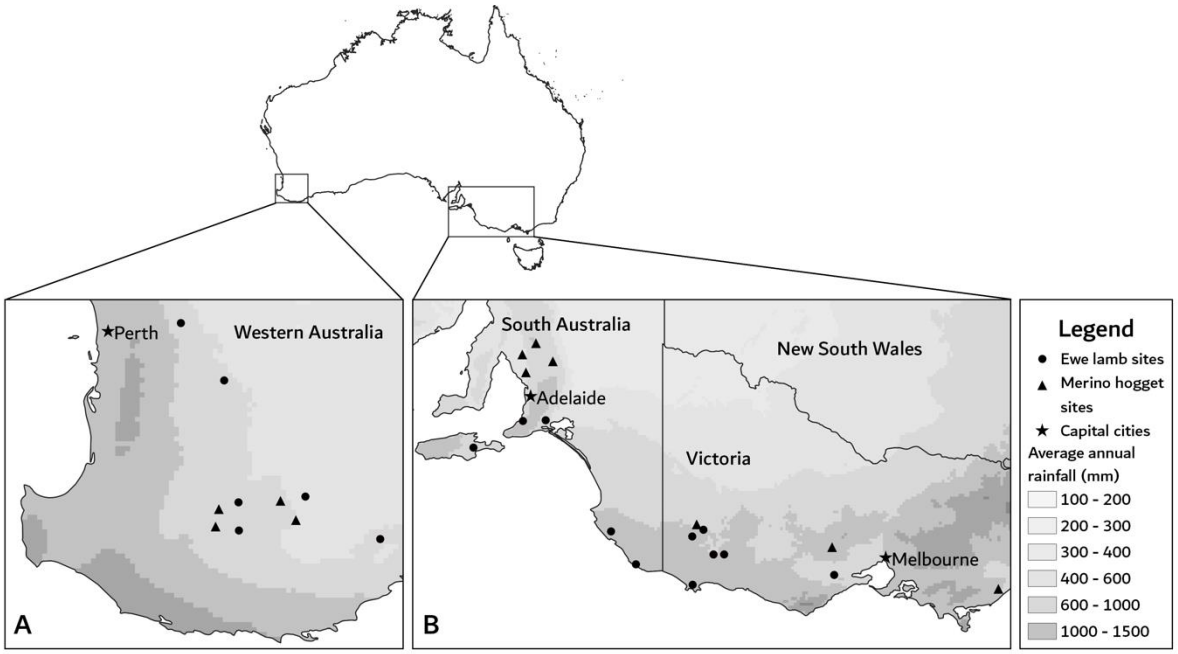

Figure 7: Location of sheep farms sampled in Western Australia (Map A), and South Australia and Victoria (Map B). Average annual rainfall data sourced from Australian Government Bureau of Meteorology (2021)

9.2.2 Animal measurements and sample collection

Maiden ewes were monitored between joining and lamb marking. Ewes were mated as either ewe lambs (7-10 months, *n* = 19 flocks) or maiden hogget ewes (18-20 months, *n* = 11 flocks). Foetal mortality was determined via sequential transabdominal pregnancy ultrasounds at 62-101 days (scan 1) and 108-136 days (scan 2) from the start of joining. Lamb mortality between birth and marking were determined for each ewe based on birth type (single, twin or triplet), birth status (lambs dead or alive at lambing rounds) and survival status (lambs dead or alive) at marking which was approximately six weeks from the start of lambing. Ewe lactation status (lactating or non-lactating) was also determined at lamb marking.

Blood samples were collected from maiden ewes by jugular venepuncture at pre- joining, scan 1, scan 2, pre-lambing and lamb marking. At each farm, 20 multiparous ewes aged three years or older that had been bred and reared on the same farm were also randomly selected for collection of blood samples. Timing of sampling relative to lambing and the reproductive outcome for pregnancy was not recorded for multiparous ewes (Appendix 5 Table A5.2). Blood samples were collected into serum vacutainer tubes with clot activator and stored on ice. Blood samples were centrifuged at 4000 rpm for 10 minutes within 72 hours of collection. Serum was then decanted into 2mL low protein-binding polypropylene screw cap micro tubes and stored at -20^{o}C prior to serological testing.

9.2.3 Serology

Coxiella burnetii serology sample size was determined using expected seroprevalence 10% (based on an apparent prevalence ranging between 0% and 19% in previous Australian studies (Derrick et al., 1959, Smith, 1962, Rowan and Keast, 1965, Tan, 2018, Banazis et al., 2010)). A sample size of 200 ewes was adequate to estimate true prevalence with 95% confidence interval (95% CI) based on assumed true prevalence 10% and precision 5% with test sensitivity 87% and specificity 98% (Lurier et al., 2021). Sample size required to detect disease was 27 ewes per flock assuming expected seroprevalence 10%, test sensitivity 87%, population size 200 ewes and 95% CI.

A sub-sample of at least 40 maiden ewes from each study flock were selected for *C. burnetii* serology (Appendix 5 Table A5.1). Where possible, selection was based on ewes that were identified as pregnant at scan 1 but failed to successfully rear a lamb. This included ewes that aborted as well as ewes for which lamb mortality occurred in the perinatal period. Ewes that had reared single or twin lambs were included for flocks with less than 40 ewes that failed to rear a lamb (Appendix 5 Table A5.1). Blood samples collected from maiden ewes at lamb marking were used for serological screening except where not available, in which case samples from the latest available timepoint were screened instead. All blood samples collected from the multiparous ewes (*n* = 20 per farm) were used for serological screening (Appendix 5 Table A5.2).

Anti-*C. burnetii* IgG serology were determined by VETPATH Laboratories (Perth, Western Australia) using a commercial indirect ELISA kit (ID Screen Q-Fever Indirect Multispecies, ID Vet, France) according to the manufacturer's instructions. The results were read at 450 nm using a Multiskan FC, Thermo Scientific spectrophotometer. Each plate included positive and negative internal controls. Optical density (OD) values were expressed as the mean percentage of sample/positive (S/P) values, as recommended by the manufacturer:

$$\text{S/P value} = (OD_{sample} - OD_{negative.control})/(OD_{positive\ control} - OD_{negative.control})$$

Serum samples were classified as positive (S/P value $\geq$50), doubtful (S/P value 40 to <50) or negative (S/P value <40) according to the manufacturer's recommendation. Lurier et al. (2021) reported this indirect ELISA kit to have median sensitivity 86.9% (95% credible interval (95% CrI) 71.2%, 93.6%) and specificity 98.5% (95% CrI 97.3%, 99.4%) for sheep.

For cases that returned positive or doubtful results using indirect ELISA, samples were re-tested with the same indirect ELISA as describe above, plus a complement fixation test (CFT) to determine the end-point titre. The CFTs were performed on serum by Department of Primary Industry and Regional Development Diagnostic Laboratory Service using methodology previously described by Ellis and Barton (2003). For the CFT, a serum tire of 1:8 or higher was considered positive (Ellis and Barton, 2003). Samples that returned one 'positive' result for either test were considered positive. Both laboratories used are NATA (National Association of Testing Authorities) accredited under ISO 17025 for veterinary testing.

9.2.4 Aborted and stillborn tissues

Tissue samples from aborted (*n* = 2) or stillborn (*n* = 33) lambs recovered from a subset of seven flocks of maiden ewes (Flocks 1, 2, 3, 7, 11, 14, 16) from six farms in Western Australia were submitted to the Department of Primary Industry and Regional Development Diagnostic Laboratory Services (Perth, Western Australia). Tissue samples were screened for *C. burnetii* using qPCR as previously described in more detail in Chapter 6.

9.2.5 Statistical analyses

Lamb mortality was calculated based on the number of foetuses identified at scan 1 and the number of lambs marked. Lamb mortality was classified as 'abortion' based on foetal loss between scan 1 and scan 2 (and validated with lambing records and ewe lactation status). Apparent *C. burnetii* seropositivity proportion was calculated using number positive samples as a proportion of samples tested, with 95% confidence interval was determined using Jeffreys method (Brown et al., 2001). Seropositivity proportion for the ewe age categories were compared using a two sample z-test to compare sample proportions (2-tailed) with *P*-value < 0.05 accepted as significant (Sergeant, 2021).

The true *C. burnetii* seropositivity proportion and 95% credible intervals (95% CrI) was estimated using Bayesian inference, considering the sensitivity and specificity and their 95% CrI derived from Lurier et al. (2021) as beta-pert distribution for priors (Speybroeck et al., 2013).

9.3 Results

9.3.1 Reproductive performance of maiden ewes

Reproductive performance for maiden ewes are described in more detail in Chapter 5. Briefly, foetal and lamb mortality between scan 1 and lamb marking for maiden ewe lambs was 36% (1567/4351 foetuses identified at scan 1; range 14 - 71%) and for maiden hoggets was 29% (582/2103 foetuses identified at scan 1; range 20 - 53%). Abortion (foetal loss between scan 1 and scan 2) was detected in 14/19 maiden ewe lamb flocks and 6/11 maiden hogget flocks. Abortion was detected in 5.2% (155/2968) ewes for maiden ewe lamb flocks, ranging 0 – 50.0% across flocks. For maiden hogget flocks, abortion was detected in 0.8% (16/1886) ewes, ranging 0 – 4.4% across flocks.

9.3.2 *Coxiella burnetii* serology

Apparent and true seropositivity to *C. burnetii* for ewe age categories are shown in Table 30. Apparent seropositivity to *C. burnetii* was 0.08% (95% CI 0.01%, 0.36%) in maiden ewes and 0.36% (95% CI 0.07%, 1.14%) in mature ewes. Seropositivity to *C. burnetii* did not differ between maiden ewes and mature multiparous ewes (*P* = 0.174), nor between maiden hoggets and ewe lambs (*P* = 0.165).

Farm-level seropositivity to *C. burnetii* (detected in at least one animal) was 10.7% (3/28) farms. Within-flock seropositivity for the three flocks where seropositivity was detected ranged 2.5%-5.0% (Appendix 5 Table A5.1). The three flocks with seropositivity to *C. burnetii* were located in Western Australia, South Australia and Victoria (Appendix 5 Table A5.1).

All three samples with seropositivity detected using indirect ELISA were negative for *C. burnetii* by CFT (Appendix 5 Table A5.3).

Table 30: Apparent *C. burnetii* seropositivity and estimated true seropositivity using indirect ELISA for maiden ewes mated as ewe lambs (approximately one-year-old at sampling) or hoggets (approximately two-years old at sampling) and mature multiparous ewes (aged 3 years or older) from 28 Australian farms

	Ewes sampled				
	Flocks (n)	Individual ewes (n)	Seropositive samples (n)	**Apparent seropositivity** % (95% CI)	**Estimated true seropositivity** % (95% CrI)
Maiden ewes					
Ewe lambs	19	839	0	0 (0, 0.30)	0.1 (0.0, 0.4)
Hoggets	11	440	1	0.23 (0.02, 1.06)	0.3 (0.0, 1.1)
Mature ewes	28	558	2	0.36 (0.07, 1.14)	0.3 (0.0, 1.0)

95% CI: 95% confidence interval
95% CrI: 95% credible interval

9.3.3 Molecular detection of *C. burnetii* in tissues from aborted and stillborn lambs

Coxiella burnetii was not detected by qPCR in tissue samples from aborted (*n* = 2) or stillborn lambs (*n* = 33) recovered from maiden ewes on the subset of seven flocks in Western (Chapter 6).

9.4 Discussion

There was no evidence to implicate *C. burnetii* as an important contributor to abortion or perinatal lamb mortality in 30 maiden ewe flocks located across southern Australia. The very low *C. burnetii* seropositivity was consistent with the absence of detection of *C. burnetii* in tissues from aborted or stillborn lambs from a subset of farms. These findings are consistent with recent reviews of veterinary laboratory investigations that reported coxiellosis to be an uncommon diagnosis in Australian sheep abortion investigations (Chapter 4)(Refshauge et al., 2020b). *Coxiella burnetii* control programs such as routine vaccination of breeding ewes are not warranted for sheep farms in southern Australia in the absence of further evidence that coxiellosis is contributing to lamb mortality. Nonetheless, *C. burnetii* should continue to be included in sheep abortion and perinatal mortality investigation protocols due to the sporadic nature of disease and important zoonotic implications.

This is the most geographically widespread serological study for *C. burnetii* in Australian sheep. Very low seropositivity to *C. burnetii* was consistent with previous studies from Western Australia (Banazis et al., 2010) and Victoria (Tan, 2018) that reported individual seroprevalence ranging from 0 - 4.1%, and flock-level seroprevalence ranging from 0 – 17.6%. Our study did not include sheep flocks from New South Wales, Queensland or Tasmania. New South Wales and Queensland have the highest rates of human Q-fever reported in Australia (Eastwood et al., 2018). The most recent studies reporting *C. burnetii* prevalence in sheep from New South Wales and Queensland are considerably dated and involve either single farms (Derrick et al., 1959, Smith, 1962) or abattoir surveys (Rowan and Keast, 1965). Increased incidence of local acquisition of human infection may be associated with high prevalence in livestock (Bond et al., 2018). Hence, investigation of *C. burnetii* seroprevalence in sheep from New South Wales and Queensland is warranted.

There are several aspects of this study that limit the generalisability of the seropositivity to *C. burnetii* observed in these flocks to the general sheep population in southern Australia. Firstly, serological testing targeted maiden ewes with evidence of abortion and perinatal lamb mortality.

Bias towards ewes that failed to rear lambs could be expected to overestimate prevalence in the general sheep population if *C. burnetii* was an important contributor to abortion and perinatal deaths. Very low seropositivity to *C. burnetii* in this sampled population suggests that coxiellosis was not an important contributor to abortion and perinatal lamb mortality in these flocks. Very low seropositivity to *C. burnetii* in the sampled population of maiden ewes was consistent with that observed for randomly selected mature ewes on these farms. Secondly, blood samples for maiden ewe samples were collected close to the time of lambing or abortion, and this may increase probability of detection of *C. burnetii* seropositivity (Muleme et al., 2017). Lastly, whilst the inclusion criteria for farms included sheep genotype and management that were generally representative of standard commercial sheep farms in the region, inclusion criteria involved ability to monitor ewes over study period and some sheep studs were included in the study. Further investigation is required to confirm if very low seroprevalence is consistently observed across the general population of breeding ewes on commercial farms in southern Australia.

Sampling younger ewes likely contributed to the low seropositivity to *C. burnetii* reported in this study. Age is recognised as an important risk factor for *C. burnetii* seropositivity, with older animals more likely to be seropositive (Klaassen et al., 2014, Muema et al., 2017, García-Pérez et al., 2009). Notwithstanding this, no apparent difference in seropositivity was observed between maiden ewe lambs (approximately 13 months old at lambing), hoggets (2 years old at lambing) and mature ewes (3 years or older).

There is no reference test for serological diagnosis of coxiellosis, and sensitivity and specificity for *C. burnetii* serological tests are not well described (Joulié et al., 2017). The commercial indirect ELISA for *C. burnetii* that was used in this study has been used in other seroprevalence studies in sheep (Joulié et al., 2017, Cruz et al., 2018, Villari et al., 2018, Conan et al., 2020, Turcotte et al., 2021, Lurier et al., 2021) and the World Organisation for Animal Health (OIE) recommends ELISA as the preferred method for *C. burnetii* seroprevalence studies (World Organisation for Animal Health, 2019). In our study, the three samples categorised as seropositive using indirect ELISA were negative by CFT. It was not possible to determine if these were false positives. Complement fixation tests are reported to have lower sensitivity than ELISA, but high specificity for elevated levels of anti-*C. burnetii* antibodies in flocks with *C. burnetii*-associated abortions (World Organisation for Animal Health, 2019). Discordant results can be observed using different ELISA kits (Horigan et al., 2011), therefore testing samples with more than one kit is an alternative option for validating animal status (World Organisation for Animal Health, 2019). Validation for commercial ELISA in Australian sheep under field conditions could better inform estimation of true prevalence. However, coxiellosis is not frequently diagnosed in Australian sheep which presents challenges for evaluating assay sensitivity and specificity under field conditions.

Seroprevalence surveys may underestimate *C. burnetii* shedding in livestock. Banazis et al. (2010) detected *C. burnetii* in Australian sheep faecal samples in the absence of *C. burnetii* seropositivity. Other studies have also demonstrated poor correlation between seropositivity and antigen detection (Berri et al., 2005, Berri et al., 2001, Arricau Bouvery et al., 2003, Joulié et al., 2017). Joulié et al. (2017) reported good correlation between high *C. burnetii* burden on vaginal swabs and seropositivity one-month post-abortion or post-lambing using the same commercial indirect ELISA kit as used in our study. However, it is possible that some ewes in our study were shedding *C. burnetii* without evidence of seropositivity, and thus represent a reservoir of *C. burnetii* infection for other sheep or humans. Nevertheless, the combination of testing methodology used and timing of blood sample collection (within 6 weeks of parturition) in conjunction with the absence of detection

of *C. burnetii* using molecular techniques on tissues from aborted or stillborn lambs suggests that coxiellosis was not a major contributor to abortion and lamb mortality observed on these farms.

This was an observational study with sheep managed extensively, reflecting standard sheep management in these regions of Australia. Although foetal and lamb mortality between scanning and lamb marking were high for some flocks, average lamb mortality in the maiden flocks was consistent with ranges previously reported in Australian studies (Hinch and Brien, 2014b). It is unclear from the current study whether reproductive performance of maiden ewes would be impacted in flocks where *C. burnetii* seroprevalence was greater.

This study focussed on *C. burnetii*, but foetal and lamb mortality are often multifactorial (Hinch and Brien, 2014b). Endemic diseases other than coxiellosis were contributing to abortions and perinatal lamb mortality in flocks in this study. *Chlamydia pecorum* was detected in aborted foetuses, stillborn lambs and lambs with evidence of polyarthritis post-weaning in a subset of flocks (Chapter 4)(Ostfeld et al., 2020). Campylobacteriosis (*Campylobacter fetus* fetus) was identified in one flock (Chapter 10). There was no evidence that infection with *Toxoplasma gondii* (Chapter 7) or *Neospora caninum* (Chapter 8) were important contributors to foetal and lamb mortality observed in these flocks. Further investigations using data from this study will include multivariable analysis to evaluate the relative importance of different pathogens on reproductive performance.

Despite low seropositivity to *C. burnetii* detected in this study, contact with sheep should still be considered a risk factor for Q-fever in humans and precautions should be taken to reduce the risk of zoonotic *C. burnetii* transmission. Sheep have been associated with cases of Q-fever in humans in Australia and overseas (Berri et al., 2001, Graves and Islam, 2016, Bond et al., 2018, Boland and Parker, 1999, O'Connor et al., 2015, Derrick et al., 1959, Tissot-Dupont et al., 2004, Webster et al., 2009). *Coxiella burnetii* shedding can occur from both symptomatic and asymptomatic sheep, and in the absence of detectable seropositivity (Jones et al., 2010, Rodolakis et al., 2007). Control strategies include use of appropriate personal protective clothing when handling birth material or lambing ewes, good hygiene practices, controlling dust and vaccination of people with an occupational risk including farm, abattoir and veterinary staff.

9.5 Conclusion

There was no evidence to implicate *C. burnetii* as an important contributor to abortions or perinatal lamb mortality observed for maiden ewes on the farms in this study. Furthermore, exposure to *C. burnetii* was not widespread in sheep from farms in southern Australia included in the study. Whilst ewes on these farms were not an important reservoir for *C. burnetii,* the occupational risk associated with transmission of *C. burnetii* from Australian sheep has public health implications and people at risk should maintain appropriate measures to avoid zoonotic transmission.

10. *Campylobacter* spp. and reproductive performance of maiden ewes

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10.1 Introduction

Infectious agents causing abortion, stillbirths and perinatal lamb mortality can cause significant production losses in sheep flocks. New Zealand studies have reported that infectious agents are an important contributor to the poor and inconsistent reproductive performance observed for maiden ewes (Kenyon et al., 2014b, Howe et al., 2012, Kenyon et al., 2004, Ridler et al., 2015). In Australia, the causes of foetal and lamb losses between pregnancy diagnosis in mid-pregnancy and lamb marking for maiden ewes are not well studied. Furthermore, it is not clear whether infectious diseases are an important contributor to the poorer reproductive performance reported for maidens compared to multiparous ewes (Allworth et al., 2017, Kilgour, 1992, Kleemann and Walker, 2005, Hutchison et al., 2022).

Ovine campylobacteriosis caused by *Campylobacter fetus* or *C. jejuni* is one of the most frequently diagnosed causes of ovine abortion in Australia (Chapter 4; (Refshauge et al., 2020a)). Transmission occurs via ingestion of feed and water contaminated by faeces or aborted material, with no evidence of venereal spread in sheep (Mearns, 2007b). Abortion due to campylobacteriosis generally occurs in the last six weeks of pregnancy and may be sporadic or associated with an abortion frequency up to 50% of ewes in previously naïve flocks (Clough, 2003). The extent of reproductive loss attributable to abortion or perinatal lamb mortality is difficult to quantify, particularly in extensively-managed sheep flocks. However, it has been estimated that sub-clinical disease could account for around 10% of foetal and lamb mortality in flocks where *Campylobacter* spp. are endemic (Anderson, 2001).

Ewes that abort due to campylobacteriosis become immune and are less susceptible to abortion or perinatal mortalities with subsequent exposure (Jensen et al., 1957, Marsh et al., 1954, Meinershagen et al., 1969). Younger ewes are less likely to have had previous exposure to *Campylobacter* spp. and are therefore considered at greater risk of abortion due to campylobacteriosis. A commercial vaccine against both *C. fetus* and *C. jejuni* is available in Australia and internationally. Higher lamb marking percentages have been reported from vaccinated compared to unvaccinated maiden ewe flocks in New Zealand (Kenyon et al., 2014b). However, Australian studies report variable responses to *Campylobacter* vaccination in maiden ewes (Kenyon et al., 2004, Glanville, 2017, Walsh, 2016). Further investigation is required to quantify the impact of

campylobacteriosis on the reproductive performance of maiden ewes in Australia and risk factors for disease.

