The Effect of Vaccination Against *Campylobacter* on Maiden Ewe Reproduction in Victoria

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Abstract

Reducing reproductive wastage is important for the Australian sheep industry. *Campylobacter fetus fetus* and *C. jejuni* infections in ewes contribute to reproductive wastage through abortions, stillbirths and the birth of small, weak neonates, potentially at greater risk of starvation-mismothering-exposure (SME).

A combined vaccine against *C. fetus fetus* and *C. jejuni* (Ovilis Campyvax®, MSD Animal Health) is registered in Australia to reduce reproductive wastage due to *Campylobacter*, but few independent field trials of the vaccine have been conducted in commercial flocks. This study described the effects of Ovilis Campyvax® on maiden ewe reproduction in a randomised controlled field trial on four winter-/spring-lambing Victorian sheep farms.

Conception and lamb marking rates were compared amongst nineteen-month-old Merino and Merino-cross ewes randomly allocated to vaccination or control groups at mating on each farm (each n = 211–249/group). Ewes were grazed together from mating until immediately before lambing, when they were set-stocked in treatment groups in matched paddocks. Antibody titres to *Campylobacter* spp. were measured at mating, mid-gestation pregnancy diagnosis and lamb marking in a subset of ewes. A cross-sectional study of cause of neonatal lamb mortality was also conducted on each farm during lambing.

Vaccination had no effect on ewe conception rate (67% to 117% depending on farm). Two of four farms had serological evidence of prior exposure to *C. fetus fetus*, and variable exposure to this organism occurred during gestation on all farms. *Campylobacter jejuni* titres were high on all farms at mating, but decreased thereafter. Despite serological evidence of a good response to *C. fetus fetus* vaccination on all farms, vaccination did not significantly increase lamb marking rates (63% to 100%, depending on farm). The main causes of lamb mortality were dystocia, starvation-mismothering-exposure and predation. There was a suggestion of a difference in the pattern of causes of neonatal lamb mortality between vaccinated and control ewes. The difference was not statistically significant, but corresponded with anecdotal observations made by the flock owners. Additional large scale studies into vaccination and the causes of neonatal lamb mortality are needed to further investigate these observations.

Vaccination appeared to prevent *Campylobacter*-associated neonatal lamb mortality and morbidity on the farm with the greatest exposure to *C. fetus fetus*. On that farm, 55% of

unvaccinated ewes that failed to rear a lamb had 'high' (≥ 1:80) *C. fetus fetus* titres, compared to 0% of ewes that successfully reared a lamb. Additionally, *C. fetus fetus* was only recovered from necropsied lambs born to unvaccinated ewes.

The results demonstrate that ewes can be vaccinated with Ovilis Campyvax® during mating without impacting conception rates. However, the effect of *Campylobacter* vaccination on reproductive output is complex and multifactorial. Vaccination effects may be obscured by other causes of reproductive loss. Vaccination may reduce the contribution of *Campylobacter* infections to lamb loss due to SME. However, the dystocia risk in protected ewes may increase depending on ewe nutrition. If this is the case, the nutrition of vaccinated ewes could be managed more economically to obtain the full benefits of vaccination. This is an avenue for future research.

Declaration

I declare that this is my original work towards the Masters of Veterinary Science, and that it complies with the University of Melbourne requirement, being fewer than 30,000 words in length, exclusive of all tables, figures, references and appendices.

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

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

I declare that this work was conducted independently at the Mackinnon Project, University of Melbourne. It was partly funded by Meat and Livestock Australia (MLA) Donor Company, the Scobie and Claire Mackinnon Trust and Coopers, MSD Animal Health Australia.

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

Elsa Jane Glanville

December 2017

“Where are we going to live,
Little Boy Blue?
Where are we going to live
Boy Blue?”
“Little Bo-Peep, Bo-Peep,
Up in the hills with the sheep”

A.A. Milne 1924

When we were very young

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1 – Literature Review

1.1 Introduction: reproductive wastage in the Australian sheep industry

Reproductive wastage is arguably the costliest disease syndrome faced by the Australian sheep industry, with neonatal lamb mortality alone estimated to cost $541 million dollars in 2015 (Young et al., 2014; Lane et al., 2015). There is considerable annual, between- and within-farm variability in the degree of reproductive wastage. However, on Australian farms, a 20-40% discrepancy between the number of lambs expected based on mid-gestation pregnancy diagnosis, and the number of lambs counted at 'lamb marking' is common (Kilgour, 1992; Fowler, 2007; Hinch and Brien, 2014; Jacobs, 2015). The discrepancy may be even greater when many multiple pregnancies are detected (Kilgour, 1992; Reed et al., 2006; Fowler, 2007; Hinch and Brien, 2014). This discrepancy is a source of great frustration for sheep producers and represents a considerable economic and welfare cost for the industry and the individual producer.

Most ovine reproductive wastage occurs within the perinatal period, extending from late-gestation through the first weeks of life (Dennis, 1972; Alexander, 1984; Hinch et al., 1986; Kleemann and Walker, 2005b). A 'normal' level of abortion of 1-2% may occur in sheep flocks without investigation (Jonker, 2004; West et al., 2009). Outbreaks of abortion, where up to 70% of ewes may abort, are less common. These may be due to infectious agents, although the cause remains undiagnosed in 18-49% of cases (Farquharson, 2003a; Jonker, 2004; West et al., 2009). However, most reproductive wastage occurs in the first 48-72 hours of life, and is referred to as neonatal lamb mortality. In Australia, the most common causes of neonatal lamb mortality are starvation, mismothering, exposure, and dystocia (Alexander, 1984; Hinch and Brien, 2014; Refshauge et al., 2016; Suter, 2016). These causes are often inter-related (Alexander, 1984). For example, low birth-weight, mismothering and/or birth trauma predispose lambs to exposure (Hinch et al., 1986; Hinch and Brien, 2014). Hence starvation, mismothering and exposure are often grouped together as a complex ('SME'). Less common causes of perinatal loss include predation, congenital malformations, and mineral deficiencies (Alexander, 1984; Broadmeadow et al., 1984; Alexander et al., 1990).

Perinatal lamb mortality in Australia has been researched intensively over the past 70 years, with the aim of describing the factors that affect lamb survival (Alexander, 1984; Kelly, 1992a; Kleemann and Walker, 2005b; Hatcher et al., 2009; Oldham et al., 2011; Hinch and Brien, 2014). Ewe live-weight and condition score (CS), breed and parity are critically important (Fogarty, 1972; Alexander et al., 1993; Hocking-Edwards et al., 2011; Hinch and Brien, 2014). For example, lamb survival may increase by 5% for singles and 20% for twins if ewes are in CS 3.0 compared to 2.0 at lambing, and the survival of both ewe and lamb is compromised when ewe CS is less than 2.0 (Ferguson et al., 2007; Oldham et al., 2011; Hinch and Brien, 2014). Important lamb factors influencing survival include birth-weight and litter size. The optimum birth-weight for survival is between 4.5 and 5.5 kg, with multiple born lambs of equal weight having a lower probability of survival (Hinch et al., 1985a; Hinch et al., 1985b; Kelly, 1992a; Holst et al., 2002). These ewe and lamb factors are influenced by management before and during lambing, including feed budget accuracy, paddock selection, mob size, and stocking rate (Alexander, 1984; Greentree et al., 2000; Anon, 2008). Environmental factors including chill index, availability of shelter, predator activity and the quality and quantity of pasture also affect lamb survival (Alexander et al., 1980; Donnelly, 1984; Hinch and Brien, 2014). Guidelines based on this research, and extension programs such as *Lifetime Ewe Management,* exist to help producers manage ewes through the reproductive cycle to improve lamb survival (Ferguson et al., 2007; Jones et al., 2011; Trompf et al., 2011). Following participation in *Lifetime Ewe Management,* producers report improvements in flock reproductive output (Trompf et al., 2011). However, despite the adoption of practices to minimise reproductive wastage, perinatal lamb mortality can remain frustratingly high (J. Webb-Ware, personal communication, August 2017).

Whilst the aforementioned factors are indisputably important determinants of perinatal lamb survival, infectious agents also contribute to reproductive wastage (Rahaley, 1984). Bacterial, viral or protozoal infection of the pregnant ewe can cause placentitis and/or foetal infection resulting in foetal death, and consequently abortion or stillbirth (West et al., 2009). Additionally, sub-lethal infection can result in placental insufficiency with reduced gas and nutrient exchange to the foetus, resulting in a low birth-weight neonate with low vigour and an increased risk of death (Kelly, 1992b).

The contribution of infectious agents to reproductive wastage is most obvious during abortion outbreaks. These are highly visible and distressing events for producers, making sample collection and investigation more likely. However, infection of the pregnant ewe

with these same agents can also contribute more subtly to reproductive wastage (Clough, 2003).

The role of infectious agents in reproductive wastage in the Australian sheep industry may be underestimated for three main reasons:

1) Difficulties in detecting infections in production systems in which the ewe is not continuously monitored (Menzies, 2011). Opportunities for disease observation are limited in the grazing systems in which most Victorian ewes are managed. Ewes may only be observed once a week during the last trimester of pregnancy
2) Poor sample retrieval. Ideally, both the placenta and foetus or neonate would be submitted for investigation (Menzies, 2011; Hovers et al., 2014). Depending on the pathogen, the likelihood of a diagnosis is increased if this is not delayed (Monke et al., 2002; Markey et al., 2013). However, finding and submitting fresh samples in a timely manner is difficult on large and remote enterprises
3) Neonatal lamb mortality complicated by infection may be attributed to the more commonly recognised causes of death, and not investigated further. For example, an infected ewe may give birth to low birth-weight and low-vigour lambs with increased susceptibility to 'SME' (Hinch et al., 1985a; Dwyer et al., 2003; Hinch and Brien, 2014). Additionally, foetal distress may result in the birth of meconium-stained stillborn or moribund lambs. These deaths may be mistakenly attributed to dystocia, as a malpresented lamb or the second of an obstructed set of twins may look similar. Thus, in the absence of a detailed investigation into the causes of neonatal lamb mortality on individual farms, the contribution of infectious agents to neonatal lamb mortality may go unrecognised

In Victoria, Australia, the most commonly diagnosed infectious agents of perinatal loss are *Campylobacter* spp.*, Listeria* spp. and *Toxoplasma gondii* (Gorrie, 1962; Hore et al., 1973; Broadbent, 1975; Suter, 2014). Less commonly diagnosed abortigenic pathogens include Border Disease Virus, *Yersinia* spp., *Coxiella burnetti* and *Samonella* spp (Farquharson, 2003a; Suter, 2014). Internationally significant abortigenic agents including *S.* Brandenburg, *Chlamydophila abortus* and *Brucella melitensis* remain exotic to Australia (Farquharson, 2003a). Historical surveys of perinatal lamb mortality in Australia found infectious agents to be responsible for between 2 and 16 percent of deaths investigated (Hughes et al., 1971; Hore et al., 1973; Broadbent, 1975). The Australian sheep industry has changed substantially since these reports in ways which could increase

both the likelihood of detection, and the risk of contact with infectious agents. For example, the adoption of ultrasound pregnancy diagnosis, meaning more producers understand the magnitude of foetal and neonatal loss (Kilgour, 1992; Fowler, 2007; Jacobs, 2015), and the change in the demographic of the Australian ewe flock. The number of self-replacing Merino ewe flocks has decreased, and the number of meat producing flocks has increased, potentially increasing the opportunity for disease introduction to naïve properties through increased sheep movements (Rowe, 2010).

Campylobacter contributes to reproductive wastage in most sheep producing regions globally, through both outbreaks of abortions and more subtle losses (West, 2002, 2003; Sahin et al., 2017). The latter, more insidious role of *Campylobacter* may be as important as its role in abortion outbreaks (Anderson, 2001; Clough, 2003). For example, in New Zealand, *Campylobacter* spp. are responsible for 6% to 10% of perinatal lamb mortality annually in endemic flocks (Anderson, 2001; West, 2003). A serological survey from 2006-2009 demonstrated considerable, widespread exposure to *Campylobacter fetus fetus* across the New Zealand ewe flock, with only 11% of 298 flocks tested completely seronegative (Dempster et al., 2011). In these circumstances, protection of ewes against the responsible *Campylobacter* spp. by vaccination has been shown to improve foetal and lamb survival relative to unprotected mobs, even in the absence of abortion outbreaks (West, 2003).

It is not known whether *Campylobacter* spp. contribute to a similar level of neonatal lamb mortality in Victorian sheep flocks (Clough, 2003). If it does, the significance of *Campylobacter* for the industry would be greater than previously believed (Clough, 2003; Lane et al., 2015). This has consequences for both the economic cost of disease and the potential to improve lamb survival, a key target for the Australian sheep industry, through vaccination of the ewe (Young et al., 2014). Thus, this thesis examines the role of *Campylobacter* spp. in reproductive wastage on sheep farms in Victoria, Australia and describes a randomised controlled field trial of a commercially available vaccine against both species of *Campylobacter* associated with ovine reproductive loss.

1.2 An overview of the Genus *Campylobacter*

Campylobacter are small, non-spore forming, gram-negative curved- or rod-shaped bacteria of the family Campylobacteraceae (Skirrow, 1994; Markey et al., 2013). Currently, there are 26 species, two provisional species and nine subspecies within this

genus, many of which naturally colonise mammals, birds and fish (Man, 2011; Kaakoush et al., 2015). Some species are particularly associated with disease. In humans, this is most commonly disease of the gastrointestinal tract (GIT) and less commonly extra-intestinal disease (Janssen et al., 2008). In production animals, this is most commonly disease of the reproductive tract (Skirrow, 1994). Infection induces innate, cell-mediated and humoral immune responses that confer some level of immunity to subsequent disease (Janssen et al., 2008; Nietfeld, 2013). However, the level of protection offered and the relative contribution of each mechanism toward immunity against subsequent re-infection is unclear (Janssen et al., 2008).

The most significant pathogenic *Campylobacter* taxa in production animals are *C. fetus* and *C. jejuni* (Table 1; Nietfeld, 2013). Other *Campylobacter* species are recovered sporadically from production animals under a range of disease conditions but their causative effect is often not well established (Sahin et al., 2017).

The interplay between the virulence of the bacteria and host susceptibility influences whether infection with *Campylobacter* spp. results in disease, and the severity of that disease (Janssen et al., 2008). In terms of bacterial virulence factors, the structure of the flagellin and the polysaccharide capsule of *Campylobacter* enable it to avoid inducing innate immunity in the intestine (Janssen et al., 2008; Nietfeld, 2013). However, some *Campylobacter* species are highly susceptible to complement-mediated killing in host serum (Blaser et al., 1985). Members of the genus with these traits can thus evade detection and potentially cause disease within the GIT, but will be inactivated if they pass into circulation when translocated across the GIT mucosa (Nietfeld, 2013). The susceptibility to serum inactivation varies both within and between *Campylobacter* species, such that there are strains of some species that are serum resistant and capable of systemic infection and localisation outside the intestines (Nietfeld, 2013). For example, strains of *C. jejuni* isolated from the GIT are often serum-sensitive, whereas isolates from extra-intestinal sites are often resistant to the killing effects of serum (Nietfeld, 2013).

The contrast between the traits possessed by the two species of greatest importance for production animals, *C. jejuni* subsp. *jejuni* (subsequently referred to as *C. jejuni*) and *C. fetus,* provides an important example of the role of virulence factors in disease. *Campylobacter jejuni* possesses neither the long, repeating polysaccharide side chains (or 'O-antigens') in their cell wall nor the microcapsule surface layer proteins ('SLPs') possessed by *C. fetus* (Nietfeld, 2013). Long 'O-antigens' confer resistance to

complement-mediated killing in serum, whilst the SLPs resist both phagocytosis and the bactericidal effects of serum (Blaser et al., 1987; Blaser et al., 1988; Nietfeld, 2013). Additionally, antigenic variation in SLPs protects against antibody-mediated killing (Dubreuil et al., 1990; Thompson, 2002). These traits are essential for systemic infection and, in their absence, the reproductive disease associated with *C. fetus* infection does not occur (Grogono-Thomas et al., 2000). They may explain why some species and strains of *Campylobacter* are more capable of causing extra-gastrointestinal disease than others.

Table 1 Species of *Campylobacter* found within ruminants that are of veterinary and/or zoonotic importance (adapted from Skirrow, 1994; Nietfeld, 2013)

***Campylobacter* species**	**Host animal**	**Disease in host**	**Zoonotic disease**
Campylobacter fetus subsp. *fetus*	Ovine (sporadically caprine & bovine)	Reproductive loss: abortion & neonatal mortality	Bacteraemia, gastrointestinal disease, reproductive loss
C. fetus subsp. *venerealis*	Bovine	Infertility and reproductive loss: early embryonic loss, sporadic abortion	Not reported
C jejuni subsp. *jejuni*	Poultry, ruminants, dogs, cats, humans	Reproductive loss: abortion & neonatal mortality; GIT disease; or asymptomatic	Gastrointestinal disease, bacteraemia & subsequent extra-intestinal disease
C. coli	Pigs, poultry, sheep	Asymptomatic; abortion	Gastrointestinal disease, bacteraemia & subsequent extra-intestinal disease
C. hyointestinalis	Ruminants, pigs, poultry, pets, birds	None reported	Gastrointestinal disease (rare)
C. sputorum	Ruminants	None reported	Gastrointestinal disease

1.2.1 Disease-causing *Campylobacter* spp. in ruminants

Campylobacter fetus, previous classified in the genus *Vibrio*, is arguably the *Campylobacter* species of greatest importance for ruminant production systems (Skirrow, 1994; Sahin et al., 2017). The two subspecies, *C. fetus* subsp. *fetus* (henceforth referred to as *C. fetus fetus*) and *C. fetus* subsp. *venerealis*, both cause disease of the reproductive tract and are genetically similar but differ in primary host, epidemiology and clinical presentation (Sahin et al., 2017). *Campylobacter jejuni* also causes reproductive disease in sheep, and has been associated with GIT disease in sheep and cattle (Skirrow, 1994).