The incidence of campylobacteriosis in Australian sheep is not well described. A serological survey by the manufacturer of a vaccine against *Campylobacter* spp. reported individual animal seroprevalence of 30% for *C. fetus* (titre ≥1:10) and 41% for *C. jejuni* (titre ≥1:80) for ewes across the major sheep production regions of Australia (Wills, 2021). This was consistent with data from veterinary laboratories indicating that *Campylobacter* abortions are diagnosed for sheep located across different states of Australia in most years (Chapter 4; (Refshauge et al., 2020a). However, interpreting the pathological significance of serology results is complicated because *Campylobacter* spp. are commonly isolated from the gastrointestinal tract of clinically healthy sheep (Bailey et al., 2003, Yang et al., 2017, Yang et al., 2014b, Milnes et al., 2008). An improved understanding of *Campylobacter* antibody dynamics in relation to sheep reproductive outcomes will improve our ability to estimate the impacts of *Campylobacter* on the health and productivity of sheep based on serological studies, and support veterinarians in making evidence-based recommendations on disease management based on serology.

The aims of this study were to: (i) investigate associations between seropositivity to *C. fetus* and *C. jejuni* and reproductive outcomes for maiden ewes, and (ii) determine appropriate strategies for estimating the impact of campylobacteriosis on abortion and lamb mortality using ewe serology.

10.2 Methodology

All procedures were conducted according to guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes and were approved by the Murdoch University Animal Ethics Committee (R3004/17). Consent to participate was provided by the owners of the sheep included in this study.

10.2.1 Animals, study sites and management

This case-control study was nested within a larger cohort study, which involved monitoring maiden ewes during pregnancy and lambing as described in Chapter 5. A subset of 22 flocks from 21 farms that had not received *Campylobacter* spp. vaccination were included in this study. Maiden ewes were joined for an average of 39 days, ranging from 17-54 days. These flocks were located across a range of geographic regions and rainfall zones across Western Australia (*n = 11*), South Australia (*n = 6*) and Victoria (*n = 5*) (Table 31 and Figure 8). Briefly, data (including condition score, liveweight and reproductive outcome) were collected for approximately 200 ewes per flock over a single breeding season between 2018 and 2020. Flock 3 (2018) and Flock 14 (2019) were located on the same farm, but all other flocks were on different farms. Farms were selected based on the following inclusion criteria: sufficient maiden ewes (approximately 200 mated), ability to monitor ewes and their progeny over the study period, and sheep genotype and management that were generally representative of standard commercial sheep farms in the region. Some stud flocks were included in the study which may have increased frequency of monitoring relative to commercial flocks, but stocking rate (density) and housing were broadly comparable to commercial sheep flocks in these regions. Flock reference codes were assigned in order of recruitment for the larger cohort study (Chapter 5). Flocks that had received *Campylobacter* spp. vaccination were subsequently excluded from this study, hence the flock reference codes are not sequential (Table 31).

Maiden ewes were mated as either ewe lambs (7-10 months, *n* = 12 flocks) or maiden hoggets (18-20 months, *n* = 10 flocks), with both Merino and non-Merino ewes included in the study (Table 31). Ewes in this study were not vaccinated against *Campylobacter* spp. However, some farms had other cohorts of ewes on the same property that had received Coopers Ovilis® Campyvax® *Campylobacter* vaccine for sheep (Coopers, MSD Animal Health, Australia). Each farm ran self-replacing flocks (*i.e.* ewes were born and raised on the study farm) and maiden ewes were managed extensively as per standard farm practice. At each farm, 10-20 unvaccinated, multiparous ewes aged three years or older, that had been bred on farm, were randomly selected for blood sampling at a single time-point during the study period.

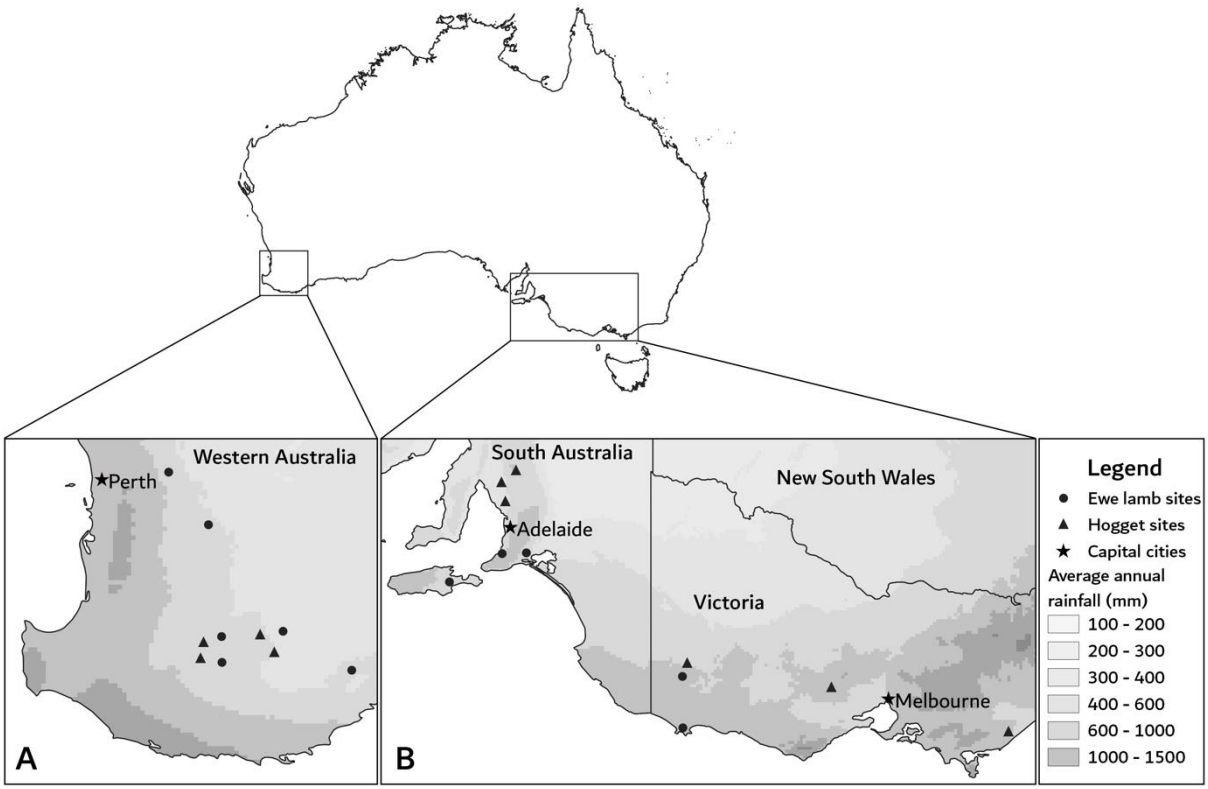

Figure 8: Approximate location of 21 farms where maiden and mature ewes were sampled in Western Australia (A) and South Australia and Victoria (B). On one farm, two flocks were sampled. Average annual rainfall data was sourced from Australian Government Bureau of Meteorology (2021)

Table 31: Location of farms, historical average annual rainfall, ewe breed, frequency of mid-pregnancy abortion between scan 1 and scan 2 and overall foetal/lamb mortality between scan 1 and lamb marking for maiden ewe lambs and hoggets in southern Australia between 2018 and 2020

Flock reference	**Location**[a]	**Rainfall** (mm/annum)	**Breed**	**Mid-pregnancy abortion** [b] (% ewes)	**Overall foetus &/or lamb mortality** [c] (% foetuses)
EWE LAMBS					
3[d]	Narrogin, WA	545	Composite	7.4	37.8[e]
4	York, WA	392	Composite	1.5	23.0
7	Kojonup, WA	530	Composite	0.7	27.7[e]
8	Katanning, WA	444	Merino	1.2	33.0
11	Kojonup WA	530	Dorper	0.0	27.0[e]
14[d]	Narrogin, WA	545	Composite	23.8	59.0[e]
16	Ongerup, WA	387	White Suffolk	2.9	33.8[e]
19	Nareen, VIC	691	Composite	8.5	50.5[e]
20	Cashmore, VIC	841	Composite	4.3	41.1
23	Kangaroo	530	Composite	1.8	18.1
25	Sellicks Hill, SA	493	Composite	1.4	65.5
30	Strathalbyn, SA	490	Border Leicester	1.3	37.7
HOGGETS					
1	Kojonup, WA	530	Merino	0	19.7[e]
2	Kojonup, WA	530	Merino	0	30.1[e]
5	Korunye, SA	364	Merino	1.1	27.4
9	Watervale, SA	650	Merino	0	21.7
10	Broomehill,	446	Merino	0	26.3
12	Tarlee, SA	469	Merino	1.1	28.6
13	Giffard West,	662	Merino	0.9	52.7
15	Katanning, WA	444	Merino	0.5	25.4
26	Culla, VIC	579	Merino	4.4	38.9
29	Ballarat, VIC	686	Merino	2.2	23.3

[a] WA: Western Australia, VIC: Victoria, SA: South Australia

[b] Ewes with mid-pregnancy abortion between scan 1 and scan 2 as the proportion (%) of ewes scanned pregnant at Scan 1. Includes all causes of mid-pregnancy abortion (*i.e.* not specific to campylobacteriosis)

[c] Overall foetal and lamb loss between Scan 1 and lamb marking expressed as proportion (%) foetuses detected at Scan 1. Includes all causes of foetal/lamb mortality (*i.e.* not specific to campylobacteriosis)

[d] Same farm – primiparous ewes tested in 2018 (Flock3) and 2019 (Flock 14)

[e] Tissues from aborted or stillborn lambs submitted for *Campylobacter* spp. microbial culture and/or qPCR

10.2.2 Determination of reproductive outcome

The reproductive outcome for maiden ewes was determined using two sequential transabdominal pregnancy ultrasounds (scans) plus observations at lambing rounds and lamb marking as previously described in Chapter 5. Briefly, pregnancy scans were conducted at approximately 85 days (range 62-101; scan 1) and 118 days (range 107-136; scan 2) from the start of mating. Pregnancy scanning for foetal number and viability were performed by experienced researchers, veterinarians or private contractors. The birth type (single, twin or triplet) and survival status (dead or alive) for lambs were recorded within 24 hours of birth. Lamb survival and ewe lactation status (lactating or not) were recorded at lamb marking approximately six weeks from the start of lambing.

Lamb mortality was calculated based on the number of foetuses identified at scan 1 and the number of lambs marked. Mortalities were classified as 'mid-pregnancy abortion' based on evidence of pregnancy loss between scan 1 and scan 2, plus validation with lambing records (no lamb allocated to ewe at lambing inspections) and ewe lactation status (ewe not lactating at lamb marking). During pregnancy, ewes were inspected by farm staff at least twice weekly by observing the ewes in their paddocks. This included observation for evidence of breech staining, foetal membranes or aborted/premature lambs**.** For flocks where mid-pregnancy abortion was detected at scan 2, farm staff were alerted to the possibility of detecting aborted foetuses and the ewes were subsequently checked at least every second day. Ewes that were pregnant at scan 1 but did not have a lamb survive to marking were determined to "failed to rear" (Table 32). "Failed to rear" included ewes that aborted or had lambs die during the perinatal period as determined by repeat ultrasound, lambing round records, lamb marking records (no live lamb allocated to ewe present at marking) and ewe lactation status at lamb marking (ewe not lactating).

Table 32: Ewe flock and *Campylobacter* spp. titre category definitions

Category	Definition
Ewe lambs	Primiparous ewe mated at 7-10 months of age
Maiden hoggets	Primiparous ewe mated at 18-20 months of age
Mature ewes	Multiparous ewes aged 3-years of age or older
Fail to rear	Maiden ewe determined to be pregnant at Scan 1 that subsequently failed to rear a lamb to lamb marking
Exposed ewe	Ewe with *C. fetus/C. jejuni* titre ≥1:10
Exposed flock	Ewe flock (ewe lamb, maiden hogget or mature ewe flock) with *C. fetus/C. jejuni* titre ≥1:10 detected in at least one ewe
Exposed farm	Farm with *C. fetus/C. jejuni* titre ≥1:10 detected in at least one ewe (regardless of age)
Positive ewe	Ewe with *C. fetus/C. jejuni* titre ≥1:80
Positive flock	Ewe flock (ewe lamb, maiden hogget or mature ewe flock) with *C. fetus/C. jejuni* titre ≥1:80 detected in at least one ewe
Positive farm	Farm with *C. fetus/C. jejuni* titre ≥1:80 detected in at least one ewe (regardless of age)

10.2.3 Blood sample collection and lamb necropsies

Blood samples were collected for maiden and mature ewes as previously described (Chapters 7, 8 and 9). Briefly, blood samples were collected for all maiden ewes at five time-points: pre-mating, scan 1, scan 2, pre-lambing (approximately 140 days from start of mating) and lamb marking. Blood samples for mature ewes (age three years or older) were collected at a single time-point during the study period. Reproductive status, timing of sampling relative to lambing, reproductive outcome and reproductive history were not recorded for mature ewes. Blood samples were not collected for mature ewes at Farm 20 because unvaccinated mature ewes were not available. All blood samples were obtained by jugular venepuncture into serum vacutainer tubes with clot activator. Samples

were stored on ice or at 2^{o}C before being centrifuged at 4000 rpm for 10 minutes. Serum was decanted into 2 mL storage tubes and stored at -20^{o}C prior to serological testing.

If abortions were observed, the aborted foetus and/or foetal membranes were collected for necropsy. Lambs that died during the lambing period were collected for necropsy from a subset of flocks from Western Australia (Flocks 1, 2, 3, 7, 11, 14, 16) as previously described in Chapter 6.

10.2.4 Sample selection for case-control study

The sample size needed for the case-control study to detect an odds ratio of 2 was 220 ewes in each group assuming 10% of control ewes had a *C. fetus* titre ≥1:80 at 95% confidence level and 80% power. As 22 farms were included, this sample size was achieved with 10 maiden ewe case-control pairs per farm.

A subsample of maiden ewes that raised lambs (*n* = 10 ewes) and failed to raise lambs (*n* ≥ 10 ewes) were selected for serological testing for each flock (Appendix 6 Tables A6.1 and A6.2). Serum samples obtained at lamb marking were used for serology except where samples at marking were not available because the ewe was removed from the study flock by the farmer after abortion was detected. In these cases, samples collected at the latest available timepoint after abortion was detected were used for serology (*i.e*. serum sample collected at scan 2 or pre-lambing).

For the flock with *C. fetus* abortions confirmed by microbial culture (Flock 19), *C. fetus* serology was also conducted for samples collected at previous timepoints for maiden ewes which failed to rear lambs and had *C. fetus* titres ≥1:10 at the latest available timepoint (Appendix 6 Table A6.3).

10.2.5 Serology

Serological testing was performed by ACE Laboratory Services, Bendigo, Victoria, Australia. Antibody titres for *C. fetus* and *C. jejuni* were determined using an Agar Gel Immunodiffusion test. Titres ≥1:10 were categorised as 'exposed' and ≥1:80 categorised 'positive' as previously described (Dempster et al., 2011, Glanville, 2017, Walsh, 2016).

10.2.6 *Campylobacter* spp. detection in tissues from aborted and stillborn lambs

Aborted (*n* = 2) and stillborn (*n* = 33) lambs were recovered from a subset of seven maiden ewe flocks (Flocks 1, 2, 3, 7, 11, 14, 16) in Western Australia (Table 31). Tissue samples were submitted to the Department of Primary Industry and Regional Development Diagnostic Laboratory Services, Perth, Western Australia and screened for *Campylobacter* spp. using qPCR and microbial culture methods as previously reported (Chapter 6). Three aborted foetuses were opportunistically recovered from one flock in Victoria (Flock 19) and submitted to the Veterinary Diagnostic Services Laboratory, Victoria (Department of Jobs, Precincts and Regions, Bundoora, Victoria).

10.2.7 Statistical analyses

Lamb mortality was calculated for each flock based on the number of foetuses identified at scan 1 and the number of lambs marked. Mid-pregnancy abortion was expressed as a proportion (%) using the number of ewes with pregnancy loss between scan 1 and scan 2 as a proportion of the number of ewes that were confirmed pregnant at scan 1.

Titres ≥1:10 were categorised as "exposed" and ≥1:80 categorised as "positive" (Table 32). A farm or flock was classified as seropositive if at least one ewe had a titre above the specified threshold.

Seropositivity proportion was calculated based on the number of samples with a titre at or above the specified titre cut-off as a proportion (%) of the samples tested. Seropositivity proportions were compared using a Pearson Chi-squared test (two-tailed). The seropositivity 95% confidence interval was determined using Jeffrey's method (Brown et al., 2001). Odds ratios were calculated for reproductive outcome (fail to rear) in exposed and positive ewes with 95% confidence interval and chi-squared P-value for significance. Correlation between seropositivity in maiden ewes and adult ewes was determined using bivariate Pearson correlation (two-tailed). For Flock 19, where serology was conducted for samples collected at multiple timepoints, titre for pre-joining and marking sample timepoints were compared using Wilcoxon matched pair-signed rank test (two-tailed).

Odds ratios for failing to raise a lamb were calculated using logistic regression for (i) "exposed" maiden ewes compared to ewes that were not exposed (titre <1:10), and (ii) "positive" maiden ewes compared to non-positive ewes (titre <1:80) using logistic regression with flock included as a fixed effect. Odds ratios were determined for failing to rear for different *Campylobacter* spp. titre categories (namely: (i) 1:10 to 1:40; (ii) 1:80; and (ii) ≥1:160) compared to titres <1:10 using logistic regression with flock included as a fixed effect. Logistic regression was performed using a generalised linear model (binomial family) with P-values calculated from the Wald statistic. Maiden ewe age category and state were not included as a fixed-factors because these were co-linear with farm (*i.e.* only one age group or state per farm).

10.3 Results

10.3.1 Abortion and lamb mortality for maiden ewe study flocks

Reproductive outcomes for each flock in the larger cohort study are described in more detail in Chapter 5. For the subset of flocks included in this study, the overall foetal and lamb mortality in maiden ewes (*i.e.* all causes of mortality between scan 1 and marking) ranged from 18 - 66% for ewe lambs and 20 - 53% for hoggets (Table 31). Mid-pregnancy abortion was detected in 11/12 ewe lamb flocks and 6/10 hogget flocks (Table 31). Mid-pregnancy abortion was detected for 5.7% of ewe lambs that were pregnant at scan 1 (220/4351). The frequency of mid-pregnancy abortion for ewe lamb flocks ranged from 0 – 23.8% (Table 31). For hogget flocks, mid-pregnancy abortion was detected in 1.5% of pregnant ewes (16/1886), with the frequency ranging from 0 – 4.4% (Table 31).

10.3.2 *Campylobacter fetus* seropositivity in maiden and mature ewe flocks

Campylobacter fetus titres ranged between zero (below detectible limit) and 1:640 in both maiden and mature ewes. Titres ranged between zero and 1:80 for ewes in most (18/22) maiden flocks, with titres ≥1:160 detected in three flocks from Victoria (Flocks 19, 20, 26) and one flock from South Australia (Flock 30). No maiden ewes with titres ≥1:10 were detected in four flocks from Western Australia (Flocks 3, 7, 8 and 14). However, all farms were "exposed" to *C. fetus* (at least one maiden or mature with titre ≥1:10) and 12/21 (66%) farms were "positive" (at least one ewe with titre ≥1:80; Appendix 6 Table A6.1).

There was a trend to a higher proportion of *C. fetus* "positive" flocks (at least one ewe in respective age category with titre ≥1:80) for mature ewes (13/20 flocks, 65%) compared to maiden ewes (8/22 flocks, 36%, $P = 0.061$, Appendix 6 Table A6.1). There was no difference in the proportion of

"positive" ewe lamb flocks (4/12 flocks, 33%) compared to maiden hogget flocks (4/10 flocks, 40%; *P* = 0.774).

The proportion of sampled maiden ewes that were *C. fetus* "exposed" and "positive" is shown in Table 33. Within maiden ewe flocks, up to 100% of sampled ewes were *C. fetus* "exposed" (Appendix 6 Table A6.1). The proportion of "positive" ewes ranged from 4.8-80% for the 8/22 flocks that had at least one "positive" ewe. The proportion of ewes "exposed" or "positive" to *C. fetus* was higher for mature ewes compared to maiden ewes at both titre thresholds (Table 33). There was no difference in proportion of "positive" ewes for ewe lambs compared to maiden hoggets (*P* = 0.163). There were trends towards weak positive correlations between the proportion of *C. fetus* "exposed" (r = 0.381, *P* = 0.088) or "positive" maiden ewes (r = 0.42, *P* = 0.057) compared to mature ewes on the same farm, noting that maiden ewes were selected based on case-control sampling and mature ewes were randomly selected.

Table 33: Individual animal seroprevalence with 95% confidence interval (CI) for *C. fetus* and *C. jejuni* in maiden ewe lambs or hoggets selected based on reproductive outcome (raised lambs or failed to rear), and randomly selected mature ewes (*C. fetus* only) across all farms.

	Tested	**Exposed** (titre ≥1:10)			**Positive** (titre ≥1:80)		
	(*n*)	*n*	%	95% CI	*n*	%	95% CI
C. fetus							
Maiden ewe lambs	260	84	32.3[a]	26.8, 38.2	37	14.2[a]	10.4, 18.9
Maiden hoggets	202	47	23.3[b]	17.8, 29.4	20	9.9[a]	6.3, 14.6
Mature ewes	210	114	54.3[c]	47.5, 60.9	65	31.0[b]	25.0, 37.4
TOTAL	**672**	**245**	**36.5**	**32.9, 40.2**	**122**	**18.2**	**15.4, 21.2**
C. jejuni							
Maiden ewe lambs	260	248	95.4[a]	92.3, 97.4	121	46.5[a]	40.5, 52.6
Maiden hoggets	202	195	96.5[a]	93.3, 98.4	83	41.1[a]	34.5, 48.0
TOTAL	**462**	**443**	**95.9**	**93.8, 97.4**	**204**	**44.2**	**39.7, 48.7**

[abc] Values within *Campylobacter* species and titre category with different superscript letters are significantly different using two sample z-test to compare sample

10.3.3 Association between seropositivity to *C. fetus* and reproductive outcome

"Exposed" maiden ewes had 2.0 higher odds of failing to rear than ewes with no evidence of exposure when adjusted for farm effects (P = 0.027; Table 34 and Appendix 6 Table A6.4). In maiden ewes that failed to rear a lamb, an extra 8.1% ewes were "exposed" to *C. fetus* compared to maiden ewes that raised lambs (32.2% vs. 24.1%; *P* = 0.054; Appendix 6 Table A6.5).

There was no evidence of increased odds for failing to rear for "positive" maiden ewes compared to ewes with *C. fetus* titre ≤1:80 (P = 0.191; Table 34 and Appendix 6 Table A6.4). For the subset of ewe lamb flocks, "positive" ewe lambs had 2.59 higher odds of failing to rear a lamb compared to ewe lambs with a titre <1:80 (P = 0.047; Table 4) and an extra 9.4% ewes were "positive" for ewe lambs that failed to rear compared to those that reared lambs (18.6% vs 9.2%; *P* = 0.03, Appendix 6 Table A6.5).

Across the farms, there was considerable variation in the proportion of "exposed" and "positive" ewes in failed to rear and reared groups (Appendix 6 Table A6.5). Flock 19 (where *C. fetus* abortions were confirmed by culture) was the only flock with a significantly higher proportion of *C. fetus*

"positive" ewes for those that failed to rear compared to ewes that raised lambs (85% vs 20%, P <0.001; Appendix 6 Table A6.5). Eight of the 22 maiden flocks had at least one "positive" ewe. In this subset of eight flocks, there was no significant increase in odds for failing to rear for "positive" ewes compared to ewes with *C. fetus* titre ≤1:80 (OR: 1.69, 95%CI: 0.77, 3.76 *P* = 0.191 adjusted for flock). However, this should be interpreted with caution due to low statistical power.

Table 34: Odds ratio and 95% Confidence Intervals (CI) for failing to rear a lamb (FTR) in ewe lambs and maiden hoggets that were "exposed" (titre ≥1:10) or "positive" (titre ≥1:80) for *C. fetus* and *C. jejuni* compared to ewes with titres below the respective thresholds determined using logistic regression with flock included as fixed effect

	Age category	Exposed[A] (*N*)	Failure to rear lamb			
			FTR *(n)*	Odds ratio	95% CI [C]	*P*-value
C. fetus						
Exposed (titre ≥1:10)	Maiden ewe lambs	84	51	2.16	0.91; 5.38	0.086
	Maiden hoggets	47	27	1.44	0.75, 2.81	0.278
	Overall	131	78	2.01	1.09, 3.77	0.027
Positive (titre ≥1:80)	Maiden ewe lambs	37	26	2.59	1.03, 6.78	0.047
	Maiden hoggets	20	9	0.55	0.10, 2.47	0.440
	Overall	57	35	1.69	0.77, 3.76	0.191
C. jejuni						
Exposed (titre ≥1 :10)	Maiden ewe lambs	249	132	0.42	0.08, 1.62	0.232
	Maiden hoggets	195	99	1.39	0.29, 7.53	0.679
	Overall	444	231	0.71	0.24, 1.93	0.506
Positive (titre ≥1:80)	Maiden ewe lambs	121	58	0.46	0.24, 0.87	0.018
	Maiden hoggets	83	38	0.69	0.29, 1.22	0.160
	Overall	204	96	0.52	0.32, 0.83	0.007

[A] Exposed: Exposed to disease risk (*e.g.* titre ≥1:10 or ≥1:80 as indicated)

Odds ratios for failing to rear a lamb in ewes with evidence of seropositivity to *C. fetus* at different titre cut-offs compared to ewes with a titre of <1:10 are shown in Table 35. There was no significant increase in the odds ratios for failing to rear a lamb at the four *C. fetus* titre cut-off levels tested compared to ewes with a titre <1:10 (Table 35).

Table 35: Odds ratio and 95% Confidence Intervals (CI) for failing to rear a lamb (FTR) in maiden ewes variable *C. fetus* and *C. jejuni* titre cut-off levels determined using logistic regression with flock included as fixed effect

	Titre cut-off category	Total tested (*N*)	Failure to rear lamb			
			FTR *(n)*	Odds ratio	95% CI [C]	*P*-value
C. fetus	Titre <1:10	331	164	reference		
	Titre 1:10 to 1:40[A]	74	31	1.41	0.85, 2.37	0.184
	Titre 1:80[B]	29	12	1.44	0.67, 3.19	0.351
	Titre ≥1:160	28	10	1.83	0.84, 4.24	0.139
C. jejuni	Titre <1:10	18	11	reference		
	Titre 1:10 to 1:40[A]	240	105	1.22	0.47, 3.42	0.689
	Titre 1:80[B]	136	74	1.88	0.70, 5.37	0.220
	Titre ≥1:160	68	34	1.57	0.55, 4.73	0.403

[A] "Exposed"
[B] "Positive"
[C] 95% confidence interval

10.3.4 Detection of *Campylobacter* spp. in tissues from aborted and stillborn lambs

Campylobacter fetus was cultured from liver, lung and abomasal content from three aborted foetuses opportunistically recovered between scan 2 and pre-lambing from one maiden ewe lamb flock in Victoria (Flock 19). Dam pedigree was not able to be determined for these aborted foetuses.

Neither *C. fetus* nor *C. jejuni* were detected by qPCR or isolated via culture from samples of aborted or stillborn lambs (*n* = 35) recovered from a subset of seven flocks in Western Australia (Chapter 6). *Campylobacter sputorum* and *Campylobacter mucosalis* were detected by qPCR and sequencing in placental samples collected from one farm. These were not detected on microbial cultures and are not considered reproductive pathogens.

10.3.5 Serial *C. fetus* titres in Flock 19

Campylobacter fetus abortions were confirmed in Flock 19 based on microbial cultures performed on aborted foetuses recovered from ewe lambs. Titres from these ewes fluctuated over time (Appendix 6 Table A6.3).

There was a significant increase in titre between mating and lamb marking in the ewes that failed to rear (Wilcoxon signed rank test, P < 0.001), but not in the ewes that raised lambs (*P* = 0.72). For the ewe lambs that failed to rear, a *C. fetus* titre ≥1:320 was detected in 4/20 (20%, 95% CI 7.2, 40.8) ewes at scan 2, 6/10 (60%, 95% CI 30.4, 84.7) ewes pre-lambing, and 2/20 (10%, 95% CI 2.1, 28.4) ewes at marking. Titres fell to <1:320 by marking for 6/8 (75%, 95% CI 40.8, 94.4) ewes that had previously had a *C. fetus* titre ≥1:320 at scan 2 or pre-lambing (Appendix 6 Table A6.3).

10.3.6 Seropositivity to *C. jejuni* in maiden ewe flocks

Campylobacter jejuni "exposure" was detected on 21/21 (100%) farms and "positive" ewes were detected on 18/21 (86%) farms (Appendix 6 Table A6.2). There was no difference in the proportion of "positive" flocks between ewe lambs (9/12 flocks) and hoggets (9/10 flocks; *P* = 0.368).

The proportion of individual ewes "exposed" and "positive" for *C. jejuni* are shown in Table 33. There was no difference in the proportion of *C. jejuni* "positive" ewe lambs compared to hoggets (*P* = 0.555). Within maiden ewe flocks, 80-100% ewes were categorised as "exposed" and 0-100% ewes were categorised as "positive'' for *C. jejuni* (Appendix 6 Table A6.2).

10.3.7 Association between seropositivity to *C. jejuni* and reproductive outcome

There was no increase in the odds of failing to rear a lamb in *C. jejuni* "exposed" maiden ewes (*P* = 0.506; Table 34) and there was no difference in the proportion of *C. jejuni* "exposed" ewes that failed to rear a lamb compared to those that raised lambs (95.0% vs 96.8%; *P* = 0.332).

Maiden ewes, and specifically maiden ewe lambs, that were *C. jejuni* "positive" had lower odds of failing to rear a lamb compared to ewes with *C. jejuni* titre ≤1:80 (Table 34 and Appendix 6 Table A6.6). In maiden ewes that failed to rear lambs, 9.4% less ewes were "positive" compared to ewes that raised lambs (39.7% vs 49.1%; *P* = 0.042). There was no evidence of increased odds of failure to rear for ewes with *C. jejuni* titres (i) 1:10 to 1:40, (ii) 1:80, and (iii) ≥1:160 compared to titre <1:10 (Table 35).

10.4 Discussion

"Exposure" to *Campylobacter* spp. was widespread across the flocks in this study. Maiden ewes that were exposed to *C. fetus* were twice as likely to fail to rear compared to ewes with no evidence of exposure. "Positive" *C. fetus* titres were inconsistently associated with failure to rear and *C. fetus* titre was a poor predictor of failure to rear for the flocks in this study. However, this was confounded by the relatively infrequent detection of titres for *C. fetus* in maiden flocks with no *C. fetus*-"exposed" maiden ewes detected in four flocks and no "positive" maiden ewes detected in 14 flocks. Additionally, there were insufficient ewes with high titres (≥1:160) in this study to determine whether high titres at lamb marking were associated with increased abortion or lamb mortality rates. *Campylobacter fetus*-associated abortion occurred on one farm, consistent with the previously reported sporadic nature of campylobacteriosis in Australian flocks (Chapter 4). Whilst *C. jejuni* was detected in all flocks, there was no evidence that seropositivity to *C. jejuni* was associated with increased odds of failing to rear at either titre threshold.

It is common practice in Australia to screen flocks with disappointing lamb marking rates for seropositivity to *Campylobacter* spp. using the serological test used in this study (Walsh, 2016). A *C. fetus* titre cut-off ≥1:80 has been used to indicate a flock as "positive" (Walsh, 2016, Wills, 2021). In our study, *C. fetus* seropositivity based on this cut-off was associated with higher odds of failing to rear but only in ewe lambs. "Exposed" ewes were detected in flocks that had no evidence of campylobacteriosis abortion or stillbirths based on monitoring ewes and necropsy of aborted and stillborn lambs (Ostfeld et al., 2020). However, lamb necropsies and testing for infectious agents were only performed on a subset of farms. Detection of "exposure" in flocks without evidence of abortion or campylobacteriosis at lamb necropsy could also reflect persistence of antibodies, infections outside of the period of risk for reproductive disease, insufficient intensity of infectious challenge, and variations in ewe immunity and strain pathogenicity (Sahin et al., 2012, Grogono-Thomas et al., 2003, Glanville, 2017). Alternative strategies for investigating the impact of *C. fetus* exposure on flock reproductive performance could include monitoring ewes for evidence of abortion (Chapter 5), lamb necropsies to determine aetiological diagnoses (Chapter 4 and Chapter 6) and/or a vaccination trial (Anderson, 2001).

Campylobacter fetus titres ≥1:320 may be associated with campylobacteriosis during abortion storms, however there were insufficient ewes in this study with titres this high at lamb marking to confidently determine an association with failure to rear. Apart from Flock 19 (where campylobacteriosis abortion was confirmed with cultures), ewes with *C. fetus* titre ≥1:320 were only detected in Flock 20 (4.3% ewes with mid-pregnancy abortion) and Flock 26 (4.4% ewes with mid-pregnancy abortion). However, aborted foetuses were not recovered from either of these flocks and lamb necropsies were not performed. Further investigation of antibody dynamics in flocks with campylobacteriosis abortions would be required to determine the positive and negative predictive value of titre ≥1:320. Such investigations should also include lamb necropsies to determine the contribution of infectious agents, including *Campylobacter* spp., to perinatal lamb mortality.

Campylobacter fetus titres fluctuated during pregnancy for ewes in the one flock with confirmed campylobacteriosis abortion. Titres had declined by marking in many ewes with titre ≤1:160 in 6/8 ewes that had *C. fetus* titre ≥1:320 at scan 2 or pre-lambing. This indicates that *Campylobacter* spp. serology for a single timepoint at lamb marking or later can result in apparent 'false negatives' (*i.e.* low or moderate titres in flocks where campylobacteriosis abortions occurred in mid-late pregnancy). Where possible, a suspected diagnosis of campylobacteriosis abortion and perinatal mortality should be based on detection of *Campylobacte*r spp. at necropsy of the foetus or lamb and not on serology from a single timepoint alone (Dempster et al., 2011). In cases where lamb necropsy is not possible, rising titres based on paired samples may be useful in supporting a presumptive diagnosis of campylobacteriosis. However, the relatively rapid change in titres observed in Flock 19 indicates that there is a short window of time during an outbreak for collection of serum samples that will demonstrate this rise.

Immunological naivety is a risk factor for campylobacteriosis abortion with previous studies indicating that convalescent ewes develop protective immunity (Jensen et al., 1957, Frank et al., 1965). This was consistent with our observations in Flock 19 where 3/10 ewes that raised lambs had *C. fetus* titre 1:160 at mating, and only 3/20 sampled ewes that subsequently failed to rear lambs were determined to be "exposed" to *C. fetus* at mating (Appendix 6 Table A6.3). Foetal or lamb mortality in the three ewes with serological evidence of exposure to *C. fetus* prior to mating could reflect other causes of abortion and perinatal mortality acting simultaneously within the same flock (Chapter 6). An alternate explanation is that prior *C. fetus* exposure was not sufficient to develop protective immunity in these ewes.

This study was not designed as a seroprevalence survey. Nonetheless, detection of seropositivity to *C. fetus* and *C. jejuni* on sheep farms and farm- and animal-level seroprevalence observed in our study were consistent with previous serological 'surveys' suggesting that exposure is common on Australian sheep farms. However, those surveys also preferentially sampled ewes that had failed to rear lambs (Walsh, 2016, Wills, 2021). Evidence of widespread exposure to *Campylobacte*r spp. for Australian sheep located over a wide geographical region was consistent with recent reviews of Australian abortion investigations that showed *Campylobacter* spp. abortions were diagnosed across southern Australian states in most years Chapter 4; (Refshauge et al., 2020a, Clune et al., 2021b). Serological evidence of "exposure" to *C. jejuni* in this study was consistent with other studies reporting that *C. jejuni* is commonly detected in Australian sheep without evidence of disease (Yang et al., 2017, Yang et al., 2014b). Notwithstanding the difference in selection criteria for maiden ewes (case control) and mature ewes (random selection), *C. fetus* seroprevalence was higher for mature ewes compared to maiden ewes. This likely reflects cumulative age-related exposure as older ewes have had more time to be exposed to infection, and potentially develop immunity.

An important limitation of serological surveys is that seropositivity does not provide information on current infection status or causality of foetal or lamb mortality. This study focussed on *Campylobacter* spp., however there are other important infectious and non-infectious causes of abortion and lamb mortality that are often multifactorial (Jacobson et al., 2020). Necropsies performed on a subset of farms in this study identified dystocia, stillbirth and starvation-mismothering as cause-of-death for majority of perinatal mortalities based on gross pathology (Clune et al., 2021c). This was consistent with other Australian studies reporting cause of death in lambs (Hinch and Brien, 2014a, Bruce et al., 2021). Apart from *Campylobacter* spp., other endemic diseases were identified in some flocks in this study. Abortions, stillbirths and polyarthritis associated with *Chlamydia pecorum* were identified in a subset of farms from Western Australia (Clune et al., 2021c, Ostfeld et al., 2020). Exposure of ewes to *Toxoplasma gondii* (Clune et al., 2022b*), Neospora caninum* (Clune et al., 2021d) and *Coxiella burnetii* (Clune et al., 2022a) were identified on farms in this study, but there was no evidence that these were important contributors to foetal and lamb mortality in these flocks. Further investigations using data from this study will include multivariable analysis to evaluate the relative importance of different pathogens on reproductive performance. Prioritisation and implementation of preventative measures for campylobacteriosis should be considered in the context of the multiple aetiologies for foetal and lamb mortality in maiden ewes, including farm and flock level risk factors. Important risk factors for clinical campylobacteriosis include the environment (*e.g.* high rainfall, short feed) and management (*e.g.* high stocking rates, confined feeding, open flocks) during pregnancy (Clough, 2003, Sahin et al., 2017).

There were several other limitations to this study. Serology was determined using Agar Gel Immunodiffusion. This method has been used by other studies (Walsh, 2016, Glanville, 2017), but sensitivity and specificity of the test is poorly defined. Further validation of the test for field investigations would improve prediction of true incidence of infection with *Campylobacter* spp.. Lamb necropsies were only performed for a subset of 8 flocks, of which 7 were sampled prospectively and one opportunistically after abortions were observed by the farmer. It is possible that foetal and lamb mortality associated with *Campylobacter* spp. occurred in the other 14 flocks but were not detected due to lack of necropsy. Some farms in this study had sheep studs. Whilst a requirement for inclusion in the study was that sheep were managed extensively at stocking rates broadly comparable to commercial sheep production in the region, risk factors for campylobacteriosis are not well defined. It is possible that differences in management of sheep between farms impacted the risk for campylobacteriosis, and additional yarding and monitoring of ewes during pregnancy for the project may have impacted the risk of exposure to *Campylobacter* spp. as well as risk of lamb mortality. Further investigation with greater number of farms in each state and expanding the number of farms tested in higher rainfall areas would be required to provide more accurate assessment of *Campylobacter*-associated abortion and lamb mortality in different farming regions. Further investigation should also consider assessment of the interaction between environmental factors and stocking rate as risk factors for disease outbreaks. This would inform region-specific recommendations relating to interpretation for *Campylobacter* spp. serology, strategies for monitoring ewes using serology and expected cost-benefit of implementing vaccination.

10.5 Conclusion

Seropositivity to *C. fetus* and *C. jejuni* were detected on most farms. Maiden ewes with serological evidence of exposure (titre ≥1:10) to *C. fetus* had twice the odds of failing to rear a lamb than non-exposed ewes. Higher odds of failing to rear were observed for positive (titre ≥1:80) ewe lambs but not maiden hogget ewes. There was no evidence that *C. jejuni* serology was a useful indicator for reproductive outcome which likely reflected the widespread distribution and commensal nature of *C. jejuni*. Abortions associated with *C. fetus* were only detected on one farm using lamb necropsy. In this flock, *C. fetus* titres fluctuated during pregnancy and lactation in ewes that both reared and failed-to-rear lambs, reinforcing the value of foetal or lamb necropsy to determine aetiological diagnosis for abortion and perinatal mortality. Campylobacteriosis is associated with reproductive loss in maiden ewes on some farms in some years. On farms with evidence of serological exposure to *C. fetus*, strategies to determine an association with reproductive disease include monitoring ewes for abortions, determining aetiological diagnoses for foetal and lamb mortality using necropsies or vaccination trial. Further investigation is warranted to inform region-specific recommendations relating to interpretation for *Campylobacter* spp. serology and preventative measures.

11. Evidence-based approach to investigating poor reproductive performance in maiden ewes and inform mitigation strategies

A protocol for on-farm investigation of poor reproductive performance in maiden ewes based on the findings from this project is outlined in **Table 36**. This protocol can be adapted for investigation of reproductive performance for multiparous ewes.

Whilst the methodology outlined in **Table 36** was developed for research investigating lamb survival, this can be readily adapted for commercial sheep farms to determine the (a) timing and (b) likely causes of foetal or lamb mortality to inform strategies targeted at addressing lamb survival. **Table 36** outlines reasoning for each recommendation and this can be used to determine the benefit for each step balanced against the associated costs or risks.

Practitioners should consider the available resources (facilities, labour), risk factors for infectious diseases specific to the farm system and location concerned, and risks/costs for monitoring activities when determining which components of the protocol are warranted. Examples of modification of the procedures for investigation on commercial farms are outlined in Table 37.

11.1.1 Disease investigations

The protocol described in **Table 36** will determine the timing of foetal or lamb loss to inform strategies targeted at addressing lamb survival. If investigation shows that abortions in mid- or late-pregnancy are contributing to poor reproductive performance, then determining whether infectious disease is contributing to foetal loss and if so, the specific aetiological agent (cause) will inform appropriate strategies to address this.

Submitting appropriate samples for testing, careful storage and transport of samples, and inclusion of placenta samples can aid in determining the aetiological diagnosis (Chapter 4).

Producers/veterinarians may be able to access subsidised testing through abortion surveillance schemes or significant disease investigations conducted by government or private veterinarians. The process for accessing subsidised testing and sampling requirements (*i.e.* number and type of samples required) varies between jurisdictions and should be discussed in advance with the relevant department or laboratory receiving samples. Significant disease and reproductive disease surveillance programs available at the time of this report are outlined in Table 38.

11.1.2 Managing potential public health risks

Several endemic disease agents associated with abortion, stillbirths and reduced neonatal viability have zoonotic potential. These include *Toxoplasma gondii*, *Campylobacter* spp., *Listeria* spp., *Leptospira* spp. and *Coxiella burnetti*. Some exotic reproductive diseases have zoonotic potential (*e.g. Chlamydia abortus*, *Salmonella abortus* and *Brucella melitensis*).