Campylobacter fetus fetus is recognised internationally as a significant cause of third-trimester abortion outbreaks in sheep flocks, as well as stillbirths and the birth of weak lambs (Sahin et al., 2017). It is also responsible for sporadic abortions in cattle, goats and camelids (Skirrow, 1994). No enduring infertility is documented following exposure and reproductive loss in sheep, in contrast to *C. fetus* subsp. *venerealis* in cattle (Skirrow, 1994). *Campylobacter fetus* subsp. *venerealis* is host-adapted to bovines and is responsible for infertility, early embryonic death and sporadic abortions in infected animals (Sahin et al., 2017). Carrier bulls are persistently infected with the bacteria, which reside in the preputial crypts of carriers (Skirrow, 1994). In contrast, *C. fetus fetus* does not persist within the reproductive tract of sheep (Jensen et al., 1957). Instead, the intestines and gall bladder are proposed to be the reservoir site for carrier ewes (Firehammer et al., 1962; Clark and Monsbourgh, 1979).

Campylobacter jejuni also causes reproductive wastage in sheep, including abortions and neonatal lamb mortality. *Campylobacter jejuni* is a common commensal organism of the gastrointestinal tract of many animal species (Skirrow, 1994). Infection of young ruminants may be accompanied by self-limiting diarrhoea, but is asymptomatic in most mature animals (Nietfeld, 2013). However, in some sheep production systems, including throughout the United States of America (USA) and in Tasmania, Australia, *C. jejuni* is more commonly diagnosed than *C. fetus fetus* in cases of reproductive loss (Diker and Istanbulluoglu, 1986; Varga et al., 1990; Elliot, 2001; Sanad et al., 2014). In the USA, a single *C. jejuni* strain adept at causing systemic infection and abortion has evolved, and is known as 'clone SA' for sheep abortion (Sahin et al., 2008; Burrough et al., 2009).

On sheep farms in Victoria, Australia, *Campylobacter* is one of the most commonly diagnosed infectious agents of perinatal mortality (Clough, 2003; Suter, 2014)}. Both *C.*

fetus fetus and *C. jejuni* have been diagnosed from cases of abortion outbreaks and investigations of neonatal lamb mortality (Clough, 2003). Thus, this thesis will focus on the role of both species on Victorian sheep farms.

1.2.2 *Campylobacter* as a zoonosis

Campylobacter spp. are a major cause of foodborne bacterial enteritis around the world (Altekruse et al., 1999). The incubation following exposure is one to seven days, and disease usually presents as self-limiting diarrhoea, fever and cramps (Altekruse et al., 1999; Man, 2011). The most common species associated with gastrointestinal disease in humans are *C. jejuni* and *C. coli.* In Australia, campylobacteriosis is the most commonly notified foodborne enteric infection with 124.9 cases per 100,000 population in 2014 (Annual Report Working Group, 2016). These figures are probably an underestimate of the frequency of human campylobacteriosis in Australia, as the disease is not notifiable in the most populous state (New South Wales), and many cases are not confirmed because the disease is usually self-limiting (Annual Report Working Group, 2016).

Human *Campylobacter* infection occurs via the oral route. The major risk factors for human infection are international travel and the consumption of undercooked poultry (Domingues et al., 2012). Travel-related infections often occur following the consumption of contaminated poultry, red meat or water (Mughini-Gras et al., 2013). Environmental exposure and direct contact with farm animals are also major risk factors for infection, highlighting the important role of non-foodborne sources of *Campylobacter* including livestock (Domingues et al., 2012; Mughini Gras et al., 2012).

Host immunocompetence is a significant contributor to the risk of disease and the severity of disease in humans following exposure to *Campylobacter.* Disease is usually restricted to the intestine in immunocompetent individuals (Janssen et al., 2008), although occasionally the consequences extend beyond enteritis (Kaakoush et al., 2015). Other gastrointestinal diseases associated with *Campylobacter* infection include irritable bowel diseases and irritable bowel syndrome (Spiller, 2007; Gradel et al., 2009; Marshall et al., 2010; Schwille-Kiuntke et al., 2011). Extra-gastrointestinal manifestations include potentially life-threatening septicaemia and autoimmune conditions such as Guillain Barre Syndrome (Nachamkin et al., 2000; Man, 2011; Kaakoush et al., 2015).

Human infection with *C. fetus* is less common than with *C. jejuni* or *C. coli*, but when it does occur, it is more often due to infection with *C. fetus fetus* than *C. fetus* subsp. *venerealis* (Wagenaar et al., 2014). Importantly, infection is more likely to be associated with bacteraemia and extra-gastrointestinal disease than infection with either of the more common zoonotic species (Guerrant et al., 1978; Fernández-Cruz et al., 2010). Infection of pregnant women with *C. fetus fetus* is associated with abortion and perinatal sepsis following maternal and foetal septicaemia (Hood and Todd, 1960; Simor et al., 1986). Pregnant and immunocompromised individuals are thus advised to avoid handling potentially high-risk livestock, such as aborting ewes and birth products, and to wear effective personal protective equipment if assisting lambing ewes.

Whilst sheep are not the major source of human campylobacteriosis, they are carriers and shedders of the *Campylobacter* species responsible for human campylobacteriosis, and contribute to the environmental reservoir of *Campylobacter* (Stanley and Jones, 2003). Additionally, live animals and carcasses can be directly infective to humans, and contact with livestock has been identified as one of the risk factors for human campylobacteriosis (Mughini Gras et al., 2012). Therefore, ovine campylobacteriosis can both directly and indirectly, via its impact on farm productivity, impact human health and wellbeing.

1.3 Ovine campylobacteriosis

Two main disease presentations are associated with *Campylobacter* infection in sheep, enteritis and reproductive loss. As discussed in Section 1.2.1, *C. jejuni* is a common commensal organism within the ruminant intestinal tract (Nietfeld, 2013). Although less common, *C. fetus fetus* is also a commensal organism of the intestine and gall bladder (Stanley and Jones, 2003). Despite these species being common on many sheep farms, most infected flocks do not experience overt disease (Stanley et al., 1998; Jones et al., 1999; Walsh, 2016; Sahin et al., 2017). This may be because exposure does not coincide with a time where there is a risk of reproductive loss, or the risk factors described below are not present.

1.3.1 Ovine campylobacteriosis: enteritis

Campylobacter jejuni has been implicated as the aetiological agent in cases of ovine enteritis (McOrist, 1985; McOrist et al., 1987). However, firmly establishing the role of *Campylobacter* spp. in ovine enteritis is complicated by the fact that *C. jejuni* has been

isolated from the faeces of sheep both with and without clinical signs of enteric disease (Skirrow, 1994; Yang et al., 2014; Yang et al., 2017). A survey of the prevalence of *Campylobacter* from sheep sent to slaughter in Scotland revealed a high prevalence of predominantly *C. jejuni* in samples cultured from faeces (64%), fleece (95%) and carcasses (90%; Garcia et al., 2010). A longitudinal study of the prevalence of *Campylobacter* shed in the faeces of Australian sheep found a lower overall prevalence of 13.3% samples positive for *C. jejuni,* by quantitative polymerase chain reaction (Yang et al., 2014). The intestinal carriage rate of *Campylobacter* is higher than the faecal shedding rate, and the latter varies with season, stress and life stage (Jones et al., 1999). Jones et al. (1999) reported considerable annual variability in the shedding of *C. jejuni* in sheep faeces in the United Kingdom, which was related to management practices. Significant results included increased shedding at lambing and weaning, and following a change in pasture (Jones et al., 1999).

Outbreaks of ovine *Campylobacter* enteritis are less common than the prevalence of shedding might suggest, and are often associated with challenging environmental or management factors. For example, outbreaks of enteritis attributed to *Campylobacter* spp. in lambs and 5-6 month old weaners have occurred in high-stress environments featuring close confinement, nutritional stress and supplementary feeding (McOrist, 1985; Glastonbury, 1990; Farquharson, 2003b). A seasonal trend in disease presentation has been reported in Australia, with disease more common when young stock are grazing short pastures over cold, wet winters, and when young sheep are grazing short dry pastures in summer (Farquharson, 2003b).

1.3.2 Ovine campylobacteriosis: reproductive loss

Compared to the ill-defined role of *Campylobacter* in enteritis, its role in ovine reproductive loss is well described (Kirkbride, 1985; Peel and Mason, 1993; Hovers et al., 2014; Sahin et al., 2017). *Campylobacter* was first identified as the causal agent of ovine abortions when isolated from a sheep foetus in 1906 (McFadyean and Stockman, 1913; Skirrow, 1994). Globally, ovine campylobacteriosis resulting in reproductive loss most commonly occurs following infection of a pregnant ewe with *C. fetus fetus* (Sahin et al., 2017). However, *C. jejuni* is also an important agent of reproductive loss (Sahin et al., 2008; Wu et al., 2014b). This is especially the case in the USA, where *C. jejuni* has replaced *C. fetus fetus* as the predominant agent of ovine abortion (Menzies, 2011; Sahin et al., 2017). Despite genetic differences between the two species, the pathogenesis,

pathology and epidemiology of reproductive loss associated with *C. jejuni* and *C. fetus fetus* infections appears to be similar (Sahin et al., 2017).

1.3.2.1 Pathogenesis and pathology of infection

In brief, oral ingestion of either *C. fetus fetus* or *C. jejuni* by a naïve, pregnant ewe results in a bacteraemia that localises in the placenta causing placentitis and potentially foetal infection (Figure 1). Foetal death and abortion typically occur 2 to 3 weeks after infection, in the third trimester. Abortion outbreaks may occur, where 5% to 50% of ewes abort (Sahin et al., 2017). Exposure later in gestation may result in stillbirth or weak lambs (Skirrow, 1994). The ewe is often clinically normal, but may have a uterine discharge and reduced milk supply. Humoral immunity is stimulated by infection, and provides some protection against subsequent reproductive loss. Some ewes become carriers following infection, and shedding from these ewes can expose naïve ewes (Figure 1).

However, there is still much to be understood concerning the finer details of the pathogenesis of reproductive loss associated with *Campylobacter* infection (Sahin et al., 2017). What triggers an otherwise commensal organism to cause reproductive loss? Host immunocompetence likely plays a role, with the immune response of immunocompetent sheep restricting infection to the intestine, limiting overt disease (Grogono-Thomas et al., 2000; Grogono-Thomas et al., 2003; Sahin et al., 2017). Immunocompetence is reduced during pregnancy (Reynolds and Griffin, 1990), and ewes are most susceptible to *Campylobacter* infection during the last three months of gestation (Lindenstruth et al., 1949; Frank et al., 1965). In a naïve, pregnant and immunocompromised ewe, ingested *Campylobacter* may be more able to translocate across the intestinal mucosa, initiating a bacteraemia that persists for 1 to 2 weeks and disseminates the organisms systemically (Grogono-Thomas et al., 2000; Grogono-Thomas et al., 2003).

If translocation across the intestinal mucosa occurs, *Campylobacter* localise in the uterus of the pregnant ewe and cause a necrosuppurative placentitis (Jensen et al., 1961; Campero et al., 2005). This reduces nutrient and gas exchange between the foetus and the dam, slowing foetal growth and development (Kelly, 1992b). The infection may extend to the foetus, resulting in suppurative bronchopneumonia, hepatitis, gastroenteritis and serositis (Skirrow, 1994; West, 2002; Campero et al., 2005; Moeller, 2012). Both the placentitis and foetal infection ultimately cause foetal death or reduced neonatal viability.

The interval between exposure and reproductive loss is often 14 to 21 days but may be as short as 13 days or as long as 113 days, depending on the timing of exposure (Skirrow, 1994; Menzies, 2011; Sanad et al., 2014).

The clinical presentation in the individual depends upon the timing of infection relative to gestation (Moeller, 2012). Infection earlier in gestation results in abortion, most commonly in the third trimester, whilst later infection results in stillbirth or the birth of a small, weak lamb (Skirrow, 1994). The ewe usually shows no clinical signs. However, infection may reduce milk production, which further threatens lamb survival (West et al., 2009). A small percentage of ewes may die from uterine sepsis and septicaemia (Nietfeld, 2013).

Gross pathology lesions in the infected foetus or lamb include serosanguinous fluid in the peritoneal and pleural cavities, subcutaneous oedema, and an enlarged, friable liver with rounded margins, which may have ruptured to cause haemoabdomen (Figure 2; Dennis, 1972; Kirkbride, 1993). In approximately 25% of cases, necrotic areas of the liver appear as multiple 2–15 mm circular to targetoid, white-yellow lesions (Figure 2). However, these lesions do not occur in all cases of campylobacteriosis (Sahin et al., 2017), and their presence is not pathognomonic for the disease (Kirkbride, 1993). Gross placental lesions include a red-brown exudate covering the cotyledons and a congested, oedematous intercotyledonary region, although these are often obscured by autolysis (Hedstrom et al., 1987; Sahin et al., 2008; Moeller, 2012).

The specific bacterial mechanisms that result in reproductive loss following *Campylobacter* infection are not fully understood (Sahin et al., 2017). However, it has been determined that the highly antigenic surface-layer proteins (SLPs) of *C. fetus fetus* are vital for pathogenesis, as strains lacking SLPs do not induce abortion (Grogono-Thomas et al., 1996; Grogono-Thomas et al., 1998; Grogono-Thomas et al., 2000; Grogono-Thomas et al., 2003). Surface layer proteins have been described that protect *C. fetus fetus* against killing by complement in serum and against phagocytosis by polymorphonuclear leukocytes, enabling bacteraemia and placental localisation (Blaser et al., 1987; Grogono-Thomas et al., 2000). These SLPs also have high antigenic diversity, important for initial protection against the host's immune response and establishing a carrier state (see Section 1.3.2.2; Blaser et al., 2008; Sahin et al., 2017).

The mechanisms facilitating *C. jejuni*-induced reproductive loss in sheep are even less well described than those of *C. fetus fetus* (Sahin et al., 2017). As mentioned in Section 1.2, *C. jejuni* does not possess SLPs but flagellar-mediated motility is thought to be important for pathogenesis (Nietfeld, 2013). Research by Iowa State University into 'clone SA', the *C. jejuni* clone responsible for most sheep abortions in the USA, has identified two important features of *C. jejuni* required for inducing reproductive loss - a major outer membrane protein and a capsular polysaccharide (Sahin et al., 2017). However, their precise function remains to be described.

1.3.2.2 Immunity following infection

The host-pathogen interactions that occur during *C. fetus fetus* and *C. jejuni* infections in sheep still require much elucidation. However, it is known that a species-specific humoral immunity develops after infection and confers 'some degree' of protection against subsequent infections and reproductive loss, although it is unclear what 'some degree' means (Skirrow, 1994; Sahin et al., 2017). No cross-protection is offered against other *Campylobacter* species (Sahin et al., 2017).

In an endemic flock, most ewes become infected and are subsequently immune, independent of pregnancy status at exposure and whether abortion occurs (Jensen et al., 1957; Meinershagen et al., 1969). Immunity is thought to persist for at least 3 years after infection (Frank et al., 1965), although there is little published information that quantifies this immunity. However, immunity following infection likely explains the epidemiological feature of abortion outbreaks every 4 to 7 years in endemic flocks (Clough, 2003; Sahin et al., 2017).

The SLPs possessed by *C. fetus fetus* and described in the preceding section are not only important for pathogenicity, but are key to the development of humoral immunity in the host (Grogono-Thomas et al., 2003). Antibodies against SLPs are formed following natural exposure, artificial challenge and vaccination with whole cell *C. fetus fetus* (Myers et al., 1970; Grogono-Thomas et al., 2003; Mannering et al., 2003b). These antibodies are associated with protection against disease, hence the potential to prevent reproductive loss by either vaccinating with inactivated whole-cells, or exposing naïve ewes to infection before they are mated (Meinershagen et al., 1969; Skirrow, 1994; Grogono-Thomas et al., 2003). Interestingly, the switching of SLPs results in antigenic diversity that temporarily allows the bacteria to avoid the hosts initial immune response, resulting

in delayed antibody development after a challenge (Grogono-Thomas et al., 2003). However, conserved antigenic regions of SLPs have been identified that induce protective immune responses independent of SLP switching (Grogono-Thomas et al., 2003).

Humoral immunity also occurs following infection with *C. jejuni.* Identifying the specific antigens involved in this response is an active field of research, with investigation into the antigens possessed by *C. jejuni* 'clone SA' receiving most attention (Delong et al., 1996; Wu et al., 2014a; Wu et al., 2014b). For example, Wu et al. (2014) used an immunoproteomic approach to identify membrane-related antigens associated with infection in sera from ewes naturally infected with 'clone SA'. Interestingly, the identified antigens were not unique to 'clone SA', but were conserved across *C. jejuni* strains and provide potential targets for targeted vaccines (Wu et al., 2014a).

1.3.2.3 Strain variation in *Campylobacter* spp. associated with reproductive loss

Strain variation exists amongst populations of *Campylobacter* species responsible for ovine reproductive loss. A strain is a genetic variation, or subtype, of a micro-organism (Baron, 1996). Understanding the strain type involved in disease is relevant for determining the epidemiology of outbreaks and the potential scope of protection offered by a vaccine. Serotyping, restriction endonuclease analysis (REA) and pulsed-field gel electrophoresis (PFGE) have all been used to identify strains of *Campylobacter* spp. associated with reproductive loss in sheep (Mannering, 2003; Mannering et al., 2003b). The number of strains identified varies inherently with the technique (Varga, 1991; Newell et al., 2000; Mannering et al., 2003b).

Serotyping was historically used to distinguish the strain of *Campylobacter* spp. involved in an outbreak (Clark and Monsbourgh, 1974; Bird et al., 1984; Varga et al., 1990). For example, Bird et al. (1984) used serotyping to investigate the strains of *Campylobacter* spp. involved in abortion outbreaks on four New Zealand farms. Two major serogroups were identified from 76 isolates (Bird et al., 1984). An Australian study also found two dominant serotypes (Clark and Monsbourgh, 1974). However, serotyping is limited in its ability to differentiate between strains, and was superseded by REA. Restriction endonuclease analysis was subsequently used to identify *C. fetus fetus* strains involved in abortion outbreaks throughout New Zealand and *C. jejuni* strains in the USA (Collins and De Lisle, 1985; De Lisle et al., 1987; Delong et al., 1996; Markey et al., 2013). Seven distinct *C. fetus fetus* REA types were identified in two New Zealand studies and five *C.*

jejuni REA types were described in the USA (Collins and De Lisle, 1985; De Lisle et al., 1987; Delong et al., 1996).