Practitioners can reduce potential public health impacts by advising clients on recommendations for hygiene when handling pregnant and periparturient ewes, neonatal lambs or any aborted tissues. This includes:

- Use disposable gloves where possible and wash hands with soap or detergent after handling affected ewes, lambs or aborted tissues
- Goggles/eye protection and mask may be warranted in high-risk scenarios

- Pregnant women should avoid handling lambing ewes or any aborted tissues
- Cuts and needlestick injuries are a potential source of zoonotic transmission
- Q-fever (*C. burnetii*) can be transmitted through aerosols and dust. Residues contaminated by birth fluids, blood, faeces, or urine may remain infectious for months to years.
    - *C. burnetii* can be dispersed by windborne spread of contaminated dust over several kilometres. Activities generating dust, such as mustering, shearing, transport may represent a risk to susceptible people.
    - Transmission can occur to people handling clothing contaminated with blood, faeces, urine, birthing fluids etc. Family members do not have to be in direct contact with livestock to be at risk of zoonotic transmission
- People working closely with livestock should discuss vaccination for Q-fever and leptospirosis with their medical practitioner.

11.1.3 Addressing non-infectious contributors to mid-pregnancy abortion

Is not clear whether management strategies can reduce mid-pregnancy abortion in flocks where an infectious aetiological diagnosis is not identified. The on-farm study (Chapter 5) found that mid-pregnancy abortion was not associated with litter size or ewe joining weight, weight change between joining and scanning or condition score. This is consistent with other ewe lamb studies in New Zealand that reported no association between foetal loss and liveweight (Kenyon et al., 2008, Morris et al., 2005, Mulvaney et al., 2010). However, lower liveweight at joining and lower liveweight gain in early pregnancy have been associated with a higher frequency of abortion in ewe lambs with lower joining weight (36kg) compared to the flocks in our study (Mulvaney et al., 2008) and in flocks that had low weight gain between joining and scanning (40g/day) plus evidence of *Leptospira* spp. concurrently associated with foetal loss (Ridler et al., 2015). There is no evidence to suggest that liveweight or liveweight change is associated with a high frequency of mid-pregnancy abortion when following current recommendations for ewe lamb joining weights (at least 42kg) and liveweight profile (100-150g/day in period between joining and scanning) (Kenyon et al., 2014b)

Table 36: Protocol for investigating poor reproductive performance at flock level, including determination of reproductive rate, abortion, and perinatal mortality

Timing	Activity	Outcome
Pre-joining	Rams: check the number provides adequate ram power (ram:ewe ratio); ram team age demographics; which rams to which ewes	Investigate potential for ram infertility contributing to poor reproductive rate
	Rams: age, breeding soundness exam including penis, prepuce, testicle circumference and palpation, body condition score, teeth and feet	
	Rams: *Brucellosis ovis* exclusion (testicular palpation ± serology)	
	If joining out of season, check teaser preparation protocol	Investigate factors that may impact ewe attainment of puberty and cycling that contribute to poor reproductive rate
	Ewes: age, weight and condition score	
	Ewes: determine farm-level risk of campylobacteriosis and instigate vaccination if warranted (2 injections at least 3 weeks apart given pre-joining). Consider a vaccination trial (treated and untreated controls).	Address campylobacteriosis risk
	Optional: test for vitamin and trace element deficiencies relevant to region (e.g. vitamin E, vitamin A, selenium, copper, zinc). Consider a trace element supplementation trial (treated and untreated controls).	Determine if vitamin or mineral deficiencies could be impacting ewe fertility
	Optional: collect blood samples for ewes to determine pre-gestation exposure to infections that require primary exposure during pregnancy for reproductive disease (*e.g. Toxoplasma* and BDV/pesti-virus). Serum and plasma can be stored at -20^{o}C.	Determine timing of primary infection in event that infectious disease is subsequently identified
Joining	Aim for 35-day joining	Schedule scanning to optimise accuracy of foetal counting Plan monitoring during lambing by estimating start and duration of lambing based on joining dates
Post-joining	Confirm joining dates to schedule pregnancy scanning within target period	Accuracy for foetal counting is highest 80-100 days from start of joining

Timing	Activity	Outcome
Scan 1	Scan with foetal count (empty, single, twin/triplet) at 50 days from removal of rams/85 days from start of joining (50-85 days gestation). Consider adjusting dates back if ewes were not teased/cycling at a=start of joining. Record litter size against ewe ID, or draft by litter size into mobs	Record number of ewes scanned, number of foetuses counted and calculate reproductive rate Determine if poor reproductive rate (poor conception/embryo survival) is contributing to overall reduced reproductive performance
	Record ewe weight and condition score	Determine if sub-optimal condition score profile or inadequate growth rate (ewe lambs) are contributing to poor reproductive rate
	Ewes with suspected recent abortion should be identified and drafted off from pregnant ewes. Consider monitor/re-check to confirm abortion.	Reduce transmission of infectious disease by affected ewes Validate accuracy of scanning in detecting abortion
	Consider collecting blood samples and vaginal/rectal swabs from ewes with evidence of recent abortion Consider collecting blood samples again in 2-3 weeks (paired serology)	Serology can be conducted for *Toxoplasma, Neospora, Coxiella, Campylobacter.* Discuss recommended tests with regional laboratory Paired serology to detect rising/falling titre or IgM/IgG may indicate timing of infection Vaginal/rectal swabs can be used for point-of-care tests or molecular tests *Chlamydia* spp.
	Check pasture for evidence of oestrogenic clover (seasonal – may need to be delayed until sufficient pasture growth for species identification)	Determine if phytoestrogens are contributing to infertility

Timing	Activity	Outcome
Scan 2	Mid-pregnancy abortion monitoring scan conducted 90 days from removal of rams/125 days from start of joining (90-125 days gestation)* to identify ewes with non-viable pregnancy (determined by lack of heartbeat or foetal movement, changed echogenicity of cotyledons) or pregnancy loss (absence of foetus). *Scan 2 should be conducted at least 30 days after scan 1. Scan 2 can be delayed to identify more ewes with foetal loss, but scanning becomes slower and less accurate later in pregnancy. Note that scanning later in pregnancy takes longer than standard pregnancy scanning – this should be considered when negotiating costs with the scanning provider and producer. Consider cost and risk associated with additional handling against potential benefit from discriminating abortion from perinatal losses.	Detect mid-pregnancy abortion
	Ewes with suspected abortion should be identified and drafted off from pregnant ewes. These ewes should be monitored over lambing to confirm abortion.	Reduce transmission of infectious disease by affected ewes Validate accuracy of scanning in detecting abortion
	Collect blood samples and vaginal/rectal swabs from ewes with evidence of mid-pregnancy abortion Consider collecting blood samples again in 2-3 weeks (paired serology)	Serology can be conducted for *Toxoplasma, Neospora, Coxiella, Campylobacter.* Discuss recommended tests with regional laboratory Paired serology to detect rising/falling titre or IgM/IgG may indicate timing of infection Vaginal/rectal swabs can be used for point-of-care tests or molecular tests *Chlamydia* spp.

Timing	Activity	Outcome
Post-Scan 2	If a high frequency of mid-pregnancy abortion is detected, increase the frequency of monitoring for all maiden ewe mobs for evidence of abortion (*e.g.* aborted lambs or foetal membranes, breech staining). Ewes with evidence of non-viable lambs at Scan 2 (*i.e.* lambs with no movement or heartbeat) should be monitored closely for evidence of abortion. If abortions are observed; • People handling aborting ewes and aborted tissues should use PPE (*i.e*. gloves and mask) and practice good hygiene. Pregnant women and immunocompromised individuals should avoid contact where possible. • Any aborted tissue (*i.e.* aborted lamb or foetal membranes) should be collected into a clean plastic bag, sealed and stored at 4-10^{o}C until submission for diagnostic testing. • If submission is likely to be delayed, discuss freezing samples with veterinarian/laboratory. Aborted tissue should be submitted for abortion surveillance testing. This should include: • Full necropsy and tissue collection where possible • Submission of fresh and fixed tissues where possible • Discussion of sample collection and submission protocol with the receiving laboratory in advance where possible	Disease investigation to determine aetiological diagnosis (cause) including infectious agents

Timing	Activity	Outcome
Optional: Scan 3	If a high frequency of mid-pregnancy abortion has been detected, a third scan can be conducted at day 120-130 gestation to identify ewes with late-pregnancy foetal loss. The benefit of quantifying late-pregnancy abortion should be considered against the risk of metabolic disease when handling ewes during late-pregnancy. Feed and water should not be withheld from ewes in late pregnancy. Treatment for metabolic disease (pregnancy toxaemia, hypocalcaemia) should be available, and ewes should be monitored carefully during and after yarding. Note that scanning later in pregnancy takes longer than standard pregnancy scanning and additional technical skills are needed to detect foetal viability (foetal movement or heartbeat) during late pregnancy. This should be considered when negotiating costs with scanning provider and producer. *NOTE: Inspections during lambing to collect dead lambs provide an alternative to quantify number of lambs born and calculate late pregnancy abortion. Lambing inspections will have less risk of metabolic disease in ewes (compared to scanning in late pregnancy) and also allow for collection of dead lambs for necropsy (post-mortem examination). However, lambing inspections risk disturbing lambing ewes and increasing risk of dystocia and mismothering if staff are not trained and ewes are not habituated to human contact. These risks should be considered based on circumstances for each enterprise, including relative benefit for identifying late term abortion.*	Detect late-pregnancy abortion
	Collect blood samples and vaginal/rectal swabs from ewes with evidence of late-pregnancy abortion Consider collecting blood samples again in 2-3 weeks (paired serology)	Serology can be conducted for *Toxoplasma, Neospora, Coxiella, Campylobacter.* Discuss recommended tests with regional laboratory Paired serology to detect rising/falling titre or IgM/IgG may indicate timing of infection Vaginal/rectal swabs can be used for point-of-care tests or molecular tests *Chlamydia* spp.
	Ewes with suspected late-pregnancy abortion should be drafted off from pregnant ewes. These ewes should be monitored over lambing to confirm abortion.	Reduce transmission of infectious disease by affected ewes Validate accuracy of scanning in detecting abortion

Timing	Activity	Outcome
Pre-lambing	Allocate ewes to lambing paddocks based on litter size (single, multiple or twins/triplets; separate dry/aborted). Record the number of ewes allocated to each lambing paddock.	Lambing/marking in litter size groups allows for calculation of single/multiple lamb survival Accurate ewe head counts and foetal count (based on scanning) allows for calculation of lamb and ewe mortality Monitoring dry/aborted ewes over lambing used to confirm scanning records
	Ewes: record weight and condition score	Determine if sub-optimal condition score at lambing or condition score/weight profile in late pregnancy are contributing to poor lamb survival
	Ewes: check for evidence of abortion or premature lambing (*e.g*. breech staining, foetal membranes, aborted foetus)	Detect late-pregnancy abortion
	Ewes: Collect blood samples and rectal/vaginal swabs from any ewes with evidence of late-pregnancy abortion Consider collecting blood samples again in 2-3 weeks (paired serology)	Serology can be conducted for *Toxoplasma, Neospora, Coxiella, Campylobacter.* Discuss recommended tests with regional laboratory Paired serology to detect rising/falling titre or IgM/IgG may indicate timing of infection Vaginal/rectal swabs can be used for point-of-care tests or molecular tests *Chlamydia* spp.
	Collect any aborted tissues or dead premature lambs for sampling (refer to post-scan 2 for procedures)	Collect samples for disease investigation to determine aetiological diagnosis (cause)
	Undertake predator control ahead of lambing	Minimise losses due to predation and disturbance
	Habituate ewes to routine inspection in lambing paddock. Use a different vehicle for inspections to that used for mustering or feeding where necessary.	Minimise disturbance from inspections during lambing rounds
	Record paddock characteristics: shelter, feed-on-offer, mob size	Determine if paddock characteristics are contributing to perinatal mortalities
	Check pasture for evidence of oestrogenic clover (seasonal)	Determine if phytoestrogens are contributing to reduced lamb survival

Timing	Activity	Outcome
Lambing	Inspect ewes in lambing paddock once or twice daily. Record the number of lambs born and/or collect dead lambs Record the number of lambs born or estimate based on the number of lambs marked and dead lambs recovered *NOTE: Inspections during lambing should be balanced against risk of disturbance of ewes. Habituation of ewes to inspection should be considered and instigated prior to the commencement of lambing.*	Use number of lambs born to calculate (a) foetal loss between scan 1 and lambing, and (b) perinatal loss
	Weigh dead lambs Optional: consider weighing and tagging lambs at birth (seedstock)	Determine whether birthweight is contributing to lamb mortality
	Necropsy dead lambs using method described by Holst [18] to assign cause of death category	Determine cause of perinatal death
	Collect appropriate samples from aborted or premature lambs, stillborn lambs, lambs with evidence of inflammatory changes at necropsy or small weak lambs for laboratory investigation to identify aetiological diagnoses. This should include • Full necropsy and tissue collection where possible • Submission of fresh and fixed tissues where possible Discuss of sample collection and submission protocol with the receiving laboratory	Disease investigation to determine aetiological diagnosis (cause) including infectious agents
	Record ewe mortalities and cause of death (if known). Consider necropsy for unexplained ewe mortalities or where mortality exceeds target	Determine cause of ewe death
	Monitor dry/aborted ewes periodically over lambing	Confirm scanning records
	Record daily temperature, wind speed and rainfall (measured or closes BOM station). Calculate chill index.	Relate chill index to lamb mortality pattern

Timing	Activity	Outcome
Marking	Record the number of lambs marked	Use number of lambs marked to estimate number of lambs born Calculate (a) foetal loss between scan 1 and lambing (b) lamb mortality between birth and marking (c) overall lamb survival scan 1 to marking
	Ewe udder inspection (wet/dry)	Identify ewes that have failed to lamb (no udder development) or failed to rear
	Ewes: Collect blood samples from sample of ewes that failed to lamb or failed to rear in mobs with (a) high proportion of ewes that fail to lamb, or (b) very high perinatal lamb mortality Consider collecting blood samples again in 2-3 weeks (paired serology)	Serology can be conducted for *Toxoplasma, Neospora, Coxiella, Campylobacter.* Discuss recommended tests with regional laboratory Paired serology to detect rising/falling titre or IgM/IgG may indicate timing of infection

Table 37: Examples of investigation protocol components outlined in Table 36 that should be prioritised for investigation of poor reproductive performance on commercial sheep farms

Measure	**Outcome**	**Priority activities**	**High priority for commercial farm field investigation?**
Pre-joining inspection	Pre-empt issues that may impact reproductive rate that season	Pre-joining inspection of rams, ewes	Rams – yes Ewes - consider for farms/flocks with history of poor reproductive rate
Reproductive rate	Potential marking rate Identify issues occurring at joining/early pregnancy	Scanning for multiples (scan 1)	Yes
Lamb survival	Quantify mortality scanning to marking without discriminating *in utero* and perinatal losses	Scanning for multiples Lambing singles and multiples in separate mobs	Yes
Mid-pregnancy abortion	Quantify contribution of mid-pregnancy abortion to overall lamb survival	Scan 2	Consider for ewe lambs or properties where abortions suspected
Late pregnancy abortion	Quantify contribution of late-pregnancy abortion to overall lamb survival	Scan 3 and/or daily inspection to collect dead lambs	Consider for ewe lambs or properties where abortions suspected
Perinatal mortality	Quantify contribution of perinatal mortality to overall lamb survival Identify cause of death	Daily inspection to collect/count dead lambs Gross post mortem ± laboratory investigation	Consider for flocks with high mortality in single and multiple lambs to inform targeted improvements in management and genetics

Table 38: Significant disease investigation and reproductive disease surveillance program programs

State	Contacts	Program	Website contact
All	Animal Health Australia	National significant disease investigations program (NSDI)	https://animalhealthaustralia.com.au/collaborative-disease-investigations/
WA	Department of Primary Industries and Regional Development (DPIRD) Diagnostics and Laboratory Services (DDLS) – Animal pathology	Ewe abortion and newborn lamb deaths surveillance program	https://www.agric.wa.gov.au/livestock-biosecurity/ewe-abortion-and-newborn-lamb-deaths-surveillance-program
		Significant Disease Investigation Program	https://www.agric.wa.gov.au/livestock-biosecurity/significant-disease-investigation-program
SA	Department of Primary Industries and Regions South Australia (PIRSA) VETLAB/ Gribbles Veterinary Pathology	Significant Disease Investigation Program	https://pir.sa.gov.au/biosecurity/animal_health/veterinarians/livestock_disease_surveillance
NSW	Department of Primary Industries (DPI NSW)	Abortion investigation	https://www.dpi.nsw.gov.au/about-us/services/laboratory-services/veterinary/abortion-investigation
		Significant Disease Investigation Program	https://www.dpi.nsw.gov.au/biosecurity/your-role-in-biosecurity/veterinary-professionals/programs-and-projects
VIC	Agriculture Victoria AgriBio Laboratories	Significant Disease Investigation Program	https://agriculture.vic.gov.au/biosecurity/animal-diseases/significant-disease-investigation-sdi-program
TAS	Departments of Natural Resources and Environment Animal Health Laboratory	Significant Disease Investigation Program	https://nre.tas.gov.au/biosecurity-tasmania/animal-biosecurity/animal-health/information-for-veterinary-practitioners

12. Conclusions

12.1 Key findings for industry

Survey of reproductive performance in maiden ewes

- A survey that represented 117,117 maiden ewes and 302,585 mature ewes identified that both lower reproductive rate and lower lamb survival contribute to lower marking rates in maiden ewes compared to multiparous (mature) ewes
- The difference in marking rates for non-Merino ewe lambs compared to multiparous ewes was 58%. This was due to 50% difference in reproductive (scanning) rate and 16% difference in lamb survival
- The gap between the reproductive performance of maiden and mature ewes was smaller for maiden Merino hoggets than non-Merino ewe lambs. The difference in marking rates for maiden Merino hoggets compared to mature Merino ewes was 22%. This was attributable to 24% difference in reproductive rate and 3% difference in lamb survival
- The survival of single-born (76% for non-Merino ewe lambs and 83% for Merino hoggets) and twin-born lambs (65% for non-Merino ewe lambs and 61% for Merino hoggets) were below current industry targets
- The reproductive performance of non-Merino ewe lambs was not correlated with mature ewes on the same farm which suggests that strategies specific to this age group may be required to improve reproductive rate, lamb survival and overall reproductive performance.
- Lamb survival for maiden Merino hoggets was moderately correlated with that of mature ewes on the same farm. Therefore, improving adoption of practices that improve lamb survival in mature ewes may also improve reproductive performance for maiden Merino ewes.

Review of submissions to state veterinary laboratories

- Abortion and stillbirth investigations were conducted for a low proportion of farms (up to 0.5% farms per year). It is not clear if low rates of submission are due to low levels of abortion being detected by farmers, low rates of voluntarily reporting of abortions to veterinarians by farmers or veterinarians choosing not to submit specimens for laboratory testing.
- A diagnosis was able to be made for 57% submissions which was consistent with overseas reports. Investigations with placenta included in the submission were twice as likely to have a diagnosis made. Autolytic changes were reported for 50% the investigations that included histopathology, but a diagnosis was still able to be reached for 57% of investigations with autolytic changes.
- 81% of abortion and stillbirth investigations with a diagnosis involved infectious disease. The three most common infections (*Campylobacter* spp., *Listeria* spp. and *Toxoplasma gondii)* accounted for 86% investigations with a diagnosis, with a wide range of infectious and non-infectious causes identified in remaining 14% investigations
- Veterinary practitioners can improve the likelihood of obtaining an aetiological diagnosis by encouraging farmers to collect any aborted or perinatal dead lambs and placenta (if available) and store these between 4-10^{O} C prior to submission.

Timing of foetal loss and lamb mortality in maiden ewes

- Lamb mortality from birth to marking represented the greatest contributor to foetal and lamb mortality after scanning, but mid-pregnancy abortion was an important contributor to lamb mortality in some ewe lamb flocks.
- Mid-pregnancy abortion was detected in 5.7% of ewes (range 0–50%) in the ewe lamb flocks and 0.9% of ewes (range 0–4.4%) in the maiden Merino hogget flocks.
- Mid-pregnancy abortion affecting ≥2% of ewes was observed in 6/19 ewe lamb flocks and 2/11 Merino hogget flocks. Notably, high levels of mid-pregnancy abortion were detected using repeat scanning in ewe lamb flocks that had no other obvious signs of illness that would alert the producer to the problem.
- Litter size (single or twin) had no effect on mid-pregnancy abortion maiden ewes
- Liveweight and condition score at joining, and liveweight change from joining to Scan 1, had no effect on mid-pregnancy abortion nor overall lamb mortality between scanning and marking in flocks in this study.
- Sequential scanning in combination with monitoring ewes during lambing to recover dead lambs, ewe udder inspection at marking and the number of lambs marked can be used to differentiate poor conception, *in utero* foetal loss and perinatal lamb mortality, and to inform strategies to improve overall reproductive performance.