More recently, PFGE has been used to identify strains involved with reproductive loss and to investigate how strain variation might influence the protection offered by whole-cell vaccines (Mannering et al., 2003b; Mannering et al., 2006). For example, 26 distinct PFGE *C. fetus fetus* types were identified from 225 New Zealand farms with *C. fetus fetus* abortions, and 12 distinct PFGE *C. jejuni* types were identified from 25 farms with *C. jejuni* abortions (Mannering et al., 2001). The dominant *C. fetus fetus* strain, PFGE type B1, was found on 66% of farms (Mannering et al., 2001; Mannering et al., 2003a; Mannering et al., 2003b). Interestingly, this PFGE type is distinct from the PFGE type used in the production of the widely used Campylovexin® vaccine (Virbac Pty Ltd, Hamilton, New Zealand; Fenwick et al., 2000; Mannering et al., 2003b).

Little has been published recently with respect to strain variation in *Campylobacter* spp. causing reproductive loss in sheep in Australia. An historical study of the strains responsible for 69 abortion outbreaks between 1956 and 1971 reported that the dominant serotype varied with region but that most outbreaks featured only one serotype (Clark and Monsbourgh, 1974). Although dated, these results are consistent with international studies reporting geographic and temporal variation in *Campylobacter* spp. strains associated with reproductive loss (Sahin et al., 2017). Sahin *et al.* 2017 conclude that generally there is a high level of genetic diversity between-farms and between-years, but that strain variation within-farm within-year is low. This is the case for both *C. fetus fetus* and *C. jejuni* in New Zealand (Fenwick et al., 2000; Mannering et al., 2003b; Mannering et al., 2006). It is also the case for *C. jejuni* in the United Kingdom (Wu et al., 2014b). In the USA, previous genetic diversity has been replaced by the dominance of *C. jejuni* 'clone SA' (Sahin et al., 2008; Wu et al., 2014b).

Strain-variation could render a single-strain vaccine ineffectual. The relationship between vaccine breakdown and strain-variation has been discussed and investigated (Fenwick et al., 2000; Mannering, 2003; Mannering et al., 2003b; Sahin et al., 2017). Despite the diversity in *Campylobacter* responsible for reproductive loss, and the differences between strains used for commercial vaccine manufacture and strains responsible for outbreaks, there are relatively few reported cases of *Campylobacter* abortions in ewes vaccinated according to label instructions (Mannering et al., 2002; Mannering et al., 2003b). For a further discussion of vaccination, see Section 1.6.2.2.

1.3.2.4 Morbidity and mortality associated with infection

The consequence of *Campylobacter* spp. infection for the individual depends on ewe reproductive status, gestation, and the extent of foetal compromise (Meinershagen et al., 1969; Skirrow, 1994; Moeller, 2012; Sahin et al., 2017). Abortion outbreaks can occur if a large proportion of a naïve flock is exposed. In such cases, the incidence of abortion is usually 10-20%, although up to 50% of ewes may abort (Plant, 2002; Clough, 2003).

Vaccine trials have shown that the reproductive output of ewes protected against *Campylobacter* may be increased compared to unprotected ewes, even in the absence of abortion outbreaks in unprotected ewes (West, 2003). This observation may be explained by infection causing subclinical disease which is less noticeable than outbreaks of abortion (Clough, 2003). Insidious reproductive wastage may not be investigated because ewes show few clinical signs, and the associated reproductive wastage may be attributed to more familiar causes of lamb mortality.

Thus, *Campylobacter* may cause a spectrum of reproductive loss, from florid outbreaks of abortion to less spectacular intermittent abortions, stillbirths and the birth of moribund neonates. The contribution of insidious reproductive loss associated with *Campylobacter* infection to reproductive wastage in sheep may be underestimated, and is discussed further in Section 1.5 (Clough, 2003).

1.3.2.5 Transmission routes and sources of infection

Infection occurs when ewes ingest birth products laden with bacteria, or feed (pasture and/or grain) and water contaminated by faeces or birth products (Figure 1; Frank et al., 1957; Jensen et al., 1957; Peel and Mason, 1993). The foetus, placenta and amniotic fluid from an infected ewe contain bacteria that contaminate the environment and are highly infective (Moeller, 2012; Sanad et al., 2014). These products provide an efficient mode of transmitting the organism to naïve ewes (Sahin et al., 2017). Hence the importance of reducing the exposure of other ewes to both the birth products of ewes suspected of aborting, and the environment (West et al., 2009). Additionally, *Campylobacter* may be shed for weeks in the uterine discharge following parturition (Sahin et al., 2017). Thus, environmental contamination from shedding ewes poses an ongoing risk for naïve ewes, if the aborted ewe is not removed from the mob.

Abortion outbreaks typically occur 2 to 3 weeks after the abortion of several ewes in the third or fourth month of gestation (Skirrow, 1994). The first ewes to abort may easily be missed. Exposure to the birth products from these ewes results in infection of many more ewes, and a subsequent increased rate of abortions (Skirrow, 1994). The duration of persistence of *C. fetus fetus* in the environment under different conditions has not been definitively determined. However, it is thought to persist longer in cool, moist conditions than in hot, dry conditions (Clough, 2003). *Campylobacter jejuni* dies within four days in 25°C water but can survive in 4°C water for four weeks (Blaser et al., 1980). In faeces, survival of up to four days has been reported (Jones et al., 1999). Thus, in some climates the threat posed by the environmental contamination, initially from the birth products and subsequently from the uterine discharge, may persist until the end of most ewe's gestation, for a 5 to 6 week lambing.

An unknown proportion of ewes become persistently infected carriers after infection, enabling *Campylobacter* spp. to become endemic in a flock (West et al., 2009). The carrier state has been demonstrated by the recovery of bacteria from the placentae of ewes birthing normal lambs and from the gall bladder, intestines, liver, faeces and mesenteric lymph nodes of sheep (Firehammer et al., 1962; Frank et al., 1965; Dennis, 1967). Carrier ewes are important for exposure and maintenance of *Campylobacter* spp. in endemic flocks, and are a source of infection for naïve flocks. As previously discussed, some degree of immunity develops following exposure (Frank et al., 1959; Meinershagen et al., 1969). Immunity likely undergoes cyclical peaks and troughs in endemic flocks, potentially resulting in abortion outbreaks every 4-7 years (Clough, 2003).

Ewes in their first pregnancy, whether they be ewe lambs first mated at 8-10 months of age, or hogget ewes first mated at 18-20 months of age, are the highest risk age cohort in a flock (Quinlivan and Jopp, 1982). Young ewes are less likely to have had sufficient prior exposure to *Campylobacter* to develop immunity (Quinlivan and Jopp, 1982). However, older ewes can also be highly susceptible if not previously exposed (Frank et al., 1965).

The age-related risk may also be associated with enterprise type, because enterprises that rely on purchasing replacement ewes introduce young ewes every year. Ewes are often introduced to Australian farms shortly before mating. If young ewes from a naïve property are introduced to an endemic farm, there is a risk of *Campylobacter*-associated reproductive loss in that age group every year. Similarly, if young ewes are introduced to

a naïve flock from an endemic farm, where they have become carriers, they pose a risk to the older age groups in the new flock (Dickason, 2012). For self-replacing sheep enterprises, where young ewes are bred and reared on-farm, young ewes may be deliberately exposed to mature ewes prior to their first pregnancy to expose them to any endemic *Campylobacter* spp. (Clough, 2003). The risk of reproductive loss associated with *Campylobacter* may be lower on these farms due to this practice.

Scavenger animals who have consumed the placenta and/or carcass of infected sheep or lambs may also introduce and spread *Campylobacter* (Dennis, 1967). Carrion eating birds, such as Australian ravens (*Corvus coronoides*) and American magpies (*Pica pica*), and foxes are proposed vectors (Waldhalm et al., 1964; Meinershagen et al., 1965; Dennis, 1967; Ogden et al., 2009). For example, ravens are widely distributed and are common predators and scavengers of weak lambs and sheep carcasses. Dennis (1967) tested whether ravens were infected after feeding on *Campylobacter* spp. infected sheep carcasses, and the consequence of exposing pregnant ewes to the faeces of infected birds. Ewes fed faeces from infected birds gave birth to compromised lambs, with evidence of infection with *Campylobacter*. This research confirmed that ravens can transmit *Campylobacter* spp., providing an explanation for how *Campylobacter* species might spread into naïve, closed ewe flocks.

1.3.2.6 Risk factors

Few published papers exist that definitively ascribe risk factors to ovine *Campylobacter*-associated reproductive loss. Proposed risk factors based on association include higher stocking densities such as rotational grazing or intensively stocked ‘containment’ areas, providing supplementary feed on the ground and both environmental and nutritional stress (Frank et al., 1965; Quinlivan and Jopp, 1982; Sykes and Morgan, 1997; Andrewartha, 1998; Clough, 2003; Shankar, 2017). Increased stress could initiate shedding from carrier ewes, increasing the opportunity for faecal-oral transmission. Additionally, if a ewe aborted in a high stocking rate environment, the opportunity for other ewes to be infected is theoretically higher than at a lower stocking rate.

The introduction of naïve animals into an endemic flock or the introduction of carriers into a naïve flock are also acknowledged risk factors, as is ewe age (as discussed in the preceding section).

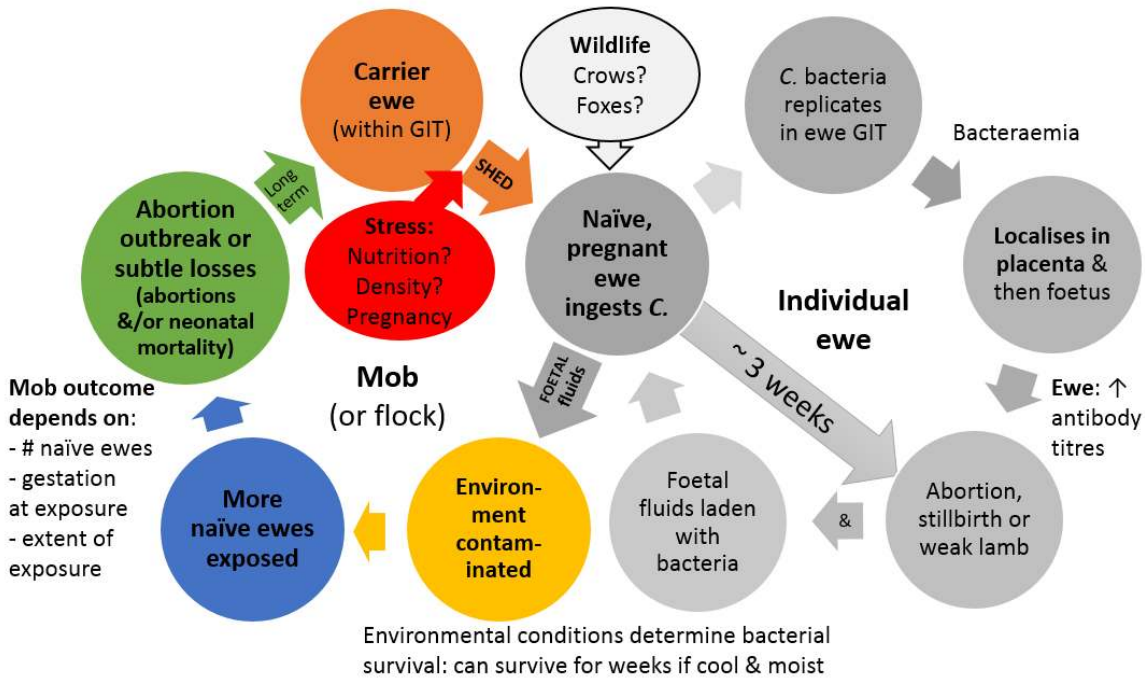

Figure 1 The potential pathogenesis and epidemiology of *Campylobacter* (*C.*)-induced reproductive loss, both in mobs and individual ewes (GIT gastrointestinal tract; adapted from Dennis, 1967; Skirrow, 1994; Sahin et al., 2017).

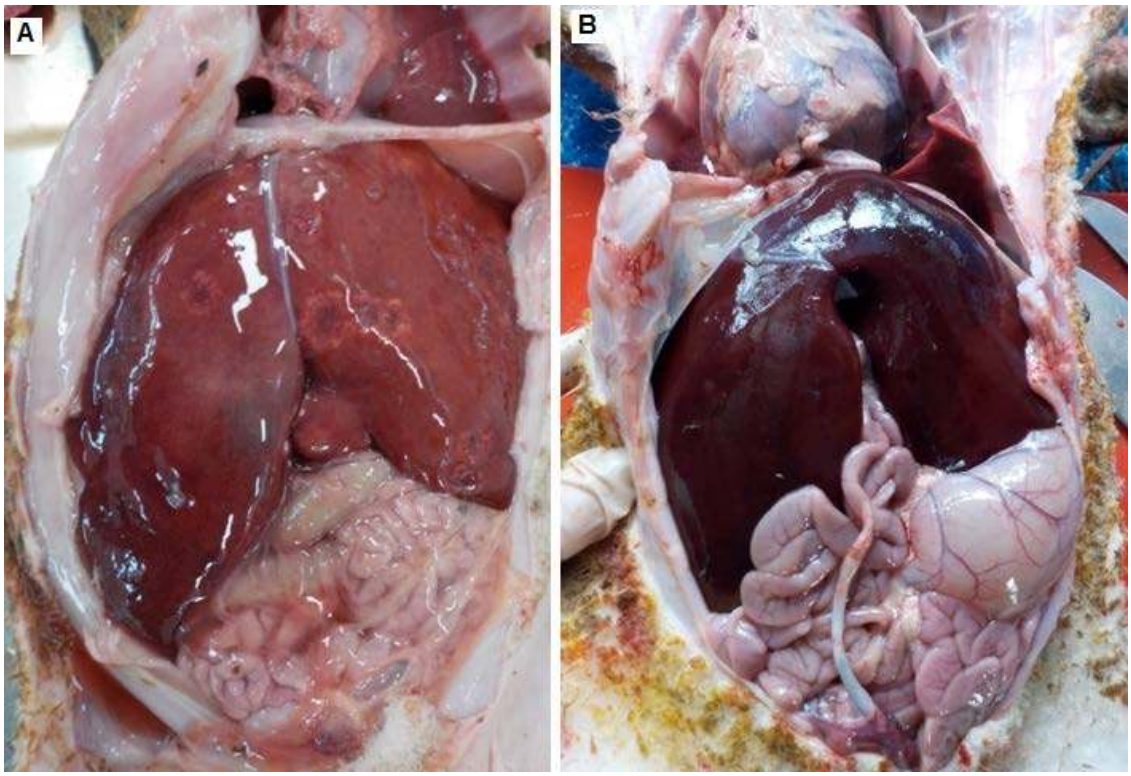

Figure 2 Campylobacteriosis in an aborted foetus (A: note target lesions in liver and oedema throughout peritoneal cavity) and a stillborn lamb (B: note hepatomegaly).

1.4 Diagnosis of *Campylobacter*-associated reproductive loss in ewes

Campylobacter infection is diagnosed by gross and histopathological post-mortem examination of the foetus or neonate, and if possible placenta, followed by culture of the causative agent (Sahin et al., 2017). Definitive diagnosis can be difficult. For example, infectious agents may not be suspected in cases of neonatal lamb mortality, with a lack of pathognomonic lesions decreasing the likelihood of microbiological investigation. If placental samples are submitted, faecal contamination and autolysis of the sample is common (R. Bushell, personal communication, September 2016). Additionally, the number of cases investigated influences the likelihood of an infectious agent being detected (Broadbent, 1975). Broadbent (1975) found infections were diagnosed twice as often when 10 or more necropsies were conducted than if five or less were conducted. Finally, *Campylobacter* spp. have fastidious growth requirements and poor survival *in vitro* (Skirrow, 1994). These difficulties compound the struggle to fully describe the role of *Campylobacter* in reproductive loss in sheep flocks.

1.4.1 Post-mortem samples

Fresh and formalin-fixed samples should be taken in cases where there is a suspicion of *Campylobacter* spp. based on the gross necropsy lesions (see Section 1.3.2.1) and/or the case history. The most reliable fresh samples for culture and isolation of *Campylobacter* are the placenta and abomasal contents (Hore et al., 1973). As previously discussed, the likelihood of a suitable placenta being submitted is low in most cases of reproductive loss in extensive sheep production systems. Samples from the abomasum are more reliable, and can be retrieved in a sterile manner by making use of a needle and syringe during a standard necropsy procedure (Holst, 2004). Samples from the lung and liver may also be rewarding for culture (Skirrow, 1994). At a minimum, samples from the lung, liver and placenta should be preserved in formalin for histology.

An impression smear of the placental cotyledons and smears of the abomasal contents made during necropsy can also be useful. *Campylobacter* spp. may be identifiable by its characteristic morphology on smears stained directly with dilute carbol fuchsin (Markey et al., 2013).

1.4.2 Culture and identification

Campylobacter have fastidious growth requirements and limited viability outside the host (Skirrow, 1994; Monke et al., 2002). The use of appropriate transport media and delivery to a laboratory within four hours may enhance the success of microbiological techniques (Monke et al., 2002; Markey et al., 2013). In the laboratory, *Campylobacter* require selective culture media and isolation procedures. They are microaerobic, requiring a low-oxygen environment to grow (3-10% oxygen; 3-15% carbon dioxide), and are variably temperature sensitive (Nietfeld, 2013). Most species will not multiply below 30°C, all grow at 37°C, but only some grow at temperatures greater than 40°C (Nietfeld, 2013). This thermotolerance is useful for microbiological isolation. For example, *C. jejuni* grows well at 42°C, but *C. fetus fetus* does not (Markey et al., 2013). Due to their temperature sensitivity, numbers are reduced by freezing and thawing (Nietfeld, 2013). However, viable bacteria can be recovered after chilling, and refrigeration of samples is advised.

Following culture, definitive diagnosis of specific *Campylobacter* species is achieved through either phenotypic tests or polymerase chain reaction (PCR; Schulze et al., 2006). Advances in molecular technology will likely facilitate more sensitive diagnosis of *Campylobacter* associated reproductive loss, with a standard PCR used successfully to detect both *C. jejuni* and *C. fetus fetus* DNA from aborted tissues (Hamali et al., 2014).