Cause of death

- Lamb necropsies (*n* = 298) identified starvation-mismothering-exposure (34%), dystocia (24%) and stillbirth (15%) as the most common causes of perinatal lamb death.
- *Chlamydia pecorum* was detected in 15/35 aborted and stillborn lambs on 5/6 farms in WA. *Chlamydia pecorum* detected in aborted and stillborn lambs were genetically identical to sequence type 23 (ST23) which has also been associated with arthritis and conjunctivitis in sheep. *Chlamydia pecorum* ST23 has also been associated with abortion and Sporadic Bovine Encephalopathy in cattle.
- *Chlamydia pecorum* ST23 associated with abortion, stillbirth, arthritis and conjunctivitis in sheep is genetically different to *C. pecorum* sequence types commonly detected in the gastrointestinal tract.
- *Chlamydia pecorum* is an “emerging” cause of reproductive disease in sheep. It is unlikely that this is a new condition but rather the use of improved molecular diagnostic tests for exotic disease exclusion testing have allowed laboratories to detect *C. pecorum*.
- The epidemiology of *C. pecorum* ST23 is not well described, including distribution on Australian sheep farms, main routes of transmission, risk factors for disease (reproductive disease, arthritis, conjunctivitis) and strategies for disease prevention

Infectious diseases: Campylobacter, Toxoplasma, Neopsora and Coxiella (Q fever)

- Abortions associated with *Campylobacter fetus* were detected on one farm confirming that *Campylobacter* causes losses on some farms in some years.
- Relationships between *Campylobacter* spp. seropositivity and reproductive outcomes were inconsistent, emphasising the importance of pathogen detection at necropsy to confirm reproductive disease and consideration of risk factors specific to the farm in planning preventive measures for campylobacteriosis.
- There was no evidence that *T. gondii, N. caninum* or *C. burnetii* were important contributors to *in utero* foetal loss or perinatal lamb mortality observed in the 30 maiden ewe flocks included in this study.
- Seropositivity to *T. gondii* was detected using indirect ELISA at 16/28 farms and 8.1% mature ewes (95% confidence interval (CI) 6.0, 10.5). However, seroconversion during pregnancy and the lambing period was detected in only 1.0% (95% CI 0.5, 1.7) of ewes that failed to raise a lamb, and 0.6% (95% CI 0.1, 2.9) of ewes with confirmed abortion.
- Sporadic outbreaks of *T. gondii* that cause considerable reproductive losses on some farms in some years are likely related to sporadic point source exposure to oocysts, *e.g.* via contaminated drinking water or feed source. Low levels of seropositivity suggest that the majority of ewes remain susceptible to reproductive disease if point source exposure occurs during gestation. In the absence of vaccination, measures to prevent contamination of feed and water sources from cat faeces can reduce the risk of toxoplasmosis outbreaks in susceptible sheep.
- *Neospora caninum* seropositivity was detected at 2/28 farms with 0.2% (95% CI 0.0, 0.5) of maiden ewes and 0% (95% CI 0.0, 0.5) of mature ewes seropositive using indirect ELISA
- *Coxiella burnetii* seropsotivity was detected at 3/28 farms, with 0.1% (95% CI 0.0, 0.4) of maiden ewes and 0.4% (95% CI 0.1, 1.1) of mature ewes seropositive using indirect ELISA
- Despite low levels of detection for *C. burnetii* seropositivity in this study, sporadic zoonotic transmission from sheep is reported in Australia and overseas and contact with sheep should still be considered a risk factor for Q-fever in humans. Precautions should be taken to reduce the risk of zoonotic *C. burnetii* transmission.

12.2 Benefits to industry

This project has:

- Provided industry data on the reproductive performance of maiden ewes
- Offered new insight into the contribution of *in utero* foetal loss and perinatal lamb mortality to the reproductive performance of maiden ewes
- Identified the need for focussed ewe management guidelines specific to maiden ewes.
- Identified *C. pecorum* as an 'emerging' cause of abortion and stillbirths in sheep, and especially ewe lambs
- Improved industry understanding on the level of exposure to important infectious diseases including *T. gondii, N. caninum* and *C. burtnetii* for maiden and mature breeding ewes in southern Australia, plus determined the contribution of these to lamb survival for maiden ewes
- Provided new insight into the public health risk of zoonotic Q-fever transmission associated with ewes in southern Australia
- Developed evidence-based strategies to investigate poor reproductive performance in ewes and inform strategies to improve ewe reproductive performance
- Provided updated recommendations for producers and veterinarians to collect and submit samples that can assist in identifying causes of abortions and stillbirth

The findings from this project have been peer-reviewed to ensure scientific validity and will be made available to existing networks such as Bred Well Fed Well, Lifetime Ewe Management (RIST), Sheep Connect, Best Wool Best Lamb, Australian Veterinary Association special interest groups to ensure that learnings are incorporated into existing programs and learning resources and shared as widely as possible.

The participatory research approach allowed producers to gain new insight into sources of reproductive losses in their maiden ewes and diseases impacting ewes on their farm. The input and feedback from host producers has been incorporated into recommendations and protocols for investigation of poor reproductive performance (Chapter 11) to ensure that these are both practical and effective.

Industry capability and expertise

This project has supported the career development of Dr Amy Lockwood, an outstanding early career researcher working in applied sheep research. Dr Lockwood was employed as a research fellow for this project, and she subsequently went on to develop and lead her own project investigating the role of supplementary feeding on lamb survival.

This project provided post-graduate training for an exceptional PhD candidate, Tom Clune. Tom Clune was subsequently awarded scholarships by Meat and Livestock Australia (Postgraduate Award) and Sheep Industry Business Innovation (Department of Primary Industries and Regional Development, Western Australia). He was awarded the 2020 Science and Innovation Awards for Young People in Agriculture, Fisheries and Forestry (Australian Government Department of Agriculture in partnership with Australian Wool Innovation). To date, Tom has authored nine journal articles related to this project which is an extraordinary achievement for a PhD candidate.

This project supported undergraduate honours training Dayna Hutchison, who has subsequently graduated with from the Murdoch University Bachelor of Science with honours and majors in animal science and animal health.

The project has fostered new collaborative networks

- A new collaboration between Murdoch University, University of the Sunshine Coast and Department of Primary Industries and Regional Development (WA) resulted in successful application for the Science and Innovation Awards for Young People in Agriculture, Fisheries and Forestry. This resulted in characterisation of “pathogenic *C. pecorum* ST23” associated with abortion, stillbirth and arthritis, plus demonstration that point-of-care testing offers opportunity for faster and cheaper *C. pecorum* screening in sheep
- This collaboration was subsequently extended to include University of Zurich which resulted in a study showing that on farms with abortions and stillbirths associated with *C. pecorum* ST23, this was also associated with in arthritis in sheep post-weaning rendering lambs unfit for transport and slaughter.
- The review of state laboratory data developed relationship between the research team and pathologists at state veterinary laboratories in Western Australia, Tasmania, Victoria and more recently, New Zealand. This has already yielded results with these groups identifying suitable case material and making this available for a follow-up study characterising molecular epidemiology of *C. pecorum* in aborted lambs from across Australia and New Zealand

13. Future research and recommendations

Benchmark reproductive rate and lamb survival for ewe lambs and maiden ewe hoggets

Chapter 3 describes a survey comparing reproductive performance of maiden ewes relative to mature ewes for 79 sheep producers. This data can be pooled with maiden ewe data from other projects to develop improved benchmarks for different maiden ewe age groups (ewe lambs and hoggets) across different production zones.

Recommendation for RD&E:

Expand maiden ewe reproductive performance datasets to provide robust benchmarks for maiden ewe reproductive rate, lamb survival and marking rates across major sheep production zones.

Strategies improving lamb survival specific to ewe lambs

Lower and more variable lamb survival in maiden ewes impacts reproductive performance.

Lamb survival for ewe lambs is not correlated with lamb survival for mature ewes on the same farm (Chapter 3). Studies that underpin recommendations to improve lamb survival have mostly used mature or mixed age ewes and it is not clear if current recommendations for addressing lamb survival (and specifically perinatal lamb mortality) for mature sheep are appropriate for ewe lambs.

In utero foetal loss has important impacts on reproductive performance in some ewe lamb flocks, with mid-pregnancy abortion impacting more than 2% ewes and loss of 3-48% of foetuses in approximately one in three ewe lamb flocks (Chapter 5). Current recommendations to address lamb survival are unlikely to address mid-pregnancy abortion given there was no association for mid-pregnancy abortion with joining liveweight, joining condition score or liveweight change from joining to scanning.

There was considerable variation in the frequency of abortion and overall lamb mortality between flocks in this study. Further investigation of the contribution of abortion to overall reproductive performance across major sheep production zones is needed to inform cost-benefit analyses for interventions to address lamb survival.

Recommendation for RD&E:

Field studies to quantify *in utero* foetal loss, perinatal lamb mortality and cause of death (necropsy) for ewe lamb flocks across different production zones, and validate observations from this project over diverse sheep production systems.

Existing recommendations for ewe management targeting lamb survival should be validated for ewe lambs, and modified if necessary.

Lamb survival for maiden Merino hoggets

The maiden ewe reproductive performance survey (Chapter 3) indicated that lamb survival for Merino hoggets was below current industry benchmarks particularly for twin-born lambs (Figure 9). Lamb survival for maiden Merino hoggets was moderately correlated with mature ewe performance on the same farm, therefore improving adoption of practices that have demonstrated to improve lamb survival are likely to result in improve reproductive performance for maiden Merino ewes.

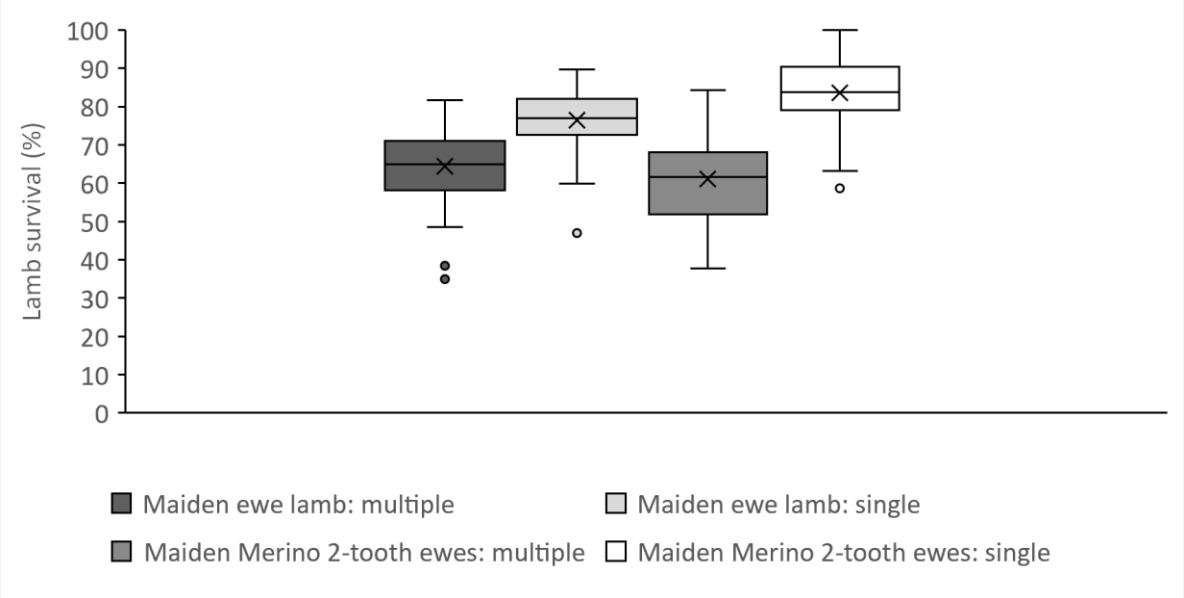

Figure 9: Box and whisker plot of for lamb survival in single- and multiple-born progeny of maiden ewe lambs and maiden Merino hoggets ewes managed according to litter size

Recommendation for RD&E:

Extension and adoption addressing lamb survival, and especially twin lamb survival, for Merinos with a focus on maiden ewes.

Emerging infectious causes of foetal and lamb mortality: *Chlamydia pecorum*

Chlamydia pecorum ST23 was detected in aborted and stillborn lambs from multiple farms in WA, plus arthritic joints of slaughter-age lambs from the same cohort of lambs on a farm with *C. pecorum*-associated abortions and stillbirths. *Chlamydia pecorum* ST23 detected in aborted, stillborn and arthritic lambs was genetically identical to *C. pecorum* associated with abortions and sporadic bovine encephalopathy in cattle. Since this study, abortions associated with *C. pecorum* have been detected in other flocks ewe lambs and ewe hoggets in other states. Diagnosis is based on molecular diagnostics including qPCR and sequencing to confirm sequence type. This process is slow, labour-intensive and costly.

There are major gaps in understanding epidemiology, disease pathogenesis and management *Chlamydia pecorum* ST23:

- It is not clear how widely *C. pecorum* ST23 is distributed on Australian sheep farms
- It is not known how sheep become infected and how infections spread between mobs
- Factors that determine whether *C. pecorum* ST23 infection impacts lamb survival are not understood
- There are no evidence-based recommendations for managing *C. pecorum* disease risk
- Genetic diversity of *C. pecorum* strains is not well understood. This can inform design of a peptide vaccine for *Chlamydia* infections in sheep

Accurate surveillance is a key step in understanding epidemiology of pathogenic infections and reducing the economic and welfare impacts. Surveillance for *C. pecorum* ST23 is currently hampered by inadequate diagnostic tools that mean infections are either (i) under-detected; (ii) misdiagnosed and over- detected; and/or (iii) diagnosed accurately but not treated appropriately. Preliminary studies have shown that point-of-care tests offer potential for faster, cheaper screening of tissue samples for *C. pecorum* (Clune et al., 2021a)

Recommendation for RD&E:

1. Further development to point-of-care screening tests to improve disease surveillance and support epidemiological studies for *C. pecorum* ST23 and quantify impacts to sheep supply chain related to reproductive disease, arthritis and pinkeye
2. Field studies to determine risk factors for abortion, lamb mortality and arthritis associated with *C. pecorum* ST23
3. Characterisation of the genetic diversity for circulating *C. pecorum* strains on sheep farms across Australia to determine the population of sheep at risk of disease and inform design of a peptide vaccine for *Chlamydia* infections in sheep

Risk factors for abortion and perinatal mortality

This study investigated the role of specific infectious agents on ewe reproductive performance. However, the prospective nature of the study and number of flocks included precluded detailed investigation of risk factors for individual diseases. The impact of pathogens on reproductive outcome (*e.g.* abortion or perinatal mortality) is usually dependent on multiple factors including strain pathogenicity, host immunity, environment, sheep management and concurrent infections. More than one aetiological cause can be occurring in a single animal or flock. Disease risk factors often cross over different infectious and non-infectious causes of death. For example, cold, wet weather is considered a risk factor for transmission of *Campylobacter* spp. or *Listeria* spp., but will also increase the likelihood of lamb mortalities due to hypothermia and starvation-mismothering.

Furthermore, endemic diseases that may cause abortion or stillbirth and also result in the birth of weak lambs that subsequently die from starvation-mismothering-exposure, but have lesions evident using gross pathology at lamb necropsy that would trigger diagnostic testing.

Recommendation for RD&E:

Future work should address the multifactorial nature of abortion and lamb mortalities including multivariable analyses that compare the relative important of different pathogens and non-infectious causes of death, plus associated risk factors.

Diagnostics for key endemic diseases: serology

Listeriosis is the most commonly diagnosed infectious cause of abortion (Chapter 4). Diagnosis is based on detection using bacterial culture and/or histopathological changes. *Listeria* serology was not used in the on-farm investigation in this project because existing methods lack sensitivity and specificity (Links, 1987). Improved diagnostic tests for acute listeriosis and to quantify *Listeria* spp. cell-mediated immunity would be useful for lamb survival research as well as investigating poor reproductive performance on-farm.

Refinement of existing serological tests for *C. pecorum* to improve sensitivity and specificity would support improved understanding of disease epidemiology (Bommana et al., 2018). Serology is unlikely to be able to differentiate pathogenic and non-pathogenic sequence types, therefore improving methodology for antigen detection and characterisation should be prioritised over serology.

Commercial indirect ELISA kits have not been well validated to determine specificity and sensitivity for key endemic diseases (toxoplasmosis, neosporosis, coxiellosis) using sera collected from naturally infected Australian sheep under field conditions. Validation of these tests will improve estimates of true prevalence in surveillance studies. Disease outbreaks are typically sporadic and often unreported, and this hampers access to sera that can be used to validate tests.

The study described in Chapter 10 used Agar Gel Immunodiffusion to determine *C. fetus* antibody titres. The sensitivity and specificity of this test is poorly defined. Further validation of the test for field investigations would improve prediction of true incidence of campylobacteriosis. The study described in Chapter 10 did not include enough animals with high *C. fetus* titres to determine positive and negative predictive value for different titre categories. Further investigation of antibody dynamics in ewes with confirmed campylobacteriosis abortions are needed to refine recommendations for interpretation of *C. fetus* titres as part of investigation of poor reproductive performance.

Recommendation for RD&E:

1. Validation of commercial ELISA kits using blood samples from sheep with evidence of disease associated with *T. gondii, N. caninum*, and *C. burnetii*
2. Characterisation of investigation of *C. fetus* antibody dynamics in ewes with confirmed campylobacteriosis abortions to refine recommendations for interpretation of titres as part of investigation of poor reproductive performance.

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Appendix 1: Survey of reproductive performance of maiden ewes

Maiden ewe reproduction performance industry benchmarking questionnaire

Producer name:

Phone:

Email:

Preferred contact method for follow-up:

Farm name:

Location:

Farm size:

Total Merino ewes on farm:

Total non-Merino ewes on farm:

Pregnancy scanner name:

I agree that the information provided in this survey may be used by Murdoch University to carry out the project. I understand that data provided will be anonymised and aggregated and then published or provided to the supporters of this project (including Meat and Livestock Australia, and Australian Wool Innovation). This consent is irrevocable, perpetual and royalty-free. Any personal information I provide will be held in accordance with Murdoch University's privacy policy who may, in the course of the project, provide this information to the above named supporters of this project.

I understand that all information provided by me is treated as confidential and will not be released by the researcher to a third party unless required to do so by law.

Name Signature Date

Do you wish to be contacted in the future:

(a) with results of the project? Yes / No

(b) with updates on future opportunities to participate in sheep production research? Yes / No

Part one: Scanning outcomes – *MAIDEN EWES ONLY*

Please note that ***maiden ewes*** are those that are being joined for the first time (youngest age group). This may refer to ewe lambs (7-9 months at joining) or hoggets (18-20 months at joining), depending on the property.

Data can be split across different mobs, or combined for all maidens.

Critical data

	Mob 1	Mob 2	Mob 3
Joining data			
Number of ewes in mob			
Average ewe age			
Ewe Breed			
Ram Breed			
Month of joining			
Scanning data			
Number of ewes scanned			
Scanned for multiples Y/N			
Number foetuses			
Number of dries			

Optional data: *information for these sections is not essential, but will give helpful information for factors that might impact reproductive performance of maiden ewes*

	Mob 1	Mob 2	Mob 3
Joining management			
Joining length (days)			
Ave ewe condition score (1-5)			
Predominant pasture type			
Management during joining & early pregnancy (day 0-50 pregnancy			
Supplementary feeding (yes/no)			
Trail feeding or self feeders			
Grain type			
Management during mid pregnancy (day 50-100 pregnancy)			
Supplementary feeding (yes/no)			
Trail feeding or self feeders			
Grain type			

Part two: Lambing outcomes – MAIDEN EWES ONLY

Please note that *maiden ewes* are those that are being joined for the first time (youngest age group). This may refer to ewe lambs (7-9 months at joining) or hoggets (18-20 months at joining), depending on the property.

If you have more than three maiden lambing mobs, an Excel version of the questionnaire with additional columns is available.

Critical data

	Paddock 1	**Paddock 2**	**Paddock 3**
Information for ewes at lambing			
Mob or Paddock name			
Number of ewes in mob			
Preg status (single/twin/triplet/mixed)			
Month of lambing			
Marking information			
Number of lambs marked			
Number of ewe deaths (if known)			

Optional data: *information for these sections is not essential, but will give helpful information for factors that might impact reproductive performance of maiden ewes*

	Paddock 1	**Paddock 2**	**Paddock 3**
Information for sheep at lambing			
Ave ewe condition score (1-5)			
Average FOO (kg DM/ha)			
Predominant pasture type			
Management in late pregnancy (day 100-150 pregnancy)			
Supplementary feeding (yes/no)			
Trail feeding or self feeders			
Grain/feed type			
Nutrition information for sheep during lambing - marking			
Supplementary feeding (yes/no)			
Trail feeding or self feeders			
Grain type			

Part three: comparative mature flock performance – ADULT EWES ONLY

Please provide a summary of your adult ewe reproductive data.

Different mobs/paddocks at marking time can be combined.

You need only provide separate groupings if you have different flocks i.e. Merino to Merino, Merino to non-Merino.

	Flock 1	Flock 2	Flock 3
Information for sheep at joining			
Number of ewes in flock			
Ewe Breed			
Ram Breed			
Scanning data			
Number of ewes scanned			
Number of foetuses scanned			
Number of dries			
Marking information			
Total number of lambs marked			
Total number of ewe deaths			

Have you attended training in condition scoring and/or assessing FOO?	
Is condition score estimated or actual (i.e. measured)?	
Is FOO estimated or actual (i.e. measured)?	