1.4.3 Ewe serology

Serology can be used to identify ewes previously exposed to or vaccinated against *Campylobacter* spp.. In Australia, an Agar Gel Immunodiffusion (AGID) test has been available commercially since 2013 as an aid in screening flocks for exposure to *C. fetus fetus* and *C. jejuni.* The AGID technique involves the diffusion of soluble antibodies and antigens toward one another, resulting in their precipitation (Walsh, 2016). It does not allow for differentiation of IgM, IgG or IgA antibody isotypes (A. Vanderfeen, personal communication, November 2017). This technique is considered to have good specificity and moderate sensitivity (A. Vanderfeen, personal communication, November 2017). The precise test specificity and sensitivity are currently being investigated by ACE Laboratory Services (Benalla, Victoria, Australia). The development of the AGID test was supported by Coopers Animal Health (MSD Animal Health), as an adjunct to the registration of a vaccine against both species produced by the same company. The test is also used in New Zealand (Walsh, 2016).

Other published methods of determining the serological status of ewes to *Campylobacter* spp. include the microagglutination test (MAT), complement fixation test (CFT), tube agglutination and an antibody enzyme-linked immunosorbent assay (ELISA; Keisler et al., 1989; Gürtürk et al., 2002; Dempster et al., 2011). The MAT was used in an extensive four-year serosurvey of *C. fetus fetus* in New Zealand ewes from 2006 to 2009, and was developed in house based on a previously described method (De Lisle et al., 1987; Dempster et al., 2011). The antibody-ELISA was used in a smaller scale serological evaluation of ewes vaccinated with commercially available *C. fetus fetus* and *C. jejuni* vaccines in the USA (Keisler et al., 1989). Antibody-ELISAs can be modified to examine isotype specific antibody responses (Grogono-Thomas et al., 2003). Antibody-ELISAs also have higher sensitivity than agglutination techniques. However, there is no commercially available sheep *Campylobacter* antibody-ELISA in Australia (A. Vanderfeen, personal communication, November 2017).

There are several complications associated with interpreting *Campylobacter* serology results from ewes. One is the lack of knowledge about how long antibodies persist. Another is the difficulty in drawing firm conclusions about disease based on antibody status alone.

The duration of persistence of *Campylobacter* antibodies following either natural exposure or vaccination of sheep is not well described. One study reports that titres to both *C. fetus fetus* and *C. jejuni* fell to near sero-negative levels 90-days after vaccination (Keisler et al., 1989). Another study examined serum IgM and IgG levels in ewes challenged with a 'wild-type' *C. fetus fetus* strain (Grogono-Thomas et al., 2003). That study found that serum IgM increased slightly and for a short time following challenge, but serum IgG antibodies had increased by 3 weeks after the challenge, and remained elevated for at least another 4 weeks. However, the challenge was artificial in that study.

Anecdotally, antibody titres detected using the AGID test remain elevated for a variable length of time after natural exposure, up to 12 weeks for *C. jejuni* and eight months for *C. fetus fetus*. Consequently, if ewes are exposed to either species 12 or more weeks before a blood sample is taken and/or have 'moderate' to 'low' antibody titres at sampling, drawing firm conclusions on the significance of the titre is difficult. Repeat samples to demonstrate rising or falling titres would be required, but are often impractical to collect in large flocks.

Finally, reproductive wastage is often not suspected until lambs are counted relative to ewes at lamb marking, which may be eight weeks after the start of lambing. In these cases, it could be difficult to achieve a reliable serological diagnosis of *Campylobacter* exposure in ewes, especially if infection occurred in mid-gestation. If a serological diagnosis of exposure to *Campylobacter* is made, the significance of antibody titres must be interpreted alongside the reproductive history and results from any foetal or lamb necropsies from that flock (Dempster et al., 2011). Information about the presence of antibodies by itself cannot estimate clinical infection (Dempster et al., 2011), but necropsy samples are also impossible to collect after the event.

1.5 Estimated prevalence and impact of *Campylobacter*-associated reproductive loss on ewe productivity in Australia

The current prevalence of *Campylobacter* spp. capable of causing reproductive loss in sheep on Australian farms is not definitively known. However, an ongoing serological survey of ewes from farms where a concern has been raised over the discrepancy between lambs expected based on pregnancy diagnosis and lamb marking rates found 62% and 97% of the 346 flocks tested have at least one ewe seropositive to *C. fetus fetus* or *C. jejuni* respectively (J. Walsh, personal communication, December 2017). The high number of flocks exposed to these species is perhaps expected, given their commensal nature. However, few serological studies of *Campylobacter* spp. have been conducted and the interpretation of the significance of titre levels is an imperfect and evolving field (Dempster et al., 2011).

In terms of the current significance of the disease to the Australian sheep industry, a 2015 Meat and Livestock Australia report estimated the annual cost of *Campylobacter* abortions to be AUD$1.63 million (Lane et al., 2015). The authors considered 5% of Tasmanian sheep flocks and 1% of sheep flocks in Victoria, New South Wales, South Australia and Western Australia to be affected by abortions (Lane et al., 2015). However, the authors emphasise that this estimate is highly uncertain due to the lack of current prevalence data and because it considers only abortion outbreaks, the more florid presentation of reproductive loss associated with *Campylobacter*.

Historical reports of the percentage of Australian farms that have at least one '*Campylobacter* positive' carcass in abortion and neonatal lamb mortality investigations

range from 7% to 27.8% (Table 2; Dennis, 1975; Clough, 2003). The historical prevalence and importance of *Campylobacter* spp. compared to other pathogens varies between reports, differing between years, regions and with farm practices (Rahaley, 1984; Clough, 2003). Study design and methodology, including the number of cases investigated per flock and the duration of data collection, also influences the reported prevalence (Broadbent, 1975; Clough, 2003). The survey results should be interpreted in consideration of these differences. Additionally, most of the published surveys of infectious causes of abortion and perinatal loss were conducted in the 1960's and 70's. The Australian sheep industry has changed over the intervening 40 years, with higher stocking rates potentially increasing the risk of *Campylobacter* infection. A more recent, longitudinal survey of causes of abortions on Victorian sheep enterprises reported *Campylobacter* spp. as the aetiological agent in 14% of 100 outbreaks investigated from 2009-2014 (Suter, 2014). Again, this report only considered outbreaks of abortion.

Over the past two decades, property specific case reports of abortion outbreaks, rather than surveys, have dominated the literature (Table 3). In the absence of the large surveys, these reports confirm that *Campylobacter* is still a problem for the Australian sheep industry. It is likely that they reflect only a proportion of the cases which occur annually, for the reasons discussed by Clough (2003), including a reluctance of producers to report or investigate reproductive loss and the potential contribution of *Campylobacter* to more subtle lamb mortality.

Describing the extent to which *Campylobacter* infection contributes insidiously to perinatal mortality is difficult, requiring detailed prospective studies. However, it may be estimated by assessing the effect of vaccination on lamb survival and lamb marking rates. For example, a simple randomised controlled trial of a killed *C. fetus fetus* vaccine was conducted on 16 New Zealand farms in 1980. In those trials, the reproductive performance of the vaccinated ewes was better than the unvaccinated ewes (Quinlivan and Jopp, 1982). Vaccinated ewes had fewer abortions (2.09% compared with 3.05%), were less likely to be dry at lamb marking (3.7% compared with 4.5%), had a higher percentage of lambs born (130.2% compared to 123.5%) and a higher marking rate (113.3% compared to 106.3%) compared to unvaccinated ewes. Interestingly, despite the 7% difference in lamb marking results and the frequency of *Campylobacter*-associated reproductive losses prior to the study being conducted, the authors concluded that vaccination may not be justifiable because of the sporadic nature of disease. Subsequent New Zealand research refers to the significance of insidious, annually repeatable neonatal

lamb mortality associated with *Campylobacter* spp. (Anderson, 2001; West, 2003). For example, even when abortions are not observed, flocks that vaccinate are reported to lose 6% to 10% less lambs than those that do not, justifying vaccination (West, 2003).

In Victoria, Australia, trials conducted by MSD Animal Health of the currently available vaccine, Ovilis Campyvax® (Coopers, MSD Animal Health, Ryde, NSW, Australia) on four farms with a serological history suggestive of endemic infection found a variable, but generally positive effect of vaccination (Walsh, 2016). Three farms had an 8% to 31% increase in lamb marking rate in vaccinated ewes compared to unvaccinated ewes, although there was no difference in marking rate on a fourth farm. An independent study of the effect of a previously available vaccine, Guardian® (Coopers, MSD Animal Health, NSW, Australia), conducted by the Glenthompson Best Wool/Best Lamb group, also found a variable but predominantly positive effect of vaccination on lamb survival across five farms, in the absence of notable abortions (Anonymous, 2011). This study included four maiden ewe mobs and one mixed-age ewe mob. In the maiden ewe mobs, vaccination increased lamb marking rates by between 6.8% and 11.1% compared to the unvaccinated ewes. There was no effect of vaccination on lamb survival in the mixed age ewes. The authors explain that this could be due to existing immunity to *Campylobacter* within this age cohort, although no serological evidence is provided to substantiate this explanation.

Despite these interesting preliminary investigations, no independent, large-scale field trial of vaccination against *Campylobacter* spp. in Victorian ewes has been published.

Table 2 Historical prevalence of *Campylobacter* spp. in Australia from abortion and perinatal lamb mortality investigations (adapted from Clough, 2003).

State & year of study	**Study details** (abortion investigations only or perinatal loss investigations including abortions)	**Farm level %** (number of farms with ≥ one positive)	**Overall %** (number of *Campylobacter* positive carcasses)	**Reference**
Tasmania 1960-65	Abortions	N/A	27% (41 of 153)	(Munday et al., 1966)
NSW 1963-64	Perinatal loss	27.8% (5 of 18)	3.6% (10 of 274)	(Haughey et al., 1967)
W.A. 1963-65	Abortions	7% (48 of 695)	2% (91 of 4650)	(Dennis, 1975)
NSW 1963-70	Abortions and perinatal loss	14.4% (53 of 368)	31% abortions (54 of 173) 18% of perinatal (33 of 184)	(Plant et al., 1972)
Victoria 1969-70	Perinatal loss	9% (4 of 44)	5.4% (12 of 222)	(Hore et al., 1973)
Victoria 1970-71	Perinatal loss	7.4% (7 of 94)	2.9% (17 of 582)	(Broadbent, 1975)
Tasmania 1986	Abortions	N/A	31% (13 of 42)	(Munday et al., 1987)

Table 3 A selection of published case reports of *Campylobacter*-associated abortion outbreaks in Australia over the last 20 years (from Quarterly Animal Health Surveillance reports, Animal Health Australia and Flock and Herd, an initiative of the District Veterinarians of New South Wales).

State & year (reference)	**Case history**	**Extent of loss & clinical findings**	***Campylobacter* species involved**	**Intervention & outcome**
Tasmania 1998 (Andrewartha, 1998)	Abortions in late pregnancy in ewes under nutritional stress	Not reported	*C. fetus fetus*	Not reported
Tasmania 2001 (Elliot, 2001)	3 flocks: late-gestation abortions	Losses: not reported Pathology: foetal hepatic necrosis one flock, inflammatory change in others	*C. jejuni* in all	Not reported
New South Wales 2008 (Arthur, 2008)	Cross-bred ewes aborting after silage feeding	Losses: 6% (80 of 1300 ewes aborted) Pathology: vaginal discharge in 'healthy' ewes	*C. fetus fetus*	Decreased stocking density, ceased ground feeding, removed aborted ewes. Advised annual vaccination.
South Australia 2010 (Dickason, 2010)	Abortions in late pregnant cross-bred maiden ewes moved to a new paddock with increased stocking density	Losses: ~ 50%	*C. jejuni*	Vaccination advised for any newly purchased maiden ewes.
South Australia 2012 (Dickason, 2012)	Abortions in late pregnant cross-bred ewes. Started in 800 newly purchased, young pregnant ewes in transport, Spread through rest of flock.	Losses: 16% abortions Pathology: histology of placenta and foetal tissues suggested a bacterial cause	*C. fetus* (identified by PCR as *C. fetus* subsp. *venerealis*)	Vaccinated ewes against *Campylobacter* during outbreak: reduced number of abortions that occurred
New South Wales 2014 (Bell, 2014)	Abortions in late pregnant, mature cross-bred ewes. No abortions in younger ewes managed separately	Total losses: 16.3% (130 of 800 aborted) Pathology: goitre, broncho-pneumonia, splenic vasculitis, placentitis, and necrosis (four foetuses)	*C. fetus fetus*	Advised to vaccinate maiden ewes for following lambing season (natural exposure or commercial vaccine)

State & year (reference)	**Case history**	**Extent of loss & clinical findings**	***Campylobacter* species involved**	**Intervention & outcome**
Tasmania 2015 (Martin, 2015)	Abortions in late pregnant ewes introduced to new property 4 weeks prior to abortions. Ewes intensively housed & supplementary fed.	Total losses: est. 5% Pathology: pneumonia and encephalitis (four foetuses)	*C. fetus* subsp. *fetus*	Abortion storm tapered off quickly & perinatal lamb mortality did not increase significantly
Tasmania 2015 (Martin, 2015)	Abortions 10 days before lambing start date in mixed age ewes grazing pasture	Total loss: not reported. Pathology: placentitis, broncho-pneumonia, hepatitis, meningoencephalitis	*C. fetus* subsp. *fetus*	Stocking rate lowered by dispersing flock, occurrence of abortions tapered off with no increase in perinatal lamb mortality
New South Wales 2017 (Shankar, 2017)	Abortions in maiden ewes 4 weeks before lambing, after routine yarding. Losses continued through gestation & lambing.	Total losses: 15% Pathology: pneumonia (two foetuses)	*C, fetus fetus*	Strict hygiene between paddocks, remove aborted foetuses; commence vaccination of maiden ewes & new introductions
New South Wales 2017 (Shankar, 2017)	Abortions started in two mixed age ewe mobs (2-7 year old) in late gestation after routine yarding.	Total loss: 25% Pathology: pneumonia (two foetuses), haemorrhagic peritonitis, hepatic lesions	*C. fetus fetus*	Strict hygiene between paddocks, remove aborted foetuses; commence vaccination of maiden ewes & new introductions

1.6 Management of *Campylobacter*-associated reproductive loss in ewes

Campylobacter-associated reproductive loss requires both short and long term responses to minimise impact, both in terms of abortion outbreaks and lamb mortality.

1.6.1 Reactive responses to control outbreaks of *Campylobacter* abortion

The control of *Campylobacter* abortion outbreaks is often aimed at reducing the severity and extent of the outbreak by reducing access to the source of infection (West, 2003). Antibiotics and vaccines are also used, to eliminate infection from carriers and those incubating the infection, and to protect naïve ewes against infection (West et al., 2009). Whilst these strategies may effectively decrease all *Campylobacter*-associated perinatal mortality, from overt abortions to more insidious neonatal lamb mortality, they are often only implemented in the event of, or following an outbreak. Additionally, as the initial abortions may be missed, infection has often spread within the mob by the time disease is suspected and the outcome may be inevitable (Mearns, 2007).

1.6.1.1 Management options for outbreak control

The high bacterial load in aborted birth tissues makes the timely removal of these tissues a critical step in minimising both the risk of ingestion by other ewes, and the contamination of feed and water (Sahin et al., 2017). Ideally, the ewe that has aborted should also be removed and the mob moved to a clean pasture (Quinlivan and Jopp, 1982). On extensive enterprises, removing the aborted ewe is usually not possible. Decreasing the stocking rate in aborting mobs is also advised (West, 2003). This decreases the opportunity for naïve ewes to interact with infected ewes and any contaminated feed and water. Removing aborted material and reducing stocking rate of paddocks is associated with the cessation of abortions in infected mobs (Arthur, 2008; Martin, 2015).

The mob in which abortions have occurred should be checked regularly and carefully from when abortions are first observed. This will allow for the removal of aborted material, and will alert the producer to any change in the rate of abortions. Vigilance should continue into lambing, allowing identification of weak lambs born to infected ewes. Other mobs on the property that have had contact with the aborting mob should also be checked carefully for abortions.

Mobs with aborting ewes should be checked last and attention should be paid to cleanliness, to minimise fomite transmission (West, 2003). Personal protective equipment including disposable gloves, glasses and masks should be used when handling aborted foetuses, placentae and aborted ewes, due to the zoonotic risk. All clothes and boots should be disinfected after contact, and exposure of pregnant or immunocompromised individuals to suspect ewes is not advisable (Quinlivan and Jopp, 1982).

1.6.1.2 Pharmaceutical options for outbreak control – antimicrobial therapy

Campylobacter spp. are susceptible to a range of antibiotics registered for use in sheep, making it technically possible to eliminate infection from ewes in the incubation phase and from carriers (West et al., 2009; Sahin et al., 2017). They are administered via both oral and parenteral routes in the face of outbreaks and preventatively, to variable effect (Mearns, 2007). Due to variable antimicrobial susceptibility, culture and susceptibility testing is recommended prior to use (Menzies, 2011; Giguère et al., 2013).

Antibiotics supplied in feed are used in countries other than Australia to reduce the risk of *Campylobacter*-associated reproductive loss, for example chlortetracycline in intensively managed ewes in the USA (ChlorMax® 50, Zoetis Inc., Kalamazoo, Michigan, USA). However, a feed based treatment is impractical in extensive enterprises, and is not justifiable given the sporadic nature of outbreaks of abortion in Australia.

A course of daily injections of penicillin-streptomycin has also been used successfully against *Campylobacter* outbreaks, again, in countries other than Australia (Giguère et al., 2013). Streptomycin is not permitted for use in food producing animals in Australia (National Registration Authority, 1999). Additionally, the daily dosing of an infected mob is impractical in most Australian sheep enterprises. A long-acting product with a sufficient duration of activity to eliminate infection is more favourable.

Selectively treating individual animals is complicated by the lack of clinical signs displayed by infected ewes prior to abortion, so options for treatment include i) aborted ewes only, to treat any metritis, reduce bacterial shed and to avoid a carrier state developing or ii) blanket treatment of the mob.