	Mob 1	Mob 2	Mob 3
Joining data			
Number of ewes in mob			
Average ewe age			
Ewe Breed			
Ram Breed			
Month of joining			
Scanning data			
Number of ewes scanned			
Scanned for multiples Y/N			
Number foetuses			
Number of dries			
	Paddock 1	Paddock 2	Paddock 3
Information for ewes at lambing			
Mob or Paddock name			
Number of ewes in mob			
Preg status (single/twin/triplet/mixed)			
Month of lambing			
Marking information			
Number of lambs marked			
Number of ewe deaths (if known)			

Appendix 2: Abortion & lamb mortality between pregnancy scanning & lamb marking

Table A2.1: Reproductive performance and timing of abortion and lamb mortalities for 19 flocks of ewe lambs

Flock reference no.	3	4	7	8	11	14	16	17	18	19	20	21	22	23	24	25	27	28	30
State	WA	WA	WA	WA	WA	WA	WA	VIC	VIC	VIC	VIC	VIC	SA	SA	SA	SA	VIC	VIC	SA
Breed	NM	NM	NM	Mo	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
Campylobacter vaccination[1]	No	No	No	No	No	No	No	Yes	Yes	No	No	Yes	Yes	No	Yes	No	Yes	Yes	No
Ewes joined (*n*)	198	200	198	189	213	197	162	196	197	203	204	206	195	201	196	302	200	202	388
Conception rate (%)[2]	74.7	65	76.3	45.5	68.5	85.3	63.6	54.1	85.8	87.2	80.4	76.7	72.3	81.6	62.2	92.4	86.5	78.7	57.7
Scanning rate (%)[3]	97.5	106.5	122.2	57.7	74.6	110.2	91.4	78.6	146.7	143.3	124.0	124.3	123.6	120.9	96.9	155.6	155.5	133.2	79.4
Marking rate (%)[4]	60.6	82.0	88.4	38.6	54.5	45.2	60.5	67.3	117.3	70.9	73.0	75.7	91.8	99.0	28.1	30.1	115.0	94.6	49.5
Aborted foetuses recovered (*n*)	1	0	0	0	0	0	0	0	0	3	0	0	0	0	1	0	0	0	0
Foetal and lamb mortality (% foetuses)[5]																			
Overall mortality (scan 1 – marking)	37.8	23.0	27.7	33.0	27.0	59.0	33.8	14.3	20.1	50.5	41.1	39.1	25.7	18.1	71.1	65.6	26.0	29.0	37.7
Mid-pregnancy abortion (scan 1 – scan 2)	6.2	1.4	1.2	1.8	0.0	21.7	3.4	0.0	0.0	8.6	4.3	0.0	0.8	2.1	48.4	1.3	0.0	1.1	1.3
Late-pregnancy abortion/loss (scan 2 – birth)[6]	6.2	6.1	17.8	16.5	8.8	9.2	8.8	0.0	3.1	27.5	11.1	25.8	2.1	4.9	7.9	NA	4.8	10.8	16.9
Perinatal mortality (birth – marking)[7]	25.4	15.5	8.7	14.7	18.2	28.1	21.6	14.3	17.0	14.4	25.7	13.3	22.8	11.1	14.7	NA	21.2	17.1	19.5
Chi-square *P*-value	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NA	<0.001	<0.001	<0.001
Mid-pregnancy abortion (ewes)																			
n	11	2	1	1	0	40	3	0	0	15	7	0	1	3	61	4	0	3	3
%	7.4	1.5	0.7	1.2	0.0	23.8	2.9	0.0	0.0	8.5	4.3	0.0	0.7	1.8	50.0	1.4	0.0	1.9	1.3

NA: not available WA: Western Australia SA: South Australia VIC: Victoria NM: non-Merino Mo: Merino

[1] Vaccination with Ovilis Campyvax™ (*Campylobacter fetus* and *Campylobacter jejuni*)

[2] Number of ewes pregnant / number of ewes joined

[3] Number foetuses scanned / number of ewes joined

[4] Number of lambs marked / number of ewes joined

[5] Number of foetuses (or lambs) lost from start to end of respective period indicated in parentheses / number of foetuses at scan 1 (%).

[6] Includes lambs that were born and not recovered at lambing rounds (e.g. lost to predation).

[7] Includes lambs dead at birth (full-term)

Table A2.2: Reproductive performance and timing of abortion and lamb mortalities for 11 flocks of maiden Merino hoggets

Flock reference no.	1	2	5	6	9	10	12	13	15	26	29
State	WA	WA	SA	SA	SA	WA	SA	VIC	WA	VIC	VIC
Campylobacter vaccination[1]	No	No	No	No	No	No	No	No	No	No	No
Ewes joined (n)	213	188	194	197	202	200	197	188	210	190	191
Conception rate (%)[2]	87.3	94.7	94.8	83.2	95.5	84.5	92.9	58.5	97.1	72.1	93.1
Scanning rate (%)[3]	116.9	116.5	109.3	90.9	113.9	89.5	113.7	59.6	135.2	85.3	121.5
Marking rate (%)[4]	93.9	81.4	79.4	NA	89.1	66.0	81.2	28.2	101.0	52.1	93.2
Foetal and lamb mortality (% foetuses)[5]											
Overall mortality (scan 1 – marking)	19.7	30.1	27.4	NA	21.7	26.3	28.6	52.7	25.4	38.9	23.3
Mid-pregnancy abortion (scan 1 – scan 2)[5]	0.0	0.0	0.9	0.0	0.0	0.0	0.9	0.9	0.7	3.7	1.7
Late-pregnancy abortion/loss (scan 2 – birth)[6]	0.0	4.1	3.8	NA	NA	15.6	3.1	40.2	1.8	21.0	3.0
Lamb mortality (birth – marking)[7]	19.7	26.0	22.6	NA	NA	10.6	24.6	11.6	22.9	14.2	18.5
Chi-square *P*-value	<0.001	<0.001	<0.001	NA	NA	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Mid-pregnancy abortion (ewes)											
n	0	0	2	0	0	0	2	1	1	6	4
%	0.0	0.0	1.1	0.0	0.0	0.0	1.1	0.9	0.5	4.4	2.2

NA: not available WA: Western Australia SA: South Australia VIC: Victoria

[1] Vaccination with Ovilis Campyvax® (*Campylobacter fetus* and *Campylobacter jejuni*)
[2] Number of ewes pregnant / number of ewes joined
[3] Number foetuses scanned / number of ewes joined
[4] Number of lambs marked / number of ewes joined
[5] Number of foetuses (or lambs) lost from start to end of respective period indicated in parentheses / number of foetuses at scan 1 (%).
[6] Includes lambs that were born and not recovered at lambing rounds (e.g. lost to predation).
[7] Includes lambs dead at birth (full-term)

Appendix 3: Necropsies and detection of infectious disease

Table A3.1: Summary data for tissues from aborted & stillborn lambs available for laboratory diagnosis

Flock code	Report case number	Case code	Tissue submitted	Necropsy interpretation
A	AS-18-2738	18-001	Brain, lung, liver	Stillborn
A	AS-18-2738	18-003	Lung, placenta, liver	Stillborn
A	AS-18-2738	18-006	Liver	Stillborn
A	AS-18-2738	18-008	Liver, placenta	Stillborn
A	AS-18-2738	18-011	Liver, placenta	Stillborn
A	AS-18-2738	18-013	Liver[C]	Stillborn
A	AS-18-2960	18-024	Liver	Stillborn
A	AS-18-2960	18-026	Liver	Stillborn
A	AS-18-2960	18-028	Liver	Stillborn
A	AS-18-2960	18-029	Liver[AB]	Stillborn
B	AS-18-2882	18-040	Liver, placenta, brain, lung, heart	Stillborn
B	AS-18-2882	18-050	Liver, brain, placenta	Stillborn
B	AS-18-2882	18-051	Liver, brain, placenta	Stillborn
F1	AS-18-2650	18-079	Liver[AB], placenta, brain, stomach contents, lung, kidney, heart	Abortion
F1	AS-18-2736	18-080	Placenta[AB]	Abortion
F1	AS-18-2881	18-081	Liver[A]	Stillborn
F1	AS-18-2881	18-082	Liver[A]	Stillborn
F1	AS-18-2881	18-083	Liver[A], placenta[AB], stomach contents	Stillborn
F1	AS-18-2881	18-084	Liver, placenta[A]	Stillborn
F1	AS-18-2881	18-085	Heart, lung[A]	Stillborn
F1	AS-18-2961	18-087	Liver[AB], placenta	Stillborn
F1	AS-18-2961	18-089	Brain, placenta	Stillborn
F1	AS-18-2961	18-091	Liver[AB], brain, lung	Stillborn
F2	AS-19-3155	19-111	Liver, lung, placenta	Stillborn
F2	AS-19-3155	19-112	Liver, lung, placenta	Stillborn
F2	AS-19-3155	19-114	Liver, lung, placenta	Stillborn
F2	AS-19-3155	19-160	Liver, lung, brain, heart, kidney	Stillborn
H	AS-19-2600	19-002	Liver, stomach, placenta[A], lung	Stillborn
H	AS-19-2600	19-003	Liver, lung	Stillborn
H	AS-19-2600	19-028	Liver[AC], placenta[AB], lung, stomach contents	Stillborn
H	AS-19-2600	19-030	Liver[AB], placenta[A], lung	Stillborn
I	AS-19-2758	19-032	Liver, lung	Stillborn
I	AS-19-2758	19-042	Liver, kidney, heart, lung	Stillborn
J	AS-19-2601	19-007	Liver[AB], lung, brain, clotted heart blood, stomach content, heart, kidney	Premature
J	AS-19-2601	19-008	Liver[A], lung, brain, clotted heart blood, stomach content, heart, kidney	Premature

[A] Tissue samples in which *C. pecorum* was detected using qPCR

[B] Tissue samples from which *C. pecorum* was characterised (MLST and *omp*A)

[C] Tissue samples where *T. pyogenes* was cultured

Table A3.2: Primer/probe final concentrations, sequences and cycling conditions for diagnostic PCRs performed during this study A

Assay	Primer/probe sequences and final PCR conc.	Cycling conditions	References
Brucella spp. PCR	ISP1 - GGT TGT TAA AGG AGA (1.0 uM) ISP2 - GAC GAT AGC GTT TCA (1.0 uM)	Hold: 95°C for 5 min 35 cycles: 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min Hold: 72°C for 6 min	Ouahrani-Bettache et al., 1996 [35]
Campylobacter spp. PCR	C412F - GGA TGA CAC TTT TCG GAG C (0.4 uM) C1288R - CAT TGT AGC ACG TGT GTC (0.4 uM)	Hold: 95°C for 5 min 30 cycles: 94°C for 30 sec, 54°C for 30 sec, 72°C for 1 min Hold: 72°C for 7 min	Linton et al., 1996 [36]
Chlamydia abortus qPCR	CpaOMP1-F - GCA ACT GAC ACT AAG TCG GCT ACA (0.5 uM) CpaOMP1-R - ACA AGC ATG TTC AAT CGA TAA GAG A (0.5 uM) CpaOMP1-S - FAM-TAA ATA CCA CGA ATG GCA AGT TGG TTT AGC G-BHQ-1 (0.2 uM)	Hold: 95°C for 5 min 40 cycles: 95°C for 15 sec, 60°C for 15 sec (acquiring green)	Pantchev et al., 2009 [32]
Chlamydia pecorum qPCR	CppecOMP1-F - CCA TGT GAT CCT TGC GCT ACT (0.5 uM) CppecOMP1-R - TGT CGA AAA CAT AAT CTC CGT AAA AT (0.5 uM) CppecOMP1-S - FAM-TGC GAC GCG ATT AGC TTA CGC GTA G-BHQ-1 (0.2 uM)	Hold: 95°C for 5 min 40 cycles: 95°C for 15 sec, 60°C for 15 sec (acquiring green)	Pantchev et al., 2010 [31]
Chlamydia psittaci qPCR	CppsOMP1-F - CAC TAT GTG GGA AGG TGC TTC A (0.5 uM) CppsOMP1-R - CTG CGC GGA TGC TAA TGG (0.5 uM) CppsOMP1-S - FAM-CGC TAC TTG GTG TGA C-BHQ-plus (0.2 uM)	Hold: 95°C for 5 min 40 cycles: 95°C for 15 sec, 60°C for 15 sec (acquiring green)	Pantchev et al., 2009 [32]
Chlamydiales PCR (1)	16SIGF - CGG CGT GGA TGA GGC AT (0.4 uM) 16SIGR - TCA GTC CCA GTG TTG GC (0.4 uM)	Hold: 95°C for 5 min 40 cycles: 94°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec Hold: 72°C for 7 min	Everett et al., 1999 [33]
Chlamydiales PCR (2)	16SIGF - CGG CGT GGA TGA GGC AT (0.4 uM) 806R - GGA CTA CCA GGG TAT CTA AT (0.4 uM)	Hold: 95°C for 5 min 40 cycles: 94°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec Hold: 72°C for 7 min	Everett et al., 1999 [33]

Table A3.2: Primer/probe final concentrations, sequences and cycling conditions for diagnostic PCRs performed during this study[A] (continued)

Assay	Primer/probe sequences and final PCR conc.	Cycling conditions	References
Coxiella burnetii multiplex qPCR	com1F - AAA ACC TCC GCG TTG TCT TCA (0.4 uM) com1R - GCT AAT GAT ACT TTG GCA GCG TAT TG (0.4 uM) com1probe - FAM-AGA ACT GCC CAT TTT TGG CGG CCA-BHQ-1 (0.2 uM)	Hold: 50°C for 3 min Hold: 95°C for 5 min 60 cycles: 95°C for 20 sec, 60°C for 40 sec (acquiring green/yellow)	Lockhart et al., 2011 [38] (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011) (Lockhart et al., 2011)
	IS1111aF - GTT TCA TCC GCG GTG TTA AT (0.2 uM) IS1111aR - TGC AAG AAT ACG GAC TCA CG (0.2 uM) IS1111aP - TET-CCC ACC GCT TCG CTC GCT AA-BHQ-1 (0.1 uM)		Banazis et al., 2010 [37] (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010) (Banazis et al., 2010))
Pathogenic *Leptospira* spp. qPCR	Lepto F - CCC GCG TCC GAT TAG (0.5 uM) Lepto R - TCC ATT GTG GCC GRA CAC (0.5 uM) Lepto P - FAM-CTC ACC AAG GCG ACG ATC GGT AGC-TAMRA (0.2 uM)	Hold: 95°C for 5 min 40 cycles: 95°C for 15 sec, 60°C for 15 sec (acquiring green)	Smythe et al., 2002 [39]
Pan-Pestivirus RT-qPCR[B]	Pesti-3F - CCT GAG TAC AGG RTA GTC GTC A (0.9 uM) Pesti-4R - GGC CTC TGC AGC ACC CTA TCA (0.9 uM)	Hold: 45°C for 10 min Hold: 95°C for 10 min 45 cycles: 95°C for 15 sec, 60°C for 45 sec (acquiring green)	Hyndman et al., 1998 [40]
	BVDV190 - GRA GTC GTC ART GGT TCG AC (0.9 uM) V326 - TCA ACT CCA TGT GCC ATG TAC (0.9 uM) TQ-pesti-P - FAM-TGC YAY GTG GAC GAG GGC ATG C-BHQ-1 (0.25 uM)		Hoffmann et al., 2006 [41]

[A] All qPCRs used Rotor-Gene Multiplex Master Mix (Qiagen) and were run on a Rotor-Gene Q (Qiagen) real-time PCR cycler except where noted. All conventional PCRs used HotStarTaq Master Mix (Quagen) and were performed on a DNA Engine (Bio-Rad) thermal cycler.

[B] AgPath-ID One-Step RT-PCR Master Mix (Applied Biosystems) was used for the Pan-Pestivirus RT-qPCR and performed on an Applied Biosystems 7500 Real-Time PCR instrument.

Table A3.3: Histopathology findings

Flock	Cause of death	Report case number	Case code	Histopathology findings
A	Stillborn	AS-18-2738	18-001, liver	NSF
			18-001, lung	NSF
			18-001, brain	NSF
A	Stillborn	AS-18-2738	18-003, liver	NSF
			18-003, placenta	NSF
			18-003, lung	NSF
F1	Abortion	AS-18-2650*	18-079, liver	Multifocal *Chlamydia* IHC staining of Kupffer cells
			18-079, placenta	Necrotising placentitis with neutrophilic vasculitis; *Chlamydia* IHC positive
			18-079, lung	Multifocal *Chlamydia* IHC staining of alveolar macrophages
			18-079, heart	Mild, multifocal histiocytic, lymphocytic & neutrophilic epicarditis
			18-079, brain	Mild, diffuse, neutrophilic, histiocytic & lymphocytic meningitis
			18-079, kidney	Autolysed. Focal histiocytic & neutrophilic pyelitis
F1	Abortion	AS-18-2736*	18-080, placenta	Autolysed. Multifocal necrotising placentitis with mineralisation and multifocal *Chlamydia* IHC staining
F1	Stillborn	AS-18-2881*	18-081, liver	NSF
F1	Stillborn	AS-18-2881*	18-083, liver	NSF
			18-083, placenta	Multifocal, necrotising placentitis with mineralisation and focal neutrophilic vasculitis
F1	Stillborn	AS-18-2881*	18-084, liver	NSF
			18-084, placenta	Mild, diffuse histiocytic placentitis with multifocal *Chlamydia* IHC staining
F1	Stillborn	AS-18-2881*	18-085, lung	NSF
			18-085, heart	NSF
F1	Stillborn	AS-18-2961*	18-091, liver	Multifocal mild histiocytic portal hepatitis
			18-091, brain	Moderate, multifocal neutrophilic encephalitis with mild histiocytic meningitis
			18-091, lung	Low numbers of neutrophils and macrophages in airways
F2	Stillborn	AS-19-3155	19-111, liver	NSF
			19-111, placenta	NSF
			19-111, lung	Meconium in small airways
F2	Stillborn	AS-19-3155	19-112, liver	NSF
			19-112, placenta	NSF
			19-112, lung	Meconium in small airways
F2	Stillborn	AS-19-3155	19-114, liver	NSF
			19-114, placenta	NSF
			19-114, lung	Meconium in small airways
F2	Stillborn	AS-19-3155	19-160, liver	Moderate, multifocal periacinar hepatic necrosis
			19-160, lung	Meconium in small airways
			19-160, heart	NSF
			19-160, brain	NSF
I	Stillborn	AS-19-2758	19-032, liver	NSF
			19-032, lung	NSF

Table 2: Histopathology findings (continued)

Flock	Cause of death	Report case number	Case code	Histopathology findings
I	Stillborn	AS-19-2758*	19-042, liver	Mild, multifocal lymphocytic & histiocytic portal hepatitis
			19-042, lung	Mild, multifocal proliferation of alveolar macrophages
			19-042, heart	NSF
			19-042, kidney	NSF
J	Premature	AS-19-2601*	19-007, liver	NSF
			19-007, lung	Mild multifocal alveolar macrophage proliferation
			19-007, heart	Multifocal, mild lymphocytic & histiocytic epicarditis & myocarditis
			19-007, brain	NSF
			19-007, kidney	Diffuse, marked lymphocytic & histiocytic pyelitis with intraepithelial, intracytocplasmic inclusion bodies indicative of *Chlamydia*
J	Premature	AS-19-2601	19-008, liver	NSF
			19-008, lung	Mild, multifocal alveolar macrophage proliferation with squames in small airways
			19-008, heart	NSF
			19-008, brain	NSF
			19-008, kidney	NSF

NSF: no significant findings

IHC: immunohistochemistry

* Cases in which *C. pecorum* was detected by qPCR

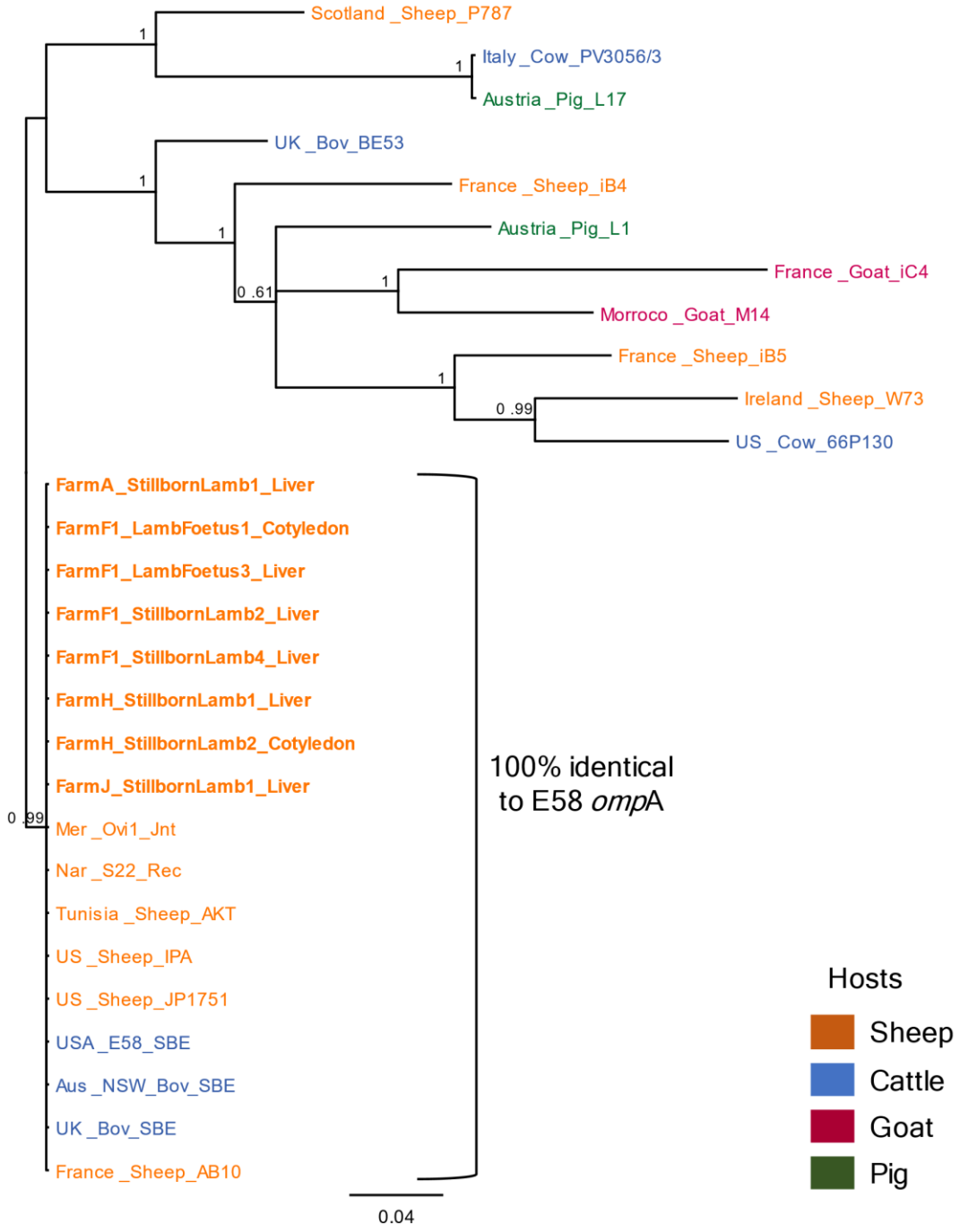

Figure A3.1: The livestock *C. pecorum ompA* phylogenetic relationships [A]

[A] The mid-point rooted Bayesian tree was constructed using the 980 bp *ompA* alignment sequences from 28 *C. pecorum* strains from livestock (sheep, goat, cattle and pig) hosts, including the lamb abortigenic *C. pecorum* strains described in this study. The lamb abortigenic strains are denoted in bold, whilst the hosts are coloured as in the legend. Posterior probabilities are displayed on the tree nodes.