If a long-acting blanket treatment is given to a mob at one point in time, it will have a variable effect because of the range of disease stages across the mob. For any ewe not yet

exposed to *Campylobacter* spp., long-acting treatment will combat any infection that occurs over the following 48-72 hours (duration of protection dependent on the product). This same protection could be afforded by moving ewes into a clean paddock. If ewes cannot be moved onto a clean paddock, or the source of infection cannot be removed from the environment, the risk of infection will return when the antibiotic ceases to be protective. Reproductive loss from ewes that are early in the incubation phase could be prevented by antibiotic treatment. However, for ewes late in the disease process, the degree of damage to the placenta and foetus may be so advanced that reproductive loss occurs regardless of antimicrobial therapy (Giguère et al., 2013). However, for these ewes and ewes that have already aborted, treatment could reduce the likelihood of them becoming carrier ewes, which might reduce future environmental contamination.

As discussed above, both tetracyclines and penicillin-streptomycin have been used to treat ewes in the face of outbreaks (West, 2003; West et al., 2009). The penicillin-streptomycin combination is not possible in Australia because the use of streptomycin is prohibited. Tetracyclines are theoretically effective against *Campylobacter* spp., are registered for use in sheep in Australia and long-acting injectable formulations are available that may be practical for blanket treatment of exposed mobs. It is important to note, however, that concerns have been raised internationally over antimicrobial resistance in *C. jejuni* (Sahin et al., 2008). For example, tetracycline resistance is commonly reported in cases of *C. jejuni* abortion in the USA (Sahin et al., 2008), and 39% of the *C. jejuni* isolates from faecal samples from Canadian flocks were resistant to tetracycline (Scott et al., 2012). Whilst no association between the usage of tetracycline and antimicrobial resistance was reported by Scott et al. (2012), the authors caution that the lack of approved products licensed for use within the sheep industry may increase selection for resistance, emphasising the importance of the judicious use of this antimicrobial. Tetracycline resistance in *C. fetus fetus* is of less concern (Sahin et al., 2017).

The current prevalence of tetracycline resistance in *C. jejuni,* or *C. fetus fetus,* isolated from Victorian sheep is not known. However, given the potential for variable success of blanket antibiotic treatment discussed above, the risk of tetracycline resistance in *C. jejuni* and the availability of non-pharmaceutical management options, there is an argument to avoid blanket treatment of mobs in which ewes have aborted, unless other control strategies are implausible.

1.6.1.3 Pharmaceutical options for outbreak control – tactical vaccine use

Tactical control of outbreaks of *Campylobacter* abortions may be achieved by vaccinating with a bacterin, if vaccination is administered early in an outbreak (Gumbrell et al., 1996). A randomised controlled trial of vaccination in the face of *C. fetus fetus* outbreaks was conducted on three farms in New Zealand (Gumbrell et al., 1996). In each case, a commercially available bacterin vaccine effective at protecting ewes against *C. fetus fetus* was used after the start of the outbreak in previously unvaccinated ewes aborting six weeks before lambing (Wallace, 1982). Two doses of the vaccine were given to the treatment groups, 10 days apart. There was a significant reduction in the incidence of abortion in vaccinated compared to control ewes on two of the farms (12.7% to 4.7% and 29.5% to 14.6%). These results confirmed earlier work, in both experimentally infected ewes in Scotland and naturally infected ewes in the USA (Gilmour et al., 1975; Jensen and Swift, 1982). On the third farm, over 15% of ewes had already aborted by the time the second vaccine was given. It was considered likely that a high level of challenge had occurred before vaccination, meaning many of the ewes were already incubating *C. fetus fetus* when vaccinated. The authors concluded that for vaccination to be successful in the face of an outbreak, the cause must be identified as *Campylobacter* spp. and the vaccine must be given early in the outbreak (Gumbrell et al., 1996). Vaccination in the face of an outbreak was also successful in a South Australian case, although no control group was available for comparison (Dickason, 2012).

1.6.2 Preventative options to minimise *Campylobacter*-associated reproductive loss

Like the responses available to combat *Campylobacter* abortion outbreaks, there are both management and pharmaceutical options available to decrease the impact of *Campylobacter* spp. on the long-term reproductive efficiency of sheep flocks.

1.6.2.1 Management options to decrease the risk of campylobacteriosis

The risk of *Campylobacter*-associated reproductive loss may be reduced by strategic management decisions that decrease the exposure of pregnant ewes to the risk factors purportedly associated with infection. For example, ewes in their first pregnancy should not co-graze with mixed age mature ewes, and no ewes should be grazed rotationally in the last month of pregnancy (Quinlivan and Jopp, 1982). Avoiding high stocking densities over the last two months of gestation is advised. High stocking densities have been

repeatedly associated with abortion outbreaks, and may result in high challenge levels that can overwhelm existing immunity (Quinlivan and Jopp, 1982; Fenwick et al., 2000).

Ewes can acquire reasonable immunity after natural exposure to *Campylobacter,* independent of their reproductive status at the time of exposure and the outcome of infection (Jensen et al., 1957; Miller and Jensen, 1961; Meinershagen et al., 1969). Early investigations into immunity following exposure found that ewes from flocks that experienced abortion outbreaks in one year lambed normally, with no evidence of disease, in subsequent years, despite presumed exposure to *Campylobacter* spp. (Baker and Stone, 1939; Marsh et al., 1954; Wiggins, 1955). Experiments designed to simulate naturally acquired immunity found that ewes fed infected foetal tissues before pregnancy were protected when challenged with *Campylobacter* during pregnancy, demonstrating protective immunity (Jensen et al., 1957). Immunity following natural exposure has been reported to last at least three years (Frank et al., 1965).

Based on these observations, protective, naturally acquired immunity can be induced in ewes on endemic farms if exposure to *Campylobacter* occurs before their first pregnancy. This can be achieved by grazing young ewes with older ewes, or intentionally mixing ewes that abort with young ewes before their first pregnancy (Farquharson, 2003a). However, there are some limitations of naturally acquired immunity, mainly the lack of knowledge about the epidemiology of infection in endemic flocks. It is not known whether there is an age cohort most likely to contain carrier ewes, or how long *Campylobacter* spp. survives on pasture under the range of conditions experienced on Australian farms. Studies from the United Kingdom indicate that shedding of *Campylobacter* varies seasonally and with management practices on a farm (Jones et al., 1999). It is likely that this variability also exists under Australian conditions, but these patterns have not been documented. Thus, it is not known when shedding of *Campylobacter* spp. is most likely to occur, hence when young ewes should be exposed to mature ewes to maximise the likelihood of transmission.

This ambiguity around naturally acquired immunity makes it potentially unreliable. Simply exposing young ewes to mature ewes cannot guarantee protection against reproductive loss associated with *Campylobacter* spp. Additionally, the level of protection provided by natural exposure cannot currently be quantified, because although antibody titres can be measured, the relationship between titres and the level of protection provided has not been definitively described (Dempster et al., 2011).

1.6.2.2 Vaccination against *Campylobacter* spp. to prevent reproductive loss

A more reliable immunity may be provided by a commercially prepared vaccine administered correctly to naïve ewes before their first pregnancy (Menzies, 2011; Menzies, 2012). The potential for effective vaccination against *Campylobacter*-associated reproductive loss has been researched for more than six decades. Vaccine trial and development began as a natural extension of the observation that ewes fed infected foetal tissue before pregnancy were immune when orally-challenged with *C. fetus fetus* infected foetuses in late pregnancy (Jensen et al., 1957). Miller and Jensen (1961) then reported trials of killed and live subcutaneous vaccines administered to ewes before their first pregnancy (Miller and Jensen, 1961). In this study, both the vaccinated and control ewes were exposed to *Campylobacter* in late gestation either through being housed with aborting ewes or oral administration of cultured *Campylobacter*. Vaccination with live *Campylobacter* organisms afforded the most robust protection in that study, as none of that group aborted.

In further work, the extent of cross-species protection afforded by vaccines was examined (Miller et al., 1964). Ewes vaccinated against *C. fetus fetus* were not protected if challenged with *C. jejuni* and vice versa. Each of the monovalent vaccines used in this research was found to be protective against the species from which it was formed. For example, no ewes vaccinated against *C. fetus fetus* aborted following intra-ruminal inoculation with *C. fetus fetus*, but 86% aborted when inoculated with *C. jejuni* (Miller et al., 1964). A bivalent vaccine comprising both species was subsequently tested, and found to be effective when ewes were challenged by either species alone or in combination. The lack of cross-species protection has been confirmed in more recent experiments, including those using guinea pigs as a model (Diker and Turutoglu, 1995).

Acknowledging the limitations of cross-species protection, the effectiveness of monovalent and bivalent bacterin based vaccines has been demonstrated both in field studies in sheep and experimental studies in sheep and guinea pigs, especially when vaccines are administered either before or during early pregnancy (Miller and Jensen, 1961; Storz et al., 1966; Williams et al., 1976; Burrough et al., 2011).

Commercially available bacterin vaccines against one or both *Campylobacter* spp. involved in reproductive loss are now routinely used in New Zealand and the USA (Menzies, 2011; Menzies, 2012). In New Zealand, two products are commercially

available, Campylovexin® (Virbac Pty Ltd, Hamilton, New Zealand) and Campyvax4® (MSD Animal Health, Wellington, New Zealand). Campylovexin® has been available since 1980 and is a single-strain, monovalent vaccine comprising killed *C. fetus fetus* (West, 2002). It has been widely used and is considered effective at protecting against both abortion outbreaks and more insidious reproductive loss in endemic flocks, as reported in published trials (Quinlivan and Jopp, 1982; West, 2002, 2003). More recently, Campyvax4®, a bivalent vaccine comprising four strains of *Campylobacter* associated with reproductive loss, has become available. It has been shown to protect against abortion outbreaks and increase lambing percent by up to 9% (Intervet, 2010). In the USA, two bivalent vaccines are available from different companies, *Campylobacter fetus-jejuni* Bacterin-Ovine (Colorado Serum Company, Denver, Colorado, USA) and *Campylobacter fetus-jejuni* Bacterin (Hygieia Biological Laboratories, Woodland, California, USA; Sahin et al., 2017). The route of delivery and recommended timing of administration of the above vaccines is similar, with two subcutaneous injections a minimum of three weeks apart before or during first mating, and an annual booster thereafter. However, many producers only vaccinate their young ewes prior to their first pregnancy (West, 2003).

There have been reports of *C. fetus fetus* abortions in vaccinated flocks in New Zealand, and of variable efficacy in the vaccines currently available in the USA (Fenwick et al., 2000; Mannering et al., 2002; Burrough et al., 2011). One possible explanation for vaccine breakdown could be a lack of cross-strain protection by single-strain vaccines (Fenwick et al., 2000; Mannering et al., 2003b). Strain variation exists in both *C. fetus fetus* and *C. jejuni.* The frequency and implications of this variation were discussed in Section 1.3.2.3. Mannering (2003) addressed concerns over strain variation in a detailed study of the effect of strain variation on the efficacy of Campylovexin® in New Zealand ewes (Mannering, 2003). The study investigated the diversity of *C. fetus fetus* PFGE strain types, the serological response of vaccinated sheep to specific strains and the protection afforded by Campylovexin® vaccination in guinea pigs challenged with two distinct strains. Incidents of apparent vaccine breakdown were also investigated.

The most common *C. fetus fetus* PFGE strain type across New Zealand differed from the strain type in Campylovexin® (Mannering et al., 2003a). Interestingly, sheep vaccinated with the single-strain vaccine produced vaccine-specific antibodies that were important for disease protection and that recognised proteins produced by different PFGE strain types (Mannering, 2003; Mannering et al., 2003b). This could explain why vaccine

breakdowns are relatively infrequent, despite the difference between the strain type in the vaccine and the most common strain type identified in the survey. Indeed, vaccinated guinea pigs were protected when challenged with both the *C. fetus fetus* PFGE strain in the vaccine and the most common strain found in the survey (Mannering, 2003; Mannering et al., 2003b). In two cases of apparent vaccine breakdown, the strain identified was identical to that in the vaccine but either a booster dose had not been given or an alternative diagnosis was made. High stocking density also featured in these cases (Mannering et al., 2002; Mannering et al., 2003b). The authors concluded that the single-strain vaccine Campylovexin® (Virbac, Hamilton, New Zealand) offers effective cross-strain protection for *C. fetus fetus* (Mannering et al., 2003b). Thus, in cases of apparent vaccine breakdown, other explanations including overwhelming challenge in ewes managed at high stocking densities and the failure to provide an annual booster, should be considered (Mannering et al., 2002; Mannering et al., 2003b).

1.7 Ovilis Campyvax®: a bivalent vaccine against *C. fetus fetus* and *C. jejuni*

In Australia, both *C. fetus fetus* and *C. jejuni* have been diagnosed in cases of reproductive loss (see Table 3). A vaccine comprising inactivated whole-cells of both species is available commercially in Australia under the name Ovilis Campyvax® (Coopers, MSD Animal Health, Ryde, NSW). The vaccine was originally developed in New Zealand and was registered with the Australian Pesticides and Veterinary Medicines Authority (APVMA; Product no. 61563) in late 2013, for use as an aid in the control of reproductive loss associated with *C. fetus fetus* and *C. jejuni* infections in sheep.

The strains used in the vaccine were isolated from cases of reproductive loss in Australia (Anonymous, 2015). The product has been designed to be used on susceptible ewes prior to the time of greatest risk, with the aim of decreasing reproductive loss associated with *Campylobacter* infections. Field trials conducted by the manufacturer have resulted in increased lamb marking rates of 0% to 31% compared to results from unvaccinated ewes (Walsh, 2016). On farms where lamb marking rates were increased by vaccination, and in the absence of outbreaks of abortion, the increase demonstrates the detrimental effect of *Campylobacter* infection on lamb survival on some farms (see Section 1.5).

However, no independent field trial testing the effect of Ovilis Campyvax® on maiden ewe reproductive performance has been published.

1.8 Objectives of this study

Ovine campylobacteriosis is a complex disease, and the epidemiology and consequences of infection on Australian sheep farms is not well understood. As described in the preceding sections, infection may contribute to the high levels of reproductive wastage in Australian flocks more frequently, and more subtly than via sporadic abortion outbreaks, through increased perinatal lamb mortality. Vaccination against the species most commonly associated with reproductive loss, *C. fetus fetus* and *C. jejuni,* provides a means to both protect against abortion outbreaks and reduce more insidious losses that may be as significant as abortions in endemic flocks (Anderson, 2001; Clough, 2003).

To establish the extent to which such losses contribute to reproductive wastage, and determine the effect of vaccination, controlled trials monitoring reproductive performance and exposure to *Campylobacter* are required. Large field trials of vaccination have been performed internationally, and similar randomised controlled field trials are required to understand the broader implications of vaccination on reproductive performance in Australian flocks. Consequently, the current study aimed to determine the effect of vaccination with Ovilis Campyvax® on maiden ewe reproduction across four sheep flocks in the state of Victoria. Vaccinated groups were compared to unvaccinated groups to test the hypothesis that there was a significant effect of Ovilis Campyvax® on

1. The reproductive performance of maiden ewes, considering
    i. The proportion of pregnant ewes
    ii. Conception rates
    iii. Foetal and lamb survival from mid-gestation pregnancy diagnosis to lamb marking, and
    iv. Lamb marking rates
2. Body condition score over one lambing season
3. The cause of death in neonatal lambs

accounting for environmental effects and exposure to *C. fetus fetus* and/or *C. jejuni*.

2 – Materials & Methods

2.1 Trial design

2.1.1 Farm selection, consent and ethics

A randomised controlled field trial of a vaccine against *C. fetus fetus* and *C. jejuni,* Ovilis Campyvax® (Coopers, MSD Animal Health, NSW, Australia), was conducted on four winter-/spring-lambing sheep enterprises in Western and North-Eastern Victoria over 2016. The trial was designed to test the effect of vaccination on the reproductive output of maiden ewes considering the other factors known to affect reproductive output (see Section 1.1) including condition score (CS), nutrition and ewe management over lambing. Hence these factors were monitored on all four farms over the course of the trial.

The farms were selected based on availability over 2016, ewe numbers and an interest in the role of infectious disease in reproductive wastage. Common to many Australian sheep enterprises, the producers had each expressed dissatisfaction with historical ewe reproductive performance despite having attended to ewe nutritional requirements and using good routine husbandry practices.

The trial was conducted in line with the annual reproductive management calendar on each farm. The four farms were allocated letters to de-identify them, and are henceforth referred to as Farm A (Yea; 37°12'S, 145°25'E), B (Meredith; 37°50'S, 144°04'E), C and D (Edenhope; 37°03S, 141°18E; Figure 3). Two of the farms had previously experienced *Campylobacter*-associated reproductive loss in the form of abortions (Farms A and C). None had previously vaccinated against *Campylobacter* spp. using Ovilis Campyvax®.

Farm A is a prime lamb enterprise with Merino-Border Leicester ewes mated to South Down rams. Farms B, C and D are self-replacing Merino enterprises. However, only Farm B is a closed flock. Both Farms C and D had purchased mobs of sheep in the last five years. Each farm had a similar timeline for reproduction, which involved rams being introduced to the ewes for mating in late-summer to early-autumn for 5 to 8 weeks to lamb in late-winter to early-spring in line with peak pasture growth. Rams were used at a 2% ratio to ewes. Lamb marking occurred on each farm two weeks after lambing end date.

Detailed discussions of the trial methodology were conducted with potential participating producers prior to the project commencing. Each participating producer subsequently formally consented to participation. The trial was carefully designed to suit the requirements of extensive sheep operations. This ensured that the trial was conducted on properties being managed in a manner representative of commercial sheep enterprises in Victoria.

The investigators could be contacted at any time throughout the trial to address any queries the producers had regarding the trial and ewe or lamb management.

Animal ethics approval was sought and granted for the trial through the University of Melbourne Animal Ethics Committee (AEC #1613848).

Figure 3 Map of Victoria, Australia, showing the approximate locations of the four farms (A to D) involved in the described vaccine trial (®University of Melbourne)

2.1.2 Trial visits

Each of the four farms enrolled in the trial was visited a minimum of five times in 2016. Each visit was scheduled to coincide with routine management activities including commencement of mating, end of mating ('rams out'), mid-gestation pregnancy diagnosis, lambing and lamb marking. A concise table describing activities that occurred at each visit is provided below (Table 4). A more detailed table is in the appendices (Appendix 1). More complete details of each of the activities that occurred at each visit can be found in the appropriate sections following this brief description.