Appendix 4: *Toxoplasma gondii*

Table A4.1: Apparent *T. gondii* seropositivity with 95% confidence interval (95% CI) in maiden ewes from Australian farms determined using indirect ELISA.

			Tested (*n*)			***T. gondii* serology**
Flock reference	**Location**	**Breed**	Aborted or FTR[C]	Reared lamb	Total	Seropositive *n* (%)
HOGGETS[A]						
1	Kojonup, WA	Merino	40	0	40	0
2	Kojonup, WA	Merino	40	0	40	0
5	Korunya, SA	Merino	40	0	40	0
6	Bagot Well, SA	Merino	NA	NA	40*	1 (2.5%)
9	Watervale, SA	Merino	30	10	40	0
10	Broomehill, WA	Merino	40	0	40	2 (5%)
12	Tarlee, SA	Merino	40	0	40	1 (2.5%)
13	Giffard West, VIC	Merino	40	0	40	1 (2.5%)
15	Katanning, WA	Merino	40	0	40	1 (2.5)
26	Culla, VIC	Merino	40	0	40	0
29	Ballarat, VIC	Merino	30	10	40	1 (2.5%)
Seroprevalence % (95% CI)			380	20	440	1.59% (0.7, 3.1)
EWE LAMBS[B]						
3 [d]	Narrogin, WA	Non-Merino	50	48	98	1 (1%)
4	York, WA	Non-Merino	31	9	40	0
7	Kojonup, WA	Non-Merino	26	14	40	1 (2.5%)
8	Katanning, WA	Merino	30	10	40	0
11	Kojonup, WA	Non-Merino	35	5	40	0
14 [d]	Narrogin, WA	Non-Merino	40	0	40	0
16	Ongerup, WA	Non-Merino	40	0	40	0
17	Hamilton, VIC	Non-Merino	20	20	40	0
18	Hamilton, VIC	Non-Merino	39	1	40	0
19 [e]	Nareen, VIC	Non-Merino	40	0	40	0
20	Cashmore, VIC	Non-Merino	40	0	40	2 (5%)
21	Coojar, VIC	Non-Merino	40	0	40	1 (2.5%)
22	Mount Gambier, SA	Non-Merino	40	0	40	1 (2.5%)
23	Kangaroo Island, SA	Non-Merino	29	11	40	0
24	Beachport, SA	Non-Merino	61	0	61	1 (1.6%)
25	Sellicks Hill, SA	Non-Merino	40	0	40	0
27 [e]	Nareen, VIC	Non-Merino	37	3	40	0
28	Inverleigh, VIC	Non-Merino	39	1	40	0
30	Strathalbyn, SA	Non-Merino	40	0	40	0
Seropositivity % (95% CI)			717	122	839	0.83% (0.37, 1.63)
Overall seropositivity (% (95% CI))			1097	142	1279	1.09% (0.63, 1.78)

[a] Maiden ewes mated at approximately 18 months of age; [b] Maiden ewes mated at approximately 8 months of age

[c] FTR: fail to rear (scanned pregnant with subsequent abortion or perinatal lamb mortality)

[d] Same property with Flock 3 (2018) and Flock 14 (2019); [e] Same property with Flock 19 (2019) and Flock 27 (2020)

WA: Western Australia SA: South Australia VIC: Victoria

*Reproductive outcome unknown

Table A4.2: Apparent *T. gondii* seropositivity for multiparous mature ewes (3 years of age or older) from Australian farms determined using indirect ELISA.

			***T. gondii* serology**	
Flock reference	**Location**	**Age**	Tested *n*	Seropositive *n* (%)
1	Kojonup, WA	4-7years	20	0
2	Kojonup, WA	5 years	20	0
3 and 14 [a]	Narrogin, WA	5 years	20	2 (10%)
4	York, WA	5-6 years	20	0
5	Korunya, SA	5+ years	20	0
6	Bagot Well, SA	5+ years	20	0
7	Kojonup, WA	5-7 years	20	2 (10%)
8	Katanning, WA	4-6 years	20	0
9	Watervale, SA	5+ years	18	0
10	Broomehill, WA	5-10 years	20	0
11	Kojonup, WA	5-6 years	20	0
12	Tarlee, SA	5+ years	20	0
13	Giffard West, VIC	5 years	20	0
15	Katanning, WA	5-7 years	20	1 (5%)
16	Ongerup, WA	4-7 years	20	0
17	Hamilton, VIC	4-5 years	20	0
18	Hamilton, VIC	6-7 years	20	1 (5%)
19 and 27 [b]	Nareen, VIC	5-8 years	20	0
20	Cashmore, VIC	4-8 years	20	7 (35%)
21	Coojar, VIC	4+ years	20	0
22	Mount Gambier, SA	3-9 years	20	1 (5%)
23	Kangaroo Island, SA	4-7 years	20	10 (50%)
24	Beachport, SA	5+ years	20	1 (5%)
25	Sellicks Hill, SA	4-7 years	20	5 (25%)
26	Culla, VIC	4-9 years	20	0
28	Inverleigh, VIC	3-6 years	20	6 (30%)
29	Ballarat, VIC	4-9 years	20	9 (45%)
30	Strathalbyn, SA	5+ years	20	0
Seropositivity (% (95% confidence interval)			558	8.06% (6.02, 10.54)

[a] Maiden ewes from property sampled in 2018 (flock 3) & 2019 (flock 14) – mature ewes sampled in one year only

[b] Maiden ewes from property sampled in 2019 (flock 19) & 2020 (flock 27) – mature ewes sampled in one year only

WA: Western Australia SA: South Australia VIC: Victoria

Table A4.3 Validation of *T. gondii* indirect ELISA (ID Screen Toxoplasmosis Indirect Multi-species, IDvet) with modified agglutination test

The indirect ELISA (ID Screen Toxoplasmosis Indirect Multispecies, ID Vet, France) was validated in-house against a panel of 28 Australian sheep serum previously tested using the modified agglutination test (Toxo-Screen Direct Agglutination kit, BioMérieux, Marcy-l'Étoile France) (Hamilton et al., 2021). Samples were analysed according to the manufacturer's instructions for both tests.

Indirect ELISA (ID Screen)	Value (%)	95% confidence interval
Sensitivity	90.5	71.1, 98.3
Specificity	100	64.6, 100
Positive Predictive Value	100	83.2, 100
Negative Predictive Value	77.8	45.3, 96

Table A4.4 Serological status category for 'doubtful' samples re-tested for anti-*T. gondii* IgG with alternate indirect ELISA

Sample ID	Flock reference	Reproductive outcome	ID-VET ELISA Result (S/P value [a])	IDEXX ELISA Result (S/P value [a])	Serological category
Maiden ewes					
3311	2	Perinatal loss	Doubtful (40.71)	Negative (6.24)	Negative
3387	2	Perinatal loss	Doubtful (41.50)	Negative (11.84)	Negative
3548	3	Perinatal loss	Doubtful (40.88)	Negative (5.21)	Negative
2240*	3	Late abortion/ perinatal loss	Doubtful (46.51)	Negative (3.90)	Negative
2804*	3	Late abortion/ perinatal loss	Doubtful (49.93)	Negative (9.41)	Negative
15115	6	Unknown	Doubtful (41.45)	Negative (3.56)	Negative
16381	7	Late abortion/ perinatal loss	Doubtful (45.92)	Negative (3.95)	Negative
16474	8	Raised twins	Doubtful (43.43)	Negative (4.24)	Negative
16476	8	Perinatal loss	Doubtful (42.84)	Negative (5.90)	Negative
16827	8	Raised single	Doubtful (48.99)	Negative (11.74)	Negative
17797	12	Perinatal loss	Doubtful (41.77)	Negative (3.56)	Negative
17870	12	Perinatal loss	Doubtful (46.29)	Negative (3.02)	Negative
11358*	15	Perinatal loss	Doubtful (43.21)	Negative (2.19)	Negative
15681*	15	Perinatal loss	Doubtful (42.55)	Negative (2.68)	Negative
18306	17	Perinatal loss	Doubtful (40.59)	Negative (3.17)	Negative
7302*	20	Perinatal loss	Doubtful (43.95)	Negative (13.60)	Negative
12499*	20	Perinatal loss	Doubtful (45.61)	Weak positive (33.67)	Positive
16178*	20	Perinatal loss	Doubtful (46.48)	Weak positive (70.52)	Positive
17987	21	Perinatal loss	Doubtful (41.26)	Negative (4.58)	Negative
7688*	21	Perinatal loss	Doubtful (43.54)	Negative (8.09)	Negative
18201	22	Perinatal loss	Doubtful (46.08)	Negative (5.95)	Negative
21895	24	Aborted	Doubtful (47.50)	Negative 0.88)	Negative
Mature ewes					
M140	11	Unknown	Doubtful (49.84)	Negative (4.24)	Negative
M549	22	Unknown	Doubtful (46.34)	Negative (2.92)	Negative
M552	22	Unknown	Doubtful (40.45)	Negative (2.58)	Negative
M554	22	Unknown	Doubtful (43.92)	Negative (2.10)	Negative
M555	22	Unknown	Doubtful (47.51)	Negative (4.87)	Negative
M288	24	Unknown	Doubtful (46.71)	Negative (9.84)	Negative
M304	25	Unknown	Doubtful (40.86)	Negative (0.97)	Negative
M308	25	Unknown	Doubtful (46.09)	Negative (1.95)	Negative
M466	26	Unknown	Doubtful (47.23)	Negative (2.92)	Negative
M525	29	Unknown	Doubtful (44.63)	Negative (14.33)	Negative
M534	29	Unknown	Doubtful (45.61)	Negative (12.33)	Negative

[a] S/P value: mean percentage of sample/positive $((OD_{sample} - OD_{negative.control})/(OD_{positive\ control} - OD_{negative.control}))$

* Serial blood sample from timepoint prior to lamb marking tested to determine timing of seroconversion

Table A4.5: Serological status category for subset of 'negative' samples (ID Screen Toxoplasmosis Indirect Multi-species, IDvet) re-tested for anti-*T. gondii* IgG with alternate indirect ELISA (IDEXX Toxotest, IDEXX)

Sample ID	**Flock reference**	**Reproductive outcome**	**ID-VET ELISA** Result	**IDEXX ELISA** Result
3056	1	Perinatal loss	Negative	Negative
3057	1	Perinatal loss	Negative	Negative
3067	1	Perinatal loss	Negative	Negative
3108	1	Perinatal death + ewe death	Negative	Negative
3111	1	Late abortion/ perinatal loss	Negative	Negative
3347	2	Perinatal loss	Negative	Negative
3348	2	Perinatal loss	Negative	Negative
3350	2	Perinatal loss	Negative	Negative
3351	2	Perinatal loss	Negative	Negative
3358	2	Perinatal loss	Negative	Negative
3426	3	Not pregnant at scanning	Negative	Negative
3427	3	Reared lamb	Negative	Negative
3429	3	Abortion	Negative	Negative
3431	3	Perinatal loss	Negative	Negative
3432	3	Suspect perinatal loss	Negative	Negative
2964	4	Perinatal loss	Negative	Negative
2995	4	Perinatal loss	Negative	Negative
2998	4	Perinatal loss	Negative	Negative
3605	4	Perinatal loss	Negative	Negative
3614	4	Perinatal loss	Negative	Negative

Appendix 5: *Neospora caninum*

Table A5.1: Seroprevalence with 95% confidence interval (95% CI) for N. caninum in maiden ewes from Australian farms determined using indirect ELISA.

Farm reference	Location	**Tested** (*n*)			***N. caninum***
		Aborted or FTR[C]	Reared lamb	Total	Seropositive (*n*)
HOGGETS [A]					
1	Kojonup, WA	40	0	40	0
2	Kojonup, WA	40	0	40	0
5	Korunya, SA	40	0	40	0
6	Bagot Well, SA	0	0	40*	0
9	Watervale, SA	30	10	40	0
10	Broomehill, WA	40	0	40	0
12	Tarlee, SA	40	0	40	0
13	Giffard West, VIC	40	0	40	1
15	Katanning, WA	40	0	40	0
26	Culla, VIC	40	0	40	0
29	Ballarat, VIC	30	10	40	0
Seroprevalence % (95% CI)		**380**	**20**	**440**	**0.23 (0.02, 1.06)**
EWE LAMBS [B]					
3	Narrogin, WA	50	48	98	0
4	York, WA	31	9	40	0
7	Kojonup, WA	26	14	40	0
8	Katanning, WA	30	10	40	0
11	Kojonup, WA	35	5	40	0
14	Narrogin, WA	40	0	40	0
16	Ongerup, WA	40	0	40	0
17	Hamilton, VIC	20	20	40	0
18	Hamilton, VIC	39	1	40	0
19	Nareen, VIC	40	0	0	0
20	Cashmore, VIC	40	0	40	0
21	Coojar, VIC	40	0	40	0
22	Mount Gambier, SA	40	0	40	0
23	Kangaroo Island, SA	29	11	40	0
24	Beachport, SA	61	0	61	0
25	Sellicks Hill, SA	40	0	40	0
27	Nareen, VIC	37	3	40	0
28	Inverleigh, VIC	39	1	40	0
30	Strathalbyn, SA	40	0	40	1
Seroprevalence % (95% CI)		**717**	**122**	**839**	**0.12 (0.01, 0.56)**
Overall seroprevalance (% (95% CI))		1097	142	1279	**0.16 (0.03, 0.5)**

[A] Maiden ewes mated at approximately 18 months of age

[B] Maiden ewes mated at approximately 8 months of age

[C] FTR: fail to rear (scanned pregnant with subsequent abortion or perinatal lamb mortality)

WA: Western Australia SA: South Australia VIC: Victoria

*Unknown reproductive status

Table A5.2: Seroprevalence for *N. caninum* for multiparous mature ewes (3 years of age or older) from Australian farms determined using indirect ELISA.

Farm reference	Location	Ewe age category	*N. caninum* serology (*n*)	
			Tested	Seropositive
1	Kojonup, WA	4-7years	20	0
2	Kojonup, WA	5 years	20	0
3 and 14 [a]	Narrogin, WA	5 years	20	0
4	York, WA	5-6 years	20	0
5	Korunya, SA	5+ years	20	0
6	Bagot Well, SA	5+ years	20	0
7	Kojonup, WA	5-7 years	20	0
8	Katanning, WA	4-6 years	20	0
9	Watervale, SA	5+ years	18	0
10	Broomehill, WA	5-10 years	20	0
11	Kojonup, WA	5-6 years	20	0
12	Tarlee, SA	5+ years	20	0
13	Giffard West, VIC	5 years	20	0
15	Katanning, WA	5-7 years	20	0
16	Ongerup, WA	4-7 years	20	0
17	Hamilton, VIC	4-5 years	20	0
18	Hamilton, VIC	6-7 years	20	0
19 and 27 [b]	Nareen, VIC	5-8 years	20	0
20	Cashmore, VIC	4-8 years	20	0
21	Coojar, VIC	4+ years	20	0
22	Mount Gambier, SA	3-9 years	20	0
23	Kangaroo Island, SA	4-7 years	20	0
24	Beachport, SA	5+ years	20	0
25	Sellicks Hill, SA	4-7 years	20	0
26	Culla, VIC	4-9 years	20	0
28	Inverleigh, VIC	3-6 years	20	0
29	Ballarat, VIC	4-9 years	20	0
30	Strathalbyn, SA	5+ years	20	0
Seroprevalance (% (95% confidence interval)			558	**0 (0, 0.45)**

[a] Maiden ewes from this property sampled in 2018 (flock 3) and 2019 (flock 14) – adult ewes sampled in one year only

[b] Maiden ewes from this property sampled in 2019 (flock 19) & 2020 (flock 27) – adult ewes sampled in one year only

WA: Western Australia SA: South Australia VIC: Victoria

Table A5.3: Classification of doubtful samples for N. caninum antibodies by indirect ELISA (ID Screen *Neospora caninum* Indirect, ID Vet, France)

Ewe ID	Farm (state)	Reproductive outcome	Initial test (S/P value)	Re-test (S/P value)	Interpretation
17180	13 (VIC)	Single-bearing ewe - perinatal lamb death	Doubtful (48.59)	Positive (98.64)	Positive
190652	30 (SA)	Single-bearing ewe - perinatal lamb death	Doubtful (42.84)	Positive (64.79)	Positive
20175977	15 (WA)	Single-bearing ewe - perinatal lamb death	Doubtful (41.79)	Negative (27.93)	Negative

WA: Western Australia SA: South Australia VIC: Victoria

S/P value: mean percentage of sample/positive $((OD_{sample} - OD_{negative.control})/(OD_{positive\ control} - OD_{negative.control}))$

Appendix 6: *Coxiella burnetii*

Table A6.1: Seropositivity to *C. burnetii* with 95% confidence interval (95% confidence interval) in maiden ewes from Australian farms determined using indirect ELISA

		Tested (*n*)			***C. burnetii* serology**
Farm reference	**Location**	Aborted or FTR[c]	Reared lamb	Total	Seropositive (*n*)
HOGGETS[a]					
1	Kojonup, WA	40	0	40	0
2	Kojonup, WA	40	0	40	0
5	Korunya, SA	40	0	40	0
6	Bagot Well, SA	-	-	40*	0
9	Watervale, SA	30	10	40	0
10	Broomehill, WA	40	0	40	0
12	Tarlee, SA	40	0	40	1
13	Giffard West, VIC	40	0	40	0
15	Katanning, WA	40	0	40	0
26	Culla, VIC	40	0	40	0
29	Ballarat, VIC	30	10	40	0
Seropositivity % (95% CI)		**380**	**20**	**440**	**0.23 (0.02, 1.06)**
EWE LAMBS[b]					
3 [d]	Narrogin, WA	50	48	98	0
4	York, WA	31	9	40	0
7	Kojonup, WA	26	14	40	0
8	Katanning, WA	30	10	40	0
11	Kojonup, WA	35	5	40	0
14 [d]	Narrogin, WA	40	0	40	0
16	Ongerup, WA	40	0	40	0
17	Hamilton, VIC	20	20	40	0
18	Hamilton, VIC	39	1	40	0
19 [e]	Nareen, VIC	40	0	40	0
20	Cashmore, VIC	40	0	40	0
21	Coojar, VIC	40	0	40	0
22	Mount Gambier, SA	40	0	40	0
23	Kangaroo Island, SA	29	11	40	0
24	Beachport, SA	61	0	61	0
25	Sellicks Hill, SA	40	0	40	0
27 [e]	Nareen, VIC	37	3	40	0
28	Inverleigh, VIC	39	1	40	0
30	Strathalbyn, SA	40	0	40	0
Seropositivity % (95% CI)		**717**	**122**	**839**	**0 (0, 0.30)**
Overall apparent seropositivity % (95% CI)		**1097**	**142**	**1279**	**0.08 (0.01, 0.36)**

WA: Western Australia SA: South Australia VIC: Victoria

*Reproductive outcome unknown

Table A6.2: Seropositivity to *C. burnetii* for multiparous mature ewes (3 years of age or older) from Australian farms determined using indirect ELISA

Flock reference	Location	Ewe age	*C. burnetii* serology (*n*)	
			Tested	Seropositive
1	Kojonup, WA	4-7years	20	0
2	Kojonup, WA	5 years	20	0
3 and 14 [a]	Narrogin, WA	5 years	20	0
4	York, WA	5-6 years	20	0
5	Korunya, SA	5+ years	20	0
6	Bagot Well, SA	5+ years	20	0
7	Kojonup, WA	5-7 years	20	0
8	Katanning, WA	4-6 years	20	1
9	Watervale, SA	5+ years	18	0
10	Broomehill, WA	5-10 years	20	0
11	Kojonup, WA	5-6 years	20	0
12	Tarlee, SA	5+ years	20	0
13	Giffard West, VIC	5 years	20	1
15	Katanning, WA	5-7 years	20	0
16	Ongerup, WA	4-7 years	20	0
17	Hamilton, VIC	4-5 years	20	0
18	Hamilton, VIC	6-7 years	20	0
19 and 27 [b]	Nareen, VIC	5-8 years	20	0
20	Cashmore, VIC	4-8 years	20	0
21	Coojar, VIC	4+ years	20	0
22	Mount Gambier, SA	3-9 years	20	0
23	Kangaroo Island, SA	4-7 years	20	0
24	Beachport, SA	5+ years	20	0
25	Sellicks Hill, SA	4-7 years	20	0
26	Culla, VIC	4-9 years	20	0
28	Inverleigh, VIC	3-6 years	20	0
29	Ballarat, VIC	4-9 years	20	0
30	Strathalbyn, SA	5+ years	20	0
Seropositivity % (95% confidence interval)			558	**0.36 (0.07, 1.14)**

[a] Maiden ewes from this property sampled in 2018 (flock 3) and 2019 (flock 14) – adult ewes sampled in one year only

[b] Maiden ewes from this property sampled in 2019 (flock 19) & 2020 (flock 27) – adult ewes sampled in one year only

WA: Western Australia SA: South Australia VIC: Victoria

Table A6.3: Results and classification of three re-tested samples for *C. burnetii* antibodies by indirect ELISA (ID Screen Q Fever Indirect Multi-species, ID Vet, France) and complement fixation test (CFT).