At the 'mating' visit, ewes were randomly allocated to either the vaccinated or control treatment groups and were individually identified. Ewes within the vaccinated treatment group were vaccinated with Ovilis Campyvax®. All ewes were condition scored, and a selection were sampled for baseline serology and bulk worm egg-counts (WEC). A feed budget was also performed and assessed relative to ewe requirements.

At 'rams out', the second 'booster' dose of vaccine was administered to all ewes within the vaccinated treatment group. Additionally, 50 random ewes were condition scored and a feed budget was conducted.

The mid-gestation pregnancy diagnosis visit occurred ~90 days after mating start date, in the week following the visit of a commercial ultrasound technician. This allowed the investigator to accurately record the required information. At the pregnancy diagnosis visit, each ewe's pregnancy status and condition score (CS) was recorded, faeces were taken for bulk WEC and repeat blood samples were taken on each ewe that had been bled at the 'rams in' visit. Blood samples were also taken from a selection of ewes that were not detectably pregnant.

The final ewe recording visit was at lamb marking, two weeks after the completion of lambing. At this visit, the total number of lambs in each group was counted and CS was recorded for all ewes. Blood samples were taken from ewes that successfully reared a lamb and those that didn't, and faeces were sampled for bulk WECs.

Additional farm visits occurred on one (Farms C & D) or two (Farms A & B) occasions throughout lambing to assess feed availability and collecting neonatal lambs for necropsy.

Table 4 Visits and activities for the randomised controlled field trial to assess the effect of Ovilis Campyvax® on maiden ewe reproduction on four Victorian sheep farms (Farms A to D; CS: condition score; SC: subcutaneous; V: vaccinated ewes; C: control ewes; WEC: worm egg count)

Activity	**Mating**	**End mating** ('Rams out')	**Pregnancy diagnosis** (90 days after rams in)	**Lambing**	**Lamb marking** (2 weeks after lambing end)
Identification & randomisation	Y	NA	NA	NA	NA
Ovilis Campyvax® (2 ml SC)	Group V	Group V	NA	NA	NA
Production measures	CS: each ewe Feed budget Bulk WEC	CS: 50 ewes Feed budget	CS: each ewe Feed budget Bulk WEC	NA Feed budget	CS: each ewe Feed budget Bulk WEC
Reproduction measures	NA	NA	Transabdominal ultrasound (empty, single, multiple)	NA	Ewe 'wet/dry' status Number of lambs marked
Serology	12 V 12 C	NA	Repeat serology (12 V & 12 C); include empty ewes (up to 12 per group)		Repeat serology (12 V & 12 C); include 'dry' ewes (up to 12 per group)
Lamb necropsies	NA	NA	NA	Two visits: A & B; one visit: C & D	NA

2.1.3 Ewe age and sample size

The trial was conducted on rising 2-year-old ewes over their first reproductive year ('maiden ewes'). This age cohort of ewe is considered most susceptible to *Campylobacter* infection (see Section 1.3.2.5).

Between 200 and 250 ewes per treatment group were needed to detect a 6 to 10% difference in lamb marking rate between the vaccinated and control groups with 95% confidence at 80% power (Sergeant, 2017). The difference in lamb marking rate used in this calculation was derived from the potential increase in lamb marking rate reported to occur as a result of vaccination against *Campylobacter* spp. in both New Zealand and Australia (West, 2003; Anonymous, 2011). As the total number of maiden ewes mated differed between farms (from 424 on Farm D to 2000 on Farm C), a treatment group size of ~250 was possible on only one of the enrolled farms (Farm C). All other farms had between 211 and 224 ewes per treatment group (Table 5). On Farm C, 500 ewes were randomly drafted off from the rest of their age cohort for participation in the trial. However, two of these sheep were ineligible for participating in the trial based on ill-health at the initial visit, reducing the sample size to 498. After the trial mob was established on Farm C, the trial ewes were managed in accordance with the trial design and separately from the rest of the maiden ewes on the farm.

Ewes that were not detected as pregnant were removed from the treatment groups after pregnancy diagnosis. Additionally, over the course of the trial, some ewes were mis-mustered, or were lost to follow up. This resulted in a decrease in the number of enrolled ewes (Table 5).

There was no opportunity for mixing of control and vaccinated ewes and their lambs prior to lamb marking on any property.

Table 5 Number of ewes in the vaccinated and control groups at mating, mid-gestation pregnancy diagnosis and lamb marking on each of the trial farms.

Farm	Treatment group	Ewe numbers (*n*)		
		Mating	Pregnancy diagnosis	Lamb marking[1]
A	Control	225	225	216
	Vaccinated	223	223	208
B	Control	221	217	202
	Vaccinated	222	222	210
C	Control	249	246	154
	Vaccinated	249	249	165
D[2]	Control	211	204	173
	Vaccinated	213	197	153

[1]Ewes that were not detected as pregnant at pregnancy diagnosis were removed from each group prior to lambing.

[2]The discrepancy between the number of ewes expected at lamb marking and the number of ewes present at lamb marking was most profound on Farm D, due to weather related fencing damage and mis-mustering. These issues were reported by the producer on Farm D.

2.1.4 Ewe randomisation and identification

At the first visit to each farm, ewes were randomly allocated to either a vaccinated or control group. On Farms A and B, every second ewe in a race was allocated to the vaccinated group. On Farms C and D, electronic sheep handlers were used. Ewes were drafted two ways prior to the commencement of the trial, with every second ewe drafted into the vaccinated group. On Farms B, C and D, ewes were identified using a unique radio-frequency identification tag (RFID) placed in the appropriate ear for each enterprise (Shearwell Pty Ltd, Bendigo, Victoria, Australia). On Farm A, ewes were identified with an individually numbered visual identification tag placed in the left ear (Allflex Pty Ltd, Capalaba, Queensland, Australia). Any ewe found to have a missing or dysfunctional RFID at subsequent visits was fitted with a new ear tag if treatment group could be established. Different coloured tags were used to enable visual identification of groups for ease of drafting in late pregnancy. Each producer was blind to the treatment group signified by tag colour.

An electronic data capture platform was used to record information against RFID tag on all farms except Farm A. An XR3000 indicator box and wand (Tru-test Pty Ltd, Eight Mile Plains, Queensland, Australia) were paired with the RFID ear tags (Shearwell Pty Ltd, Bendigo, Victoria, Australia). Tru-Test Data Link (version 5.1, 27/10/2015) software was used to download data files from the indicator box for use in Microsoft Excel 2016® (Microsoft Office, 2016).

2.1.5 Vaccination procedure

Ewes in the treatment group were vaccinated with two doses, 3 to 5 weeks apart, of the commercially available Ovilis Campyvax® (APVMA 61563). The vaccine is a water-in-oil emulsion adjuvanted preparation of inactivated whole cells of *C. jejuni* and *C. fetus fetus,* and the preservative Thiomersal (0.1 mg/ml). On Farms B, C and D, the first dose was given immediately prior to rams being introduced to ewes. This coincided with when ewes were yarded for standard farm management practices. The second dose was given when rams were removed 5 to 6 weeks later. On Farm A, the first dose was given several weeks after the rams were first introduced to the ewes. The second dose was given when rams were removed three weeks later.

The vaccine was administered per label directions, with a standard dose of 2.0 ml injected subcutaneously, high on the neck, using a ¼ inch needle attached to an adjustable vaccinator set at 2.0 ml (Coopers Animal Health, MSD Animal Health, NSW, AUS). The needle was changed every 50 ewes. Un-opened vials were stored refrigerated at 2 to 8°C until required. A new, well-shaken vaccine vial and administration gun were used at each visit to minimise contamination and ensure the product was used within 24 hours of opening, per product directions. No adverse effects were associated with vaccination.

2.2 Ewe management

On each of the four farms, the two treatment groups (vaccinated and control) grazed and were managed together throughout mating until late pregnancy. This avoided any ram or paddock effects on pregnancy and conception rates, nutritional differences over gestation and ensured similar exposure to any sources of *Campylobacter* for most of gestation.

At pregnancy diagnosis, ewes that were not detected as pregnant were removed from the mob to enable nutritional management according to reproductive status (Ferguson et al., 2007). The small number of empty ewes on Farm A meant that these ewes remained with their respective treatment groups until the completion of the trial but were excluded from the analysis of results from lamb marking to ensure consistency with Farms B, C and D.

Two-to-three weeks prior to the expected start of lambing, ewes received a routine pre-lambing vaccine against *Clostridial* diseases. The product used differed between farms, but each was a subcutaneous injection administered as per label instructions. Following pre-lambing vaccination, each producer drafted the ewes into treatment group based on ear tag colour. Each treatment group was then allocated to a separate paddock during late pregnancy and lambing to allow observation of each treatment group over lambing, including recording causes of death in neonates and number of lambs marked.

Throughout the trial, producers were asked to report any ewe that was observed to be unwell, had a blood-stained breech or vaginal discharge, or was found dead for any reason to the investigator. Each was also asked to observe treatment groups immediately before lambing, and report any abortions for investigation. No outbreaks of abortion were observed on any of the four farms.

2.2.1 Bulk worm egg counts

Bulk worm egg counts (WECs) were conducted on both treatment groups on each farm at three time points (mating, mid-gestation pregnancy diagnosis and lamb marking) to help guide decision making around ewe management. The results were provided as soon as possible to each producer to facilitate good animal health management.

Fresh faeces were taken per rectum from 10 randomly selected ewes per treatment group. The modified McMaster technique was used to determine the average worm egg count for each group (WEC; eggs per gram: epg). The WEC results were provided to each farmer, with an interpretation and recommendation for how to proceed. Where egg counts breached tolerable thresholds, administration of an anthelmintic treatment was recommended for both groups (~50 epg for a second summer drench following the first visit and ~250-300 at all other times; Love and Hutchinson, 2007).

Ewes were last drenched 1.5 to 9 months before mating started, depending on the farm. Long-acting drench capsules were administered prior to lambing to most maiden ewes in the trial on Farm D. At the producer's discretion, some ewes were not capsuled. The ewes that did not receive a capsule were not permanently identified, and therefore were not able to be excluded from the random sampling at the lamb marking visit.

Descriptive results only are provided for the bulk WEC results, with no statistical analysis performed due to a lack of within-group replication (one bulk WEC was performed per treatment group per time point).

2.3 Paddock selection and management

Lambing paddocks were carefully selected to reduce potentially confounding paddock factors. Feed on offer, paddock area hence stocking rate, aspect and the availability of shelter were all considered by the investigator and the producers. The lambing paddocks were adjacent to one another, or met on one corner.

On Farm A, smaller paddock sizes and higher conception rates caused the producer concern over high stocking rates at lambing and possible increased risks of mismothering. To address this, two sets of paired paddocks were selected; one set on the eastern side of the farm (127 control ewes in paddock 1, 130 vaccinated ewes in paddock 2), the other

on the western side (91 control ewes in paddock 3, 91 vaccinated ewes in paddock 4). The multiple bearing ewes were equally allocated between the paddocks.

To minimise predation, predator control was recommended prior to lambing on each property. The degree to which this was conducted was at the discretion of the producer.

2.3.1 Rainfall

The Australian Government Bureau of Meteorology monthly rainfall (mm) records were accessed to graph the rainfall for the recording stations closest to each farm (Farm A: Yea, Farm B: Meredith and Farms C and D: Edenhope). This was overlaid on historical data from the Bureau of Meteorology reporting mean, 10^{th} and 90^{th} percentiles of monthly rainfall for all years on record at each of the three locations (Anonymous, 2017).

2.4 Production traits in ewes

2.4.1 Body condition scoring

All ewes were condition scored from 1 to 5 to the nearest quarter, according to standard practice, at three time points: mating, mid-gestation pregnancy diagnosis and lamb marking (Jefferies, 1961; Russel et al., 1969; Russel, 1984). At the second visit, a random 50 ewes were condition scored to monitor nutrition and condition during autumn.

One operator was responsible for condition scoring all ewes except for during visit one to Farm C, when two operators were unavoidably responsible for condition scoring. One operator scored the first 99 ewes, whilst the second operator, who condition scored at all other visits, scored from ewe 100 to ewe 500. Later analysis revealed a difference between the two operators, despite attempts to standardise. Consequently, the first 99 condition scores from visit one, Farm C were removed from the analysis as they were deemed unreliable. Removing these records did not substantially change the output of the multivariable models.

After the lamb marking visit, the CS of any ewe that was not pregnant at pregnancy diagnosis but was present at lamb marking was removed from the analysis to allow fair comparison between farms. This was especially the case on Farm A, where ewes that were not detected as pregnant were intentionally retained in the mob by the producer.

2.4.2 Estimated metabolisable energy requirements and intakes of ewes

Ewe nutritional requirements were compared with available feed at four time points (mating, end-mating, mid-gestation pregnancy diagnosis and lambing). Feed–budgeting tools developed for the Lifetime Ewe Management project were used to calculate daily feed intake, as all producers were familiar with these tools and their output (Ferguson et al., 2007). Feed on offer (FOO; kg DM/ha) was estimated by a single observer (Curnow et al., 2011).

Poor pasture growth in the spring preceding this trial (2015), and a late autumn break in 2016, meant supplementary feed was required on all farms through mating and early gestation. Barley was offered through to mid-gestation on Farms A and B, but stopped one month before mid-gestation pregnancy diagnosis on Farms C and D. Straw was included in the ration on Farm B, and hay was included in the ration at one point on Farm D. The quantity and quality of any supplementary feed offered was included in the feed budget, including the results of any available independent feed tests.

Daily intake (MJ ME DM/day) was compared to daily energy requirements (MJ ME/ewe/day) to determine whether the nutritional requirements of the pregnant or lactating ewe was met. The daily requirements were flock and visit specific, and were calculated based on the stage of gestation or lactation (dry and early pregnant ewes 8.3 MJ ME/day, day 80 pregnant ewes 9.3 MJ ME/day, day 10 lactation for Farms A & B 18.7 MJ ME/day, day 28 lactation for Farms C & D 20.2 MJ ME/day) multiplied by a factor determined by the frame-size of the ewe (Table 6; Anonymous, 2014). A frame-size dependent intake multiplier was also used (Table 6; Anonymous, 2014).

The difference between requirement and intake was used together with the Lifetime Ewe Management CS change tables to estimate the likely change in condition over the following 30 days (Anonymous, 2014). If the feed budget results suggested that ewes were likely to lose more than 0.1 CS over the coming month, recommendations were made to adjust the feed on offer to maintain ewe condition.

Table 6 Requirement and intake multipliers for the average ewe frame-size on each of the four farms, used to calculate whether energy requirements were met (from Anonymous, 2014)

Farm	Average ewe frame-size (kg)	Requirement multiplier[1]	Intake multiplier[1]
A	62.5	1.2	1.275
B	45	0.92	0.89
C	35	0.76	0.67
D	40	0.84	0.78

[1]Lifetime Ewe Management: Ewe Condition Manager. Tables 2 (requirement multiplier for different live-weight ewes) and 4 (intake multiplier for ewes of different live-weight).

2.5 Reproduction traits

2.5.1 Pregnancy status and conception rate

The pregnancy status of each ewe was determined 80 to 90 days after mating started using trans-abdominal ultrasound. To fit in with the management practices on each farm, three different but highly experienced commercial contractors were used. Each contractor diagnosed the pregnancy status of each ewe, and an indelible stock-marker was applied to the back of the ewe to designate whether she was detected as not-pregnant, or carrying a single foetus or multiple foetuses. Within one week of pregnancy diagnosis, each ewe's pregnancy status was recorded against her RFID following the contractor's diagnosis.

The number of pregnant ewes divided by the number of ewes present at pregnancy diagnosis was used to calculate the proportion of pregnant ewes (Table 7).

The number of foetuses conceived divided by the number of ewes present at pregnancy diagnosis was used to calculate the conception rate (Table 7).

The denominator for each of these measures differed from convention, i.e. the number of ewes mated. This allowed for a fair comparison between farms, as it accounted for complications with mis-mustering on Farm D, which resulted in fewer ewes present at pregnancy diagnosis than at mating.

Table 7 Reproductive rates used in the analysis of reproductive output of ewes in both vaccinated and control groups on each of the trial farms.

Reproductive measurement	Numerator	Denominator[1]
Proportion pregnant[1]	Number of pregnant ewes	Number of ewes present at pregnancy diagnosis
Conception rate[1]	Number of foetuses ((# single-bearing ewes) + (# multiple bearing ewes)*2)	Number of ewes present at pregnancy diagnosis
Lamb marking rate[2]	Number of lambs present at lamb marking	Number of ewes present at lamb marking
Lamb survival	Number of lambs present at lamb marking	Number of foetuses expected based on mid-gestation pregnancy diagnosis

[1]The denominator differs from convention, which is the number of ewes mated, due to mis-mustering on Farm D prior to pregnancy diagnosis. The pregnancy status of mis-mustered ewes was not known, and to allow for fair comparison between farms, the decision was made to utilise the same denominator for each farm.

[2]The denominator differs from convention, which is the number of ewes mated, due to fencing and mis-mustering on Farm D before lamb marking.

2.5.2 Cross sectional study of cause of neonatal lamb mortality

A cross-sectional study of cause of neonatal lamb death in each treatment group on each farm was conducted. Each producer was asked to pick up all dead neonates from each trial paddock for three consecutive days for submission for necropsy. Two necropsy days per farm, a minimum of one week apart, were planned to gather a representative sample of causes of death and minimise any bias contributed to by daily variation in weather. Carcasses were refrigerated on farm prior to submission, if collected more than 12 hours beforehand. The number of lambs necropsied on each farm is presented in Table 8.

The necropsy procedure followed that described in the NSW Department of Primary Industries Lamb Autopsy guide (Holst, 2004). Likely cause of death was determined based on gross lesions, and where appropriate, microbiology and histology. Cause of death was allocated to one of five categories: the starvation-mismothering-exposure ('SME') complex; dystocia; primary predation; congenital malformation or prematurity and infection. 'Unknown' was included as a sixth category. Gender and weight were also recorded, and are reported within the necropsy results (Section 3.2.2).

Regardless of treatment group, if an infectious agent was suspected based on the presence of any of the lesions described in Section 1.3.2.1, or there was no obvious cause of death based on gross post-mortem findings, fresh abomasal content was aspirated using sterile technique (sterile 18 gauge needle attached to a 5 ml syringe). Additionally, fresh liver and lung were sampled. Samples were submitted as soon as possible to the University of Melbourne Clinical Microbiology Laboratory for inoculation on selective media (Skirrows agar, Media Preparation Unit, University of Melbourne, Australia; R. Bushell, personal communication, November 2017). The samples were incubated at 37°C and 42°C in micro-aerophilic conditions (5% O_2, 10% CO_2, 85% N_2, CampyGen™, Oxoid Ltd., United Kingdom). Suspect colonies were stained using dilute carbol fuchsin and any morphologically similar to *Campylobacter* were sub-cultured for species identification using phenotypic testing (R. Bushell, personal communication, November 2017).

In suspicious cases, impression smears of liver and smears of the abomasal contents were made during necropsy. Smears were later stained with gram stain or dilute carbol fuchsin for examination under light microscopy to check for the presence of bacteria morphologically similar to *Campylobacter* spp. (R. Bushell, personal communication, November 2017).

Lambs were collected for necropsy from Farms A and B twice during lambing, at least one week apart and spanning the anticipated peak of lambing during week 2. Lambs could only be collected at one time point on Farms C and D, which coincided with week three of lambing (see Section 4.5). Necropsy kits, recording sheets and sampling equipment were sent out to the latter farms to facilitate collection of information on lamb mortality from these farms (Appendix 2). Following instruction from the investigating veterinarian, the producers on Farms C and D safely opened the peritoneal cavity of any deceased lamb with no obvious cause of death, or where there was a suspicion of an infectious agent, and swabbed the liver with a gel swab on advice from the laboratory (R. Bushell, personal communication, September 2016). This included lambs that were small, meconium stained, and/or had swollen abdomens. Swabs were appropriately packaged and express-posted to the University of Melbourne for microbiological culture and sensitivity.

On the day Farm D was visited, the number of deceased lambs collected for necropsy was small (Table 8). However, the producer conducted lamb pick-ups every second day throughout peak lambing when weather permitted. Guided by the investigator, prior experience and the NSW DPI Autopsy manual, the producer attributed cause of death to one of five categories: stillborn, dystocia, exposure (included mismothering and starvation), major predation or no obvious cause. The producer also recorded the total number of lambs picked up. These additional results were not included in the investigators necropsy results, as they could not be verified. However, they are included as a supplementary set of results (see Section 3.3.1.1).

On the days when lambs were collected for necropsy on Farms A, B and C, there were fewer dead lambs in the vaccinated paddocks than the control paddocks. Farmers observed that the vaccinated ewes started lambing slightly later than the control ewes, which may explain why fewer lambs were available from the vaccinated groups on the days when necropsy collections took place (discussed further in Section 4.4.1). All dead lambs that were found in each paddock were submitted for necropsy, except for four lambs from the vaccinated group on Farm A. These were omitted by the producer, who believed they were clear cases of dystocia and so did not require necropsy. They could not be retrieved in a timely manner for necropsy and hence are not included in the results.

Farm specific findings were reported to each producer, and future actions to attempt to minimise major causes of loss were discussed.

Table 8 Total number of lambs necropsied from each treatment group on each of the trial farms.

	Number of lambs necropsied	
Farm	**Control group**	**Vaccinated group**
A	11	4[1]
B	27	17
C	11	4
D[2]	1	1
Total	**50**	**26**

[1]**Farm A**: on the sampling days, the producer observed but did not submit four cases of dystocia in lambs from the vaccinated group. The implications of this are addressed in the discussion. [2]**Farm D** picked up lambs every second day and based on the producers' previous experience and Lamb Autopsy guidelines, attributed cause of death based on gross findings. These results were not included in the overall set of necropsy results but are included as a separate set of results.

2.5.3 Lamb marking rate, lamb survival and 'wet' and 'dry' ewes

At lamb marking, each treatment group of ewes and lambs was processed individually, allowing the number of lambs produced by each group to be accurately counted. The number of lambs was divided by the number of ewes present at lamb marking on all farms, to give the lamb marking rate (Table 7). The denominator used differs from convention, i.e. the number of ewes mated. Complications with mis-mustering in the vaccinated ewes on Farm D meant that to enable a fair comparison between each farm, the number of ewes present at lamb marking was used as the denominator.

The number of lambs counted at lamb marking was also used in a calculation of lamb survival. Lamb survival from mid-gestation pregnancy diagnosis was determined by dividing the number of lambs present in each of the two treatment groups at marking by the number of foetuses expected based on the results of pregnancy diagnosis.

The udder of every ewe was examined in the race by an assistant or by the producer. Ewes that were detected as pregnant at pregnancy diagnosis but had no udder or a very dirty udder at lamb marking were recorded as 'dry'. It is likely that these ewes either lost lambs in late pregnancy or had lambs that succumbed as neonates. Ewes with developed udders and clean teats were recorded as 'wet'.

2.6 Ewe antibody titres to *C. fetus fetus* and *C. jejuni*

Twelve ewes from both treatment groups on each of the four farms were randomly selected at the initial visit to monitor both the exposure to *Campylobacter* (control ewes) and the humoral response to vaccination (vaccinated ewes), at three time points over the course of the trial. At each time point, jugular venepuncture was conducted using a new 18 gauge 1 inch needle for each ewe. Around 5 ml of blood was collected into a plain vacutainer. The sample size was calculated to give a 95% confidence that exposure would be detected if present at a given prevalence of 30% given the use of an imperfect test (assuming 75% sensitivity; Stevenson and Firestone, 2015).

To investigate links between gestational *Campylobacter* exposure and reproductive output, a sub-sample of ewes that were not-pregnant at pregnancy diagnosis (≤12 ewes per group) and a sub-sample of those that were pregnant but did not have an udder ('dry') at lamb marking (12 ewes per group) were also sampled for serological testing (Table 9).

All serum samples were sent to an external, independent laboratory for serology (ACE Laboratory Services, Bendigo, Victoria, Australia). An Agar Gel Immunodiffusion (AGID) test was used to determine the titres to both *C. fetus fetus* and *C. jejuni* prior to vaccination, at pregnancy diagnosis and at lamb marking. The AGID is reported to have good specificity and moderate sensitivity (A. Vanderfeen, personal communication, November 2017).

The AGID results are semi-quantitative, and require interpretation, although there is no definitive peer-reviewed information regarding the interpretation of serology results. However, guidelines have been recommended that are used extensively by members of the Australian and New Zealand Coopers Animal Health Technical Services teams (Anonymous, 2015; Walsh, 2016). In line with these recommendations, each ewes' serological status for both *Campylobacter* species was categorised as 'low' (<1:10, naïve animals with no previous exposure), 'moderate' (1:10–1:60; background or historic exposure, likely endemic in flock) or 'high' (≥1:80; recent exposure likely associated with reproductive loss or vaccination).

Table 9 Sampling strategy for ewe serology on each of the trial farms, with sample size (*n*) according to visit, treatment group and ewe reproductive status.

Visit	Group	Reproductive status	*n*
Pre-vaccination	Control	Empty	12
(mating)	Vaccinated	Empty	12
Pregnancy diagnosis	Control	Not-pregnant & pregnant	≤12[1]
	Vaccinated	Not-pregnant & pregnant	12
Lamb marking	Control	'dry' (no detectable udder) & 'wet'	12
	Vaccinated	'dry' (no detectable udder) & 'wet'	12

[1]The serostatus of up to 12 ewes that were not detected as pregnant by ultrasound was determined. The number of ewes sampled here was dictated by the number of ewes that were not-pregnant in each group. Hence on farm A, at pregnancy diagnosis, only three ewes were sampled in the control group and 10 in the vaccinated group.

2.7 Statistical analysis

Continuous variables were analysed using simple t-tests assuming equal variances to test for significant differences between treatment groups at each time point on each farm. Results for continuous variables are presented as the mean ± standard error of the mean (SEM).

Categorical data was analysed using Chi-square tests where cell values were ≥5, or Fisher's exact test if cell values were <5, to compare between groups within farm. Lamb survival between mid-gestational pregnancy diagnosis and lamb marking was analysed using a two-tailed two-sample Z-test of proportions to test for a difference between-groups within-farm (Sergeant, 2017). Count data were analysed using univariable or multivariable Poisson regression. Categorical and count data are presented as the mean (lower 95% confidence interval (CI); upper 95% CI).

Two-tailed Fisher's exact tests were also used to compare the numbers of ewes in different antibody titre categories between vaccinated and control groups within-farm at each time point, and within-treatment groups between time points within-farm. As described previously, titres were classified as 'high', 'medium' or 'low' based on cut-offs of ≥1:80, 1:10-1:60 and <1:10. The difference in the proportion of ewes 'exposed' to each *Campylobacter* species (i.e., those with either 'high' or 'moderate' titres) was analysed separately in pregnant or not-pregnant control ewes, and in 'wet' or 'dry' controls, on each farm also using two-tailed Fisher's exact tests.

In the cross-sectional study of neonatal lamb mortality, for each cause of death, two-tailed Fisher's exact tests were used to compare whether there was any difference in the proportion of lambs born to vaccinated ewes and lambs born to control ewes recorded as having died of that cause. Two-tailed t-tests assuming unequal variance were used to test for an effect of treatment on the body-weight of necropsied lambs within farm and over all farms. Two-tailed Fisher's exact tests were also used to analyse the difference in proportions of lambs recorded as having died due to a specific cause of death on Farm D.

Multivariable regression models were used to test for the effect of treatment on various reproduction parameters, accounting for the concurrent effect of farm, CS and CS change between mating and pregnancy diagnosis. The specific multivariable models used are reported in Table 10. The odds ratio (OR) represents the ratio of the odds of ewes being

pregnant between vaccinated and control groups. Similarly, the incidence rate ratio (IRR) in these analyses corresponds to the ratio of lamb marking rates (number of lambs marked divided by number of ewes present at lamb marking) between vaccinated and control ewes. A term for 'farm' was included as a fixed or random effect in separate models for each reproductive outcome.

Purposeful forward selection of covariates was used to build the models, using all terms in univariate tests significant at an alpha level of 0.20. All first-order interactions between model terms were tested and included if significant at an alpha level of 0.05. Where these conditions were met but several interaction terms were eligible for inclusion in the statistical model, the 'treatment' term was preferentially retained to explore its associations with the outcome variables. Condition score at lamb marking was used as a covariate in the lamb marking analyses, as it is the best summary measure of a ewe's total nutritional status in later pregnancy and during lambing, and absolute CS has a strong association with reproductive outcomes (Kleemann and Walker, 2005a; Kleemann et al., 2006; Behrendt et al., 2011). Where competing models were constructed with different parameters, the most biologically plausible model with the smallest Akaike Information Criterion (AIC) score was chosen (Dohoo et al., 2009).

A Poisson regression model was chosen over a zero-inflated, zero-truncated or negative binomial model for the following reasons. The negative binomial model was rejected because the variances of the independent variables being analysed did not exceed their means. The zero-truncated model was rejected because of the presence of zeros in the dataset, representing ewes that failed to conceive or raise a lamb (Dohoo et al., 2009). The denominator against which lambs present at marking were analysed is a count of ewes present at marking and some of the ewes had no lamb and are therefore 'zero' records. The zero-inflated model was rejected because all ewes had the opportunity to be mated by a ram, and therefore a zero for conception meant only that a ewe failed to conceive (Dohoo et al., 2009). With respect to marking rates, the ewes that failed to conceive were removed from each mob, and from the dataset, and so a zero at lamb marking meant only that a ewe failed to rear a lamb. The decision was made separately for each variable.

Statistical analyses were conducted using Microsoft Excel 2016®, EpiTools (Sergeant, 2017), WinPepi (Abramson, 2016) and STATA (StataCorp, 2017). For all statistical analyses, a P-value < 0.05 was considered statistically significant.

Table 10 The regression model and statistical measure used for each reproductive measure.

Reproductive measure	Regression model	Statistical measure
Pregnancy proportion (pregnant/not pregnant)	Logistic	Odds ratio (OR)
Conception rate (no. foetuses ÷ no. ewes at pregnancy diagnosis)	Poisson	Incidence rate ratio (IRR)
Lamb marking rate (no. lambs marked ÷ no. ewes present at marking)	Poisson	Incidence rate ratio (IRR)

3 – Results

3.1 Rainfall

On all farms, rainfall in the first third of the year was at or below mean monthly rainfall (Figure 4). However, rainfall was higher than the monthly mean over lambing on Farms C and D, with consequences for flock management and trial outcome (see Appendix 3). Lambing coincided with monthly rainfall exceeding the 95th percentile on Farms C and D.

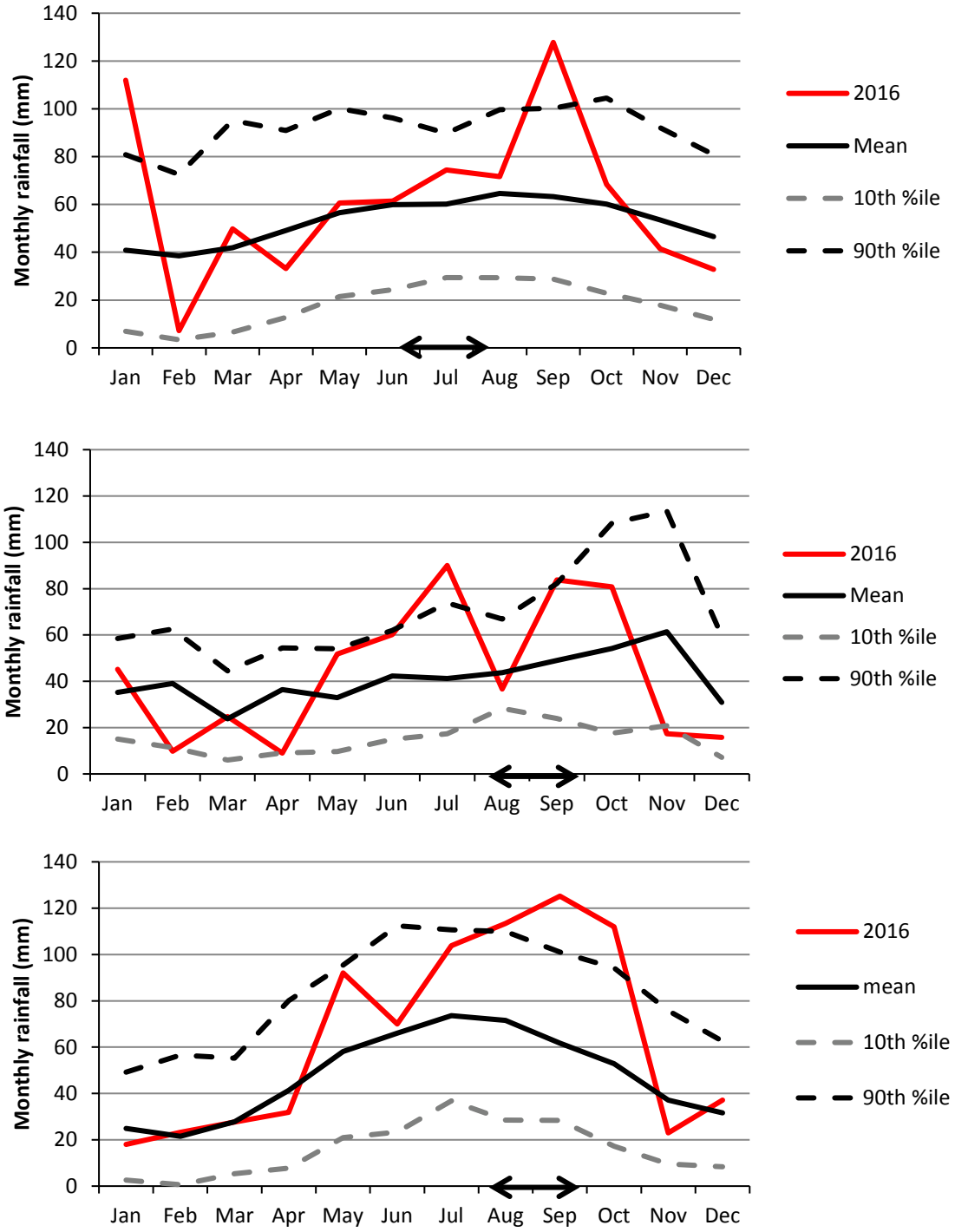

Figure 4 Monthly rainfall for 2016 compared with mean, 10th and 90th percentiles on Farms A (top), B (middle), C and D (bottom). Approximate time of lambing is represented by a dark arrow (rainfall data from Bureau of Meteorology 2017; http://www.bom.gov.au/climate/data; Yea, Meredith, Edenhope).

3.2 Production traits

3.2.1 Ewe condition score

Ewes on all farms lost condition over the course of the trial, from mating through to lamb marking (Figure 5). The difference in condition score (CS) between vaccinated and control groups, and the trajectory of CS over the reproductive year varied between farms. However, there was less than 0.1 CS difference between groups at all time points except for lamb marking on Farm C, as discussed below.

3.2.1.1 Mating

Mean ewe CS at mating approached the target condition for mating (CS 3.0) on all but one farm (Farm C; Figure 5). There was no significant difference between the CS of control and vaccinated groups at the initial visit on Farms A ($P = 0.10$) and B ($P = 0.31$; Figure 5). There was a difference of 0.08 of a CS between control and vaccinated ewes at mating on Farm C ($P < 0.001$). On farm D, there was a difference of 0.03 of a CS between control and vaccinated ewes at mating ($P = 0.05$). At mating, ewes ranged from CS 2.5 to 4.0 on Farm A, 2.5 to 3.5 on Farm B, 2.0 to 3.5 on Farm C and 2.5 to 3.25 on Farm D (Figure 6).

3.2.1.2 Pregnancy diagnosis

There was no significant difference in mean ewe CS between the treatment groups on any farm at pregnancy diagnosis (A: $P = 0.34$; B: $P = 0.43$; C: $P = 0.97$; D: $P = 0.63$; Figure 5).