Ewe ID	Farm (state)	Ewe category	Reproductive outcome	Initial ELISA test (S/P value)	ELISA Re-test (S/P value)	CFT
4689	12 (SA)	Maiden hogget ewe	Single-bearing ewe, perinatal lamb death	Positive (86.97)	Positive (113.45)	Neg @ 1:8
140551	13 (VIC)	Mature ewe (6 years old)	Unknown	Positive (105.51)	Positive (116.07)	Neg @ 1:8
130495	8 (WA)	Mature ewe (6 years old)	Unknown	Doubtful (44.05)	Positive (62.06)	Neg @ 1:8

WA: Western Australia SA: South Australia VIC: Victoria

S/P value: mean percentage of sample/positive [$(OD_{sample} - OD_{negative.control})/(OD_{positive\ control} - OD_{negative.control})$]

Appendix 7: *Campylobacter* spp.

Table A7.1: Flock-level *C. fetus* seropositivity in maiden ewe lambs or hoggets based on reproductive outcome and randomly selected mature ewes on the same farms

Flock reference	Location[a]	***Maiden - abortion, fail to rear*** *n* (%)			***Maiden - reared all lambs*** *n* (%)			***Mature ewes*** *n* (%)			
		Tested	Exposed [b]	Positive [c]	Tested	Exposed [b]	Positive [c]	Age	Tested	Exposed [b]	Positive [c]
EWE LAMBS											
3[d]	Narrogin, WA[e]	20	0 (0)	0 (0)	10	0 (0)	0 (0)	5 years	10[d]	10 (100)[e]	9 (90)[e]
4	York, WA	10	1 (10)	0 (0)	10	1 (10)	0 (0)	5-6 years	10	2 (20)	1 (10)
7[d]	Kojonup, WA	10	0 (0)	0 (0)	10	0 (0)	0 (0)	5-7 years	10	6 (60)	4 (40)
8	Katanning, WA	10	0 (0)	0 (0)	10	0 (0)	0 (0)	4-6 years	10	4 (40)	0 (0)
11[d]	Kojonup WA	10	0 (0)	0 (0)	10	2 (20)	0 (0)	5-6 years	10	3 (30)	0 (0)
14[d]	Narrogin, WA[e]	10	0 (0)	0 (0)	10	0 (0)	0 (0)	5 years	10[d]	10 (100)[e]	9 (90)[e]
16[d]	Ongerup, WA	10	3 (30)	0 (0)	10	2 (20)	0 (0)	4-7 years	10	1 (10)	1 (10)
19[d]	Nareen, VIC	20	20 (100)	17 (85)	10	10 (100)	2 (20)	5-8 years	20	19 (95)	15 (75)
20	Cashmore, VIC	10	10 (100)	6 (60)	10	8 (80)	2 (20)	4-8 years	NA[f]	-	-
23	Kangaroo Island, SA	10	6 (60)	1 (10)	10	5 (50)	4 (40)	4-7 years	10	3 (30)	2 (20)
25	Sellicks Hill, SA	10	8 (80)	2 (20)	10	5 (50)	3 (30)	4-7 years	10	8 (80)	2 (20)
30	Strathalbyn, SA	10	3 (30)	0 (0)	10	0 (0)	0 (0)	5+ years	10	9 (90)	1 (10)
HOGGETS											
1[d]	Kojonup, WA	10	0 (0)	0 (0)	10	1 (10)	0 (0)	4-7years	10	1 (10)	0 (0)
2[d]	Kojonup, WA	10	2 (20)	0 (0)	10	0 (0)	0 (0)	5 years	10	3 (30)	0 (0)
5	Korunye, SA	10	2 (20)	0 (0)	10	1 (10)	0 (0)	5+ years	10	6 (60)	4 (40)
9	Watervale, SA	10	3 (30)	0 (0)	10	0 (0)	0 (0)	5+ years	10	4 (40)	0 (0)
10	Broomehill, WA	10	0 (0)	0 (0)	10	1 (10)	0 (0)	5-10 years	10	1 (10)	0 (0)
12	Tarlee, SA	11	2 (18)	0 (0)	10	3 (30)	1 (10)	5+ years	10	5 (50)	2 (20)
13	Giffard West, VIC	10	6 (60)	0 (0)	10	3 (30)	2 (20)	5 years	10	7 (70)	6 (60)
15	Katanning, WA	11	1 (9)	0 (0)	10	0 (0)	0 (0)	5-7 years	10	3 (30)	0 (0)
26	Culla, VIC	10	9 (90)	8 (8)	10	10 (100)	8 (80)	4-9 years	10	9 (90)	8 (80)
29	Ballarat, VIC	10	2 (20)	1 (10)	10	1 (10)	0 (0)	4-9 years	10	10 (100)	10 (100)

[a] SA: South Australia, VIC : Victoria, WA : Western Australia [b] Exposed = *C. fetus* titre ≥1:10 [c] Positive = *C. fetus* titre ≥1:80

[d] Tissues from aborted or stillborn lambs submitted for *Campylobacter* spp. microbial culture and/or qPCR [e] Same farm – ewe lamb flocks tested in 2 years, mature ewes tested once only

[f] NA: Not available (mature ewes vaccinated for *Campylobacter* spp. therefore not tested)

Table A7.2: Flock-level *Campylobacter jejuni* seropositivity in maiden ewes lambs or hoggets based on reproductive outcome

		Maidens - abortion, fail to rear (*n* (%))			**Maidens - reared all lambs** (*n* (%))		
Farm	**Location**[a]	Tested	Exposed [b]	Positive [c]	Tested	Exposed [b]	Positive [c]
EWE LAMBS							
3	Narrogin, WA	20	20 (100)	20 (100)	10	10 (100)	10 (100)
4	York, WA	10	7 (70)	6 (60)	10	9 (90)	7 (70)
7	Kojonup, WA	10	10 (100)	3 (30)	10	10 (100)	7 (70)
8	Katanning, WA	10	10 (100)	6 (60)	10	8 (80)	6 (60)
11	Kojonup WA	10	10 (100)	0 (0)	10	10 (100)	0 (0)
14	Narrogin, WA	10	10 (100)	8 (80)	10	10 (100)	9 (90)
16	Ongerup, WA	10	10 (100)	4 (40)	10	10 (100)	6 (60)
19	Nareen, VIC	20	16 (80)	0 (0)	10	10 (100)	0 (0)
20	Cashmore, VIC	10	10 (100)	2 (20)	10	10 (100)	6 (60)
23	Kangaroo Island, SA	10	9 (90)	3 (30)	10	10 (100)	5 (50)
25	Sellicks Hill, SA	10	10 (100)	5 (50)	10	10 (100)	4 (40)
30	Strathalbyn, SA	10	19 (95)	1 (10)	10	10 (100)	3 (30)
TOTAL		140	131 (94)	58 (41)	120	117 (97)	63 (52)
HOGGETS							
1	Kojonup, WA	10	10 (100)	10 (100)	10	9 (90)	8 (80)
2	Kojonup, WA	10	10 (100)	10 (100)	10	10 (100)	10 (100)
5	Korunye, SA	10	10 (100)	0 (0)	10	10 (100)	2 (20)
9	Watervale, SA	10	9 (90)	2 (20)	10	10 (100)	2 (20)
10	Broomehill, WA	10	10 (100)	1 (10)	10	10 (100)	4 (40)
12	Tarlee, SA	11	11 (100)	5 (45)	10	9 (90)	3 (30)
13	Giffard West, VIC	10	9 (90)	0 (0)	10	8 (80)	0 (0)
15	Katanning, WA	11	11 (100)	2 (18)	10	10 (100)	6 (60)
26	Culla, VIC	10	9 (90)	5 (50)	10	10 (100)	4 (40)
29	Ballarat, VIC	10	10 (100)	3 (30)	10	10 (100)	6 (60)
TOTAL		102	99 (97)	38 (37)	100	96 (96)	45 (45)
OVERALL		**242**	**230 (95)**	**96 (40)**	**220**	**213 (97)**	**108 (49)**

[a] SA : South Australia VIC : Victoria WA : Western Australia [b] Exposed = *C. fetus* titre ≥1:10 [c] Positive = *C. fetus* titre ≥1:80

Table A7.3: Serial *Campylobacter fetus* titres for maiden ewes in Flock 19

Ewe ID	Mating	Scan1	Scan 2	Pre-lambing	Marking
Mid-pregnancy abortion (scan 1 – scan 2)					
18650	≤1:10	≤1:10	1:320	1:320	1:160
18621	≤1:10	≤1:10	1:160	1:320	1:160
18698	≤1:10	≤1:10	1:160	1:160	1:80
18648	≤1:10	≤1:10	1:80	1:160	1:80
18551	≤1:10	≤1:10	1:80	1:320	1:160
18654	≤1:10	≤1:10	1:40	1:320	1:320
18557	≤1:10	≤1:10	1:40	1:320	1:40
18611	≤1:10	≤1:10	1:20	1:20	1:10
18595	≤1:10	1:40	1:160	1:160	1:160
18538	1:20	1:160	1:320	1:640	1:80
Fail to rear (late abortion or perinatal lamb death)					
18546	<1:10	NT[A]	1:320	NT	1:160
18549	<1:10	NT	1:80	NT	1:80
18548	<1:10	NT	1:80	NT	1:80
18563	<1:10	NT	1:80	NT	1:80
18553	<1:10	NT	1:40	NT	1:80
18643	<1:10	NT	1:40	NT	1:80
18610	<1:10	NT	1:20	NT	1:160
18691	<1:10	NT	<1:10	NT	1:80
18571	1:80	NT	1:320	NT	1:320
18615	1:160	NT	1:80	NT	1:40
Raised lambs					
18628	≤1:10	NT	NT	NT	1:160
18690	≤1:10	NT	NT	NT	1:80
18632	≤1:10	NT	NT	NT	1:40
18703	≤1:10	NT	NT	NT	1:20
18708	≤1:10	NT	NT	NT	1:20
18709	≤1:10	NT	NT	NT	1:20
18663	≤1:10	NT	NT	NT	1:10
18555	1:160	NT	NT	NT	1:40
18642	1:160	NT	NT	NT	1:40
18625	1:160	NT	NT	NT	1:10

[A] NT: not tested

Table A7.4: Odds ratios (OR) for failing to rear a lamb in maiden ewe lambs or hoggets above and below different C. fetus titre cut-offs with 95% confidence interval (95% CI) and two-tailed Fisher's exact test for significance

Flock reference	Location[a]	*C. fetus* titre ≥1:10 [b]			*C. fetus* titre ≥1:80 [b]			*C. fetus* titre ≥1:160 [b]			*C. fetus* titre ≥1:320 [b]		
		OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
EWE LAMBS													
3	Narrogin, WA	-	-	-	-	-	-	-	-	-	-	-	-
4	York, WA	1.00	0.01, 87.05	1.000	-	-	-	-	-	-	-	-	-
7	Kojonup, WA	-	-	-	-	-	-	-	-	-	-	-	-
8	Katanning, WA	-	-	-	-	-	-	-	-	-	-	-	-
11	Kojonup WA	-	-	0.474 [c]	-	-	-	-	-	-	-	-	-
14	Narrogin, WA	-	-	-	-	-	-	-	-	-	-	-	-
16	Ongerup, WA	1.70	0.14, 25.60	1.000	-	-	-	-	-	-	-	-	-
19	Nareen, VIC	-	-	-	19.3	2.42, 278.15	0.001	5.7	0.58, 294.8	0.203	-	-	0.54
20	Cashmore, VIC	-	-	0.474 [c]	5.43	0.60, 79.83	0.170	-	-	0.211 [c]	-	-	1.000 [c]
23	Kangaroo Island, SA	1.47	0.19,12.4	1.000	0.18	0.00, 2.45	0.303	-	-	0.087	-	-	-
25	Sellicks Hill, SA	3.72	0.40,53.81	0.350	0.60	0.04, 6.94	1.000	-	-	-	-	-	-
30	Strathalbyn, SA	-	-	0.211 [c]	-	-	-	-	-	-	-	-	-
HOGGETS													
1	Kojonup, WA	-	-	1.000 [c]	-	-	-	-	-	-	-	-	-
2	Kojonup, WA	-	-	0.474 [c]	-	-	-	-	-	-	-	-	-
5	Korunye, SA	2.16	0.10,147.09	1.000	-	-	-	-	-	-	-	-	-
9	Watervale, SA	-	-	0.211 [c]	-	-	-	-	-	-	-	-	-
10	Broomehill, WA	-	-	1.000 [c]	-	-	-	-	-	-	-	-	-
12	Tarlee, SA	0.54	0.04, 6.09	0.635	-	-	0.476 [c]	-	-	-	-	-	-
13	Giffard West, VIC	3.27	0.41, 33.27	0.370	-	-	0.474 [c]	-	-	-	-	-	-
15	Katanning, WA	-	-	1.000 [c]	-	-	-	-	-	-	-	-	-
26	Culla, VIC	-	-	1.000 [c]	1.00	0.06, 17.08	1.000	2.23	0.27, 21.94	0.65	5.49	0.41, 327.0	0.303
29	Ballarat, VIC	2.16	0.10, 147.1	1.000	-	-	1.000 [c]	-	-	-	-	-	-
OVERALL[d]		**2.01**	**1.09, 3.77**	**0.027**	**1.69**	**0.77, 3.76**	**0.191**	**1.89**	**0.79 ,5.43**	**0.217**	**8.75**	**1.29, 179.07**	**0.058**

[a] SA: South Australia, VIC : Victoria, WA : Western Australia

[b] Odds ratio for failure to rear calculated for ewes with specified *C. fetus* titre compared to ewes with titre below specified threshold [c] Odds ratio not calculated due to empty cell

[d] Overall odds ratio calculated using logistic regression (flock included as fixed effect)

Table A7.5: Within-flock comparison for *Campylobacter fetus* seroprevalence in maiden ewes that failed to rear (FTR) and ewes that reared lambs (rear) with two-way Pearson Chi-square test

Flock reference	Location[a]	Exposed (titre ≥1:10)			Positive (titre ≥1:80)		
		FTR *n* (%)	Rear *n* (%)	P-value	FTR *n* (%)	Rear *n* (%)	P-value
EWE LAMBS							
3[b]	Narrogin, WA	0 (0)	0 (0)	NA	0 (0)	0 (0)	NA[e]
4	York, WA	1 (10)	1 (10)	1	0 (0)	0 (0)	NA
7[b]	Kojonup, WA	0 (0)	0 (0)	NA	0 (0)	0 (0)	NA
8	Katanning, WA	0 (0)	0 (0)	NA	0 (0)	0 (0)	NA
11	Kojonup WA	0 (0)	2 (20)	0.136	0 (0)	0 (0)	NA
14[b]	Narrogin, WA	0 (0)	0 (0)	NA	0 (0)	0 (0)	NA
16[b]	Ongerup, WA	3 (30)	2 (20)	0.6056	0 (0)	0 (0)	NA
19[b]	Nareen, VIC	20 (100)	10 (100)	NA	17 (85)	2 (20)	**0.0005**
20	Cashmore, VIC	10 (100)	8 (80)	0.136	6 (60)	2 (20)	0.0679
23	Kangaroo Island, SA	6 (60)	5 (50)	0.6531	1 (10)	4 (40)	0.1213
25	Sellicks Hill, SA	8 (80)	5 (50)	0.1596	2 (20)	3 (30)	0.6056
30	Strathalbyn, SA	3 (30)	0 (0)	0.0603	0 (0)	0 (0)	NA
TOTAL		51 (36)	33 (28)	0.126	26 (19)	11 (9)	**0.031**
HOGGETS							
1[b]	Kojonup, WA	0 (0)	1 (10)	0.3049	0 (0)	0 (0)	NA
2[b]	Kojonup, WA	2 (20)	0 (0)	0.136	0 (0)	0 (0)	NA
5	Korunye, SA	2 (20)	1 (10)	0.5312	0 (0)	0 (0)	NA
9	Watervale, SA	3 (30)	0 (0)	0.0603	0 (0)	0 (0)	NA
10	Broomehill, WA	0 (0)	1 (10)	0.3049	0 (0)	0 (0)	NA
12	Tarlee, SA	2 (18)	3 (30)	0.5185	0 (0)	1 (10)	0.3049
13	Giffard West, VIC	6 (60)	3 (30)	0.1775	0 (0)	2 (20)	0.136
15	Katanning, WA	1 (9)	0 (0)	0.3311	0 (0)	0 (0)	NA
26	Culla, VIC	9 (90)	10 (100)	0.3049	8 (80)	8 (80)	1.000
29	Ballarat, VIC	2 (20)	1 (10)	0.5312	1 (10)	0 (0)	0.3049
TOTAL		27 (28)	20 (20)	0.241	9 (9)	11 (11)	0.600
OVERALL		**78 (32)**	**53 (21)**	**0.054**	**35 (15)**	**22 (10)**	**0.142**

[a] SA : South Australia VIC : Victoria WA : Western Australia [b] Tissues from aborted or stillborn lambs submitted for *Campylobacter* spp. microbial culture and/or qPCR

Table A7.6: Odds ratios (OR) for failing to rear a lamb in maiden ewe lambs or hoggets above and below different *C. jejuni* titre cut-off with 95% confidence interval (95% CI) and two-tailed Fisher's exact test for significance

Flock reference	Location[a]	*C. jejuni* titre ≥1:10 [b]			*C. jejuni* titre ≥1:80 [b]			*C. jejuni* titre ≥1:160 [b]			*C. jejuni* titre ≥1:320 [b]		
		OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
EWE LAMBS													
3	Narrogin, WA	-	-	-	-	-	-	0.34	0.01, 3.85	0.633	-	-	-
4	York, WA	0.28	0.00, 4.35	0.582	0.66	0.08, 5.78	1.000	0.46	0.05, 3.61	0.656	-	-	1.000 [c]
7	Kojonup, WA	-	-	-	0.20	0.02, 1.68	0.179	-	-	-	-	-	-
8	Katanning, WA	-	-	0.474[c]	1.00	0.11, 8.42	1.000	-	-	-	-	-	-
11	Kojonup WA	-	-	-	-	-	-	-	-	-	-	-	-
14	Narrogin, WA	-	-	-	0.46	0.01, 10.51	1.000	-	-	1.000 [c]	-	-	-
16	Ongerup, WA	-	-	-	0.46	0.05, 3.61	0.656	-	-	-	-	-	-
19	Nareen, VIC	-	-	0.272 [c]	-	-	-	-	-	-	-	-	-
20	Cashmore, VIC	-	-	-	0.18	0.01, 1.66	0.170	-	-	1.000 [c]	-	-	-
23	Kangaroo Island, SA	-	-	1.000 [c]	0.45	0.05, 3.67	0.650	-	-	-	-	-	-
25	Sellicks Hill, SA	-	-	-	1.47	0.19, 12.40	1.000	-	-	0.474 [c]	-	-	1.000 [c]
30	Strathalbyn, SA	-	-	-	0.28	0.00, 4.35	0.582	-	-	-	-	-	-
HOGGETS													
1	Kojonup, WA	-	-	1.000 [c]	-	-	0.474 [c]	1.00	0.10, 10.33	1.000	-	-	-
2	Kojonup, WA	-	-	-	-	-	-	0.27	0.02, 2.47	0.350	-	-	-
5	Korunye, SA	-	-	-	-	-	0.474 [c]	-	-	-	-	-	-
9	Watervale, SA	-	-	1.000 [c]	1.00	0.06, 17.8	1.000	-	-	-	-	-	-
10	Broomehill, WA	-	-	-	0.18	0.00, 2.45	0.303	-	-	-	-	-	-
12	Tarlee, SA	-	-	0.476 [c]	1.83	0.23, 17.7	0.659	-	-	0.476 [c]	-	-	-
13	Giffard West, VIC	2.16	0.10,147.1	1.000	-	-	-	-	-	-	-	-	-
15	Katanning, WA	-	-	-	0.16	0.01, 1.45	0.080	-	-	-	-	-	-
26	Culla, VIC	-	-	1.000 [c]	1.47	0.19, 12.40	1.000	-	-	1.000 [c]	-	-	-
29	Ballarat, VIC	-	-	-	0.30	0.00, 2.46	0.370	-	-	1.000 [c]	-	-	1.000 [c]
OVERALL[d]		**0.71**	**0.24, 1.93**	**0.506**	**0.52**	**0.32, 0.83**	**0.007**	**0.51**	**0.22, 1.14**	**0.108**	**0.48**	**0.02, 5.31**	**0.561**

[a] SA: South Australia, VIC : Victoria, WA : Western Australia

[b] Odds ratio for failure to rear calculated for ewes with specified *C. fetus* titre compared to ewes with titre below specified threshold [c] Odds ratio not calculated due to empty cell

[d] Overall odds ratio calculated using logistic regression (flock included as fixed effect)