Between mating and pregnancy diagnosis, there was a small increase in CS of ewes on Farm A. The increase was significantly greater in the control ewes (0.07 ± 0.02) compared to vaccinated ewes (0.02 ± 0.02; $P < 0.05$).

Condition score fell over the three months between mating and pregnancy diagnosis on Farms B, C and D. On Farm B, both vaccinated and control ewes lost 0.08 of a condition score ($P = 0.78$). On Farm D, the vaccinated ewes lost more condition (-0.16 ± 0.02) than the control ewes (-0.11 ± 0.02), which was marginally significant ($P = 0.06$). The vaccinated ewes on Farm C lost significantly more condition (-0.16 ± 0.02) than the control ewes (-0.09 ± 0.02) between mating and pregnancy diagnosis ($P < 0.01$).

3.2.1.3 Lamb marking

Two sets of results are presented. Firstly, the overall CS of treatment groups independent of ewe 'wet' or 'dry' status at lamb marking, to analyse the overall effect of treatment on ewe condition. Secondly, an analysis of CS of ewes detected as having an udder ('wet') compared with those that were not detected as having an udder ('dry').

At lamb marking, there was no significant difference in the CS of vaccinated compared to control ewes on either Farm A ($P = 0.21$), B ($P = 0.17$) or D ($P = 0.34$; Figure 5). Condition score in both vaccinate and controls decreased between pregnancy diagnosis and lamb marking, but there was no difference in the magnitude of change on Farm A (vaccinated ewes lost -0.25 ± 0.02; control ewes lost -0.21 ± 0.03; $P = 0.35$) or B (vaccinated ewes lost -0.13 ± 0.02; control ewes lost -0.15 ± 0.02; $P = 0.45$). Condition score increased by a similar amount in both treatment groups on Farm D (vaccinated ewes increased by 0.09 ± 0.04; control ewes increased by 0.15 ± 0.04; $P = 0.39$). On Farm C, the CS of vaccinated ewes (2.42 ± 0.02) as significantly lower than that of control ewes (2.61 ± 0.02; $P < 0.001$). Condition score did not change in control ewes from pregnancy diagnosis to lamb marking, but fell in vaccinated ewes by 0.17 ± 0.02 CS ($P < 0.001$).

When the effect of a ewe's reproductive status at lamb marking, i.e. whether she was 'wet' or 'dry', was analysed, there remained no effect of treatment on condition score on Farms A, B and D (wet vaccinated versus wet control: $P = 0.36, 0.65, 0.19$, respectively; dry vaccinated versus dry control: $P = 0.67, 0.13, 0.27$, respectively; Figure 7). Ewes that reared a lamb, as determined by the presence of an udder, were between 0.22 and 0.45 lower in CS than ewes that were detected as pregnant but failed to rear a lamb (Table 11).

However, on Farm C, vaccinated 'wet' ewes were on average 0.19 of a CS lighter than control 'wet' ewes ($P < 0.001$; Figure 7). Vaccinated 'dry' ewes were also significantly lower in CS than control 'dry' ewes (-0.21 CS; $P < 0.001$). The 'dry' ewes were on average 0.25 CS heavier than the 'wet' ewes in each treatment group (vaccinated 'dry' versus 'wet': $P < 0.001$; control 'dry' versus 'wet': $P < 0.001$).

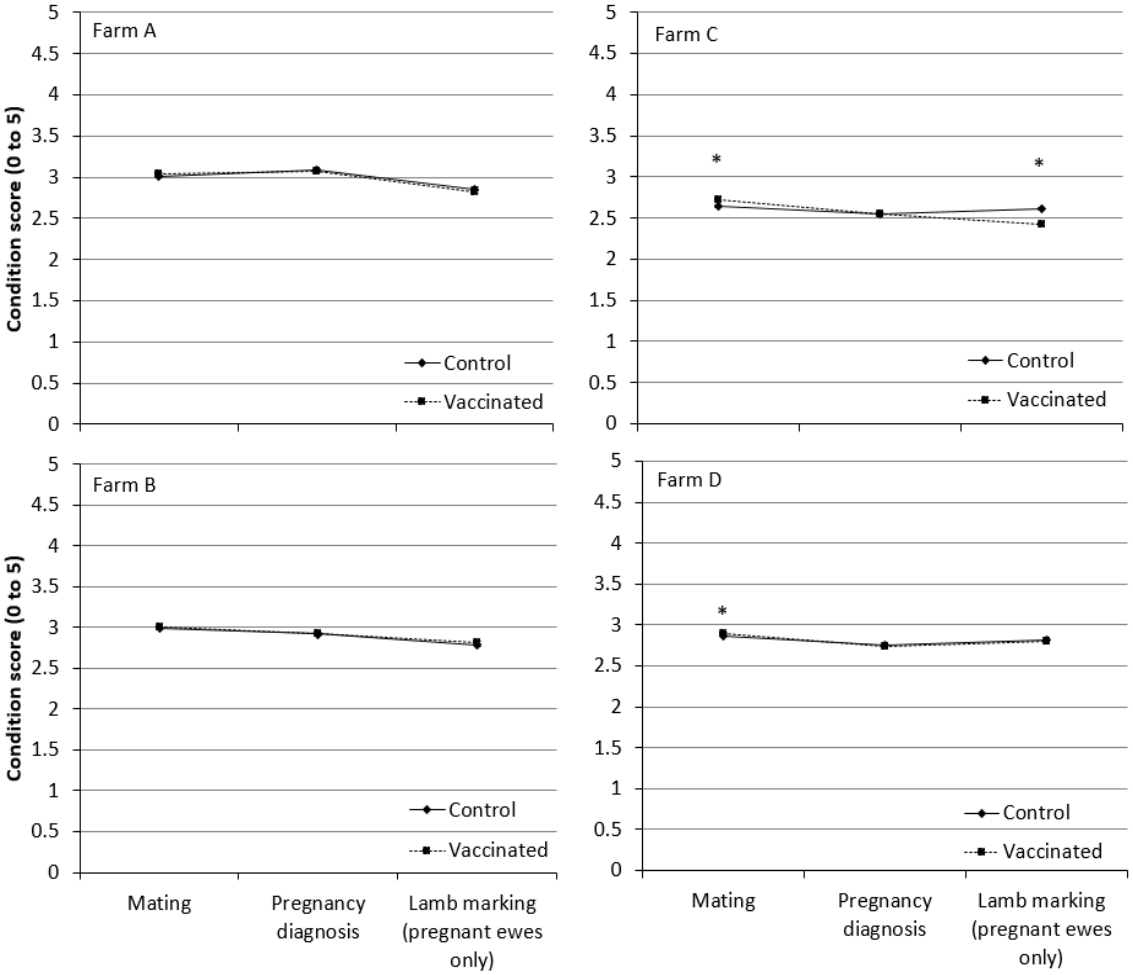

Figure 5 Condition score of vaccinated (broken black line) and control (solid black line) ewes on each trial farm at mating, mid-gestation pregnancy diagnosis and lamb marking. Standard error of mean condition score was ≤ 0.02 for all points. '*' denotes a statistically significant difference between treatment groups at a point in time ($P < 0.05$).

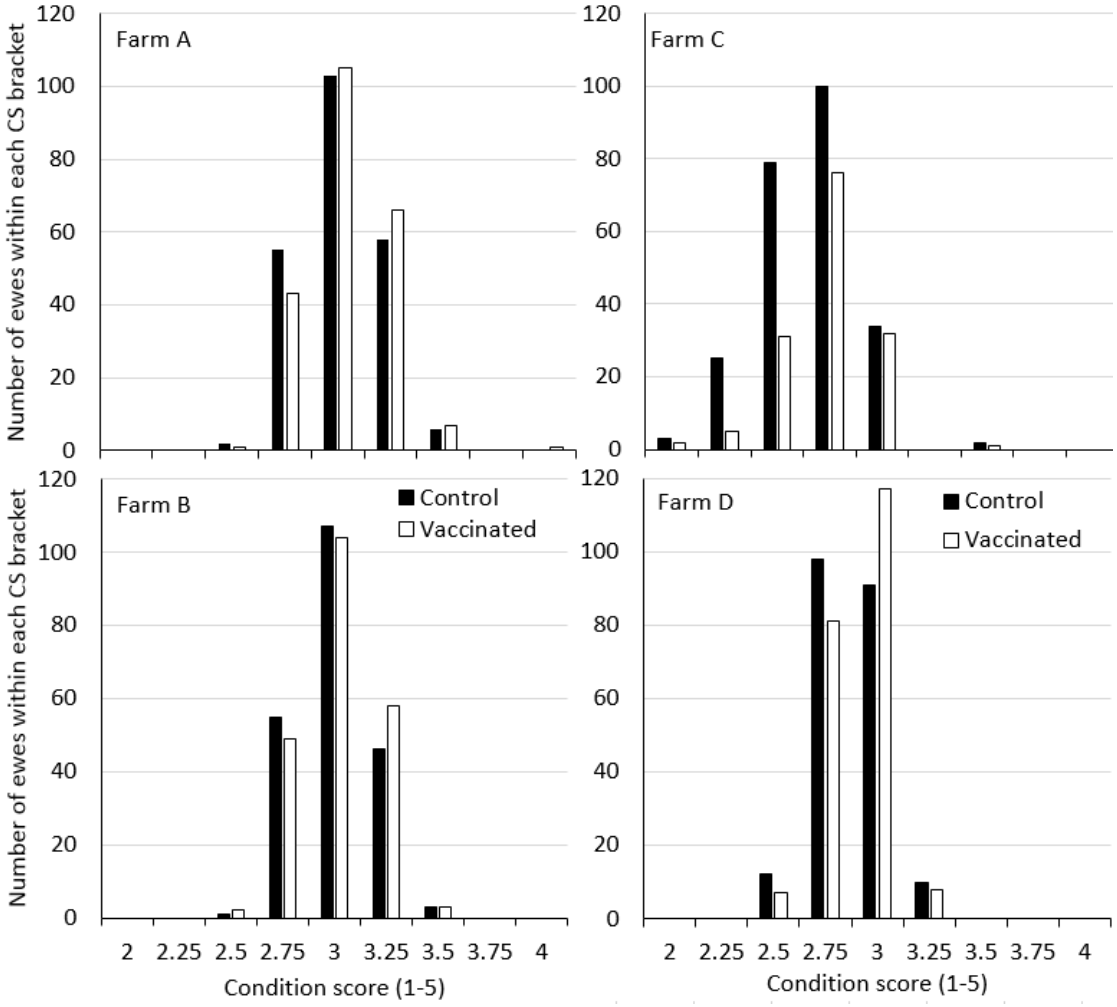

Figure 6 Frequency histogram of condition score (CS) at mating of maiden ewes in the control (black column) and vaccinated (white column) groups on each trial farm.

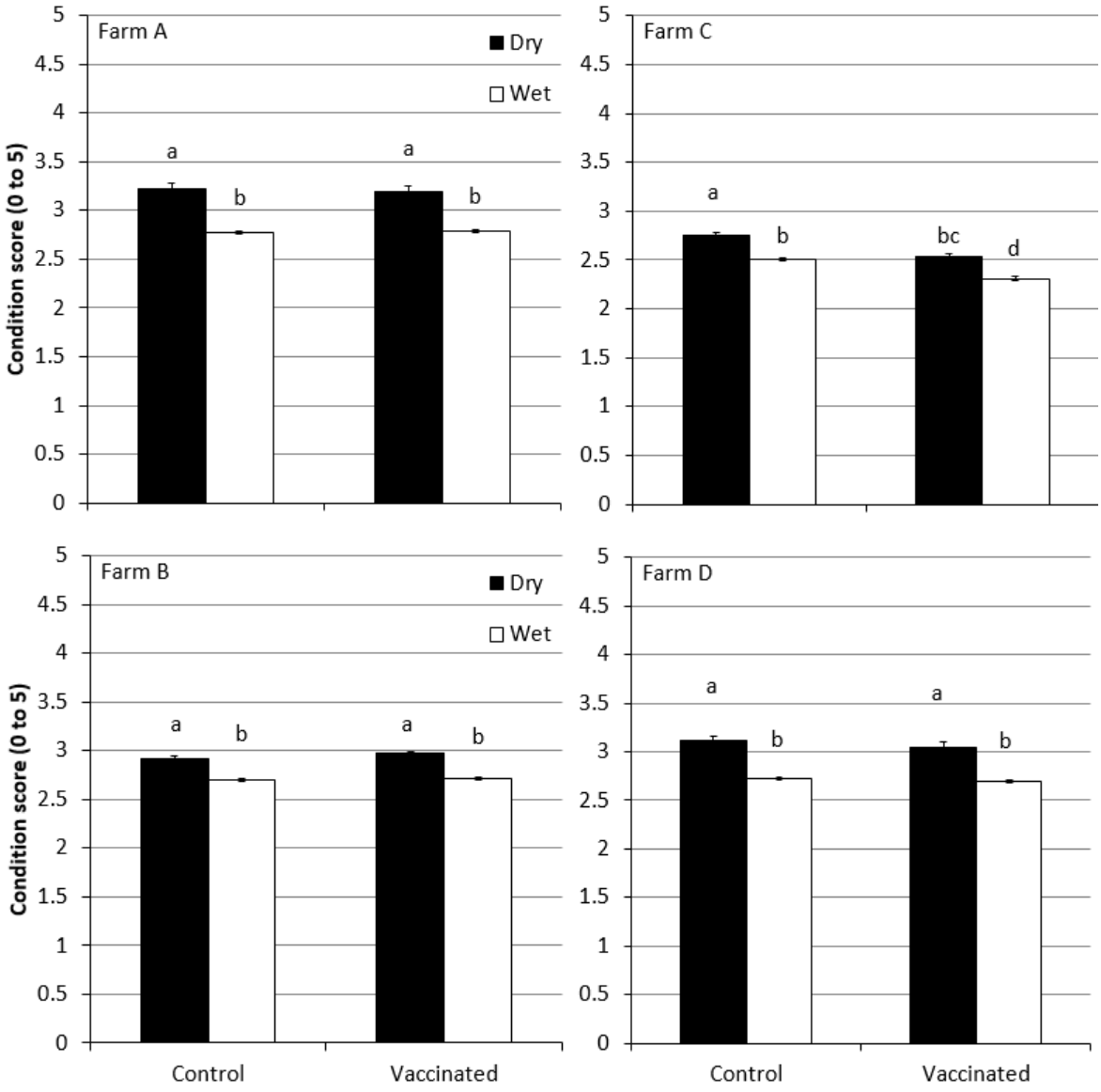

Figure 7 Condition score (mean ± SEM) at lamb marking of control and vaccinated ewes diagnosed pregnant in mid-gestation that failed to rear a lamb ('dry': black columns) and that reared a lamb ('wet': white columns) on each trial farm. Different letters show a statistically significant difference between-groups within-farms ($P < 0.05$).

Table 11 Mean difference in condition score (CS) at lamb marking between ewes that were detected as pregnant at mid-gestation pregnancy diagnosis and were either 'wet' or 'dry' at lamb marking.

Farm	Treatment group	Absolute CS difference between 'wet' ewes and 'dry' ewes at lamb marking
A	Control	0.45
	Vaccinated	0.40
B	Control	0.22
	Vaccinated	0.26
C	Control	0.25
	Vaccinated	0.23
D	Control	0.40
	Vaccinated	0.35

3.2.2 Estimated metabolisable energy requirements and intakes of ewes

Before and during mating, ewes on all four farms consumed supplementary barley and dry standing pasture, estimated to be sufficient to maintain condition throughout mating (Table 12). By the end of mating, the quality of the standing dry feed was predicted to have further declined (Court et al., 2010). At the discretion of each producer, the daily barley ration offered to the trial ewes increased on Farm A, was maintained on Farms B and D but decreased on Farm C. Consequently, the discrepancy between daily intake and ewe requirements was less on Farm A than on any other farm. The decreased barley ration on Farm C resulted in the estimated intake being 1.32 MJ metabolizable energy (MJ ME) less than requirements. This deficit was estimated to cause a loss of 0.14 CS in one month, from the end of mating to mid-gestation pregnancy diagnosis.

At pregnancy diagnosis, ewes on both Farms A and B were still being fed supplementary barley. The feed-on-offer (FOO) on Farm A was estimated to exceed requirements by 56% (Table 12), which was reflected in the measured CS change (Figure 5), and so supplementation was reduced. Estimated ewe requirements at pregnancy diagnosis were met on Farm B, but ewe CS decreased slightly from mating to mid-gestation (Figure 5). Ewe ME intake on Farm C was estimated at 80% of requirement (Table 12), associated with a measured decrease in CS (Figure 5). Estimated ewe ME intake on Farm D was slightly below requirement and was also associated with a decrease in CS (Figure 5).

Ewes on Farm A were allocated to two pairs of paddocks prior to lambing: one pair on the east side of the farm, and the other on the west. FOO in both pairs of paddocks was estimated to exceed requirements for late pregnancy and lactation (Table 12). There was no difference in the estimated FOO in vaccinate and control paddocks within each pair.

On Farm C, estimated FOO at day 28 of lactation for both vaccinate and control groups was less than requirements (vaccinate: -6.0 MJ ME DM/day; control: -3.9 MJ ME DM/day). Flooding occurred on Farms C and D during lambing (Figure 4), reducing FOO in both control and vaccinated paddocks. However, the effect was worse in the vaccinate paddock on Farm C, where estimated ME intake was 39% less than requirements. However, no alternative paddocks were available, and the paddock could not be accessed throughout the second half of lambing.

Table 12 Estimated energy requirement and intake (MJ ME/day), predicted monthly change in condition score (CS), and feed source throughout pregnancy and lactation on trial farms. Red text indicates differences in estimated feed availability between control (C) and vaccinated (V) groups within a farm.

Time point	**Farm**	**Requirement** (MJ ME/day)	**Intake** (MJ ME /d)	**Deficit / surplus** (ME/d)	**Predicted CS change** (30 d)	**Feed source**
Mating	A	9.96	9.04	-0.92	-0.07	Barley & dry pasture
	B	7.64	8.18	+0.55	0.03	Barley & straw
	C	6.31	6.39	+0.08	0.05	Barley & dry pasture
	D	6.97	7.26	+0.29	0.02	Barley & hay
End-mating	A	9.96	10.74	+0.78	0.05	Barley & dry pasture
	B	7.64	7.68	+0.05	0.00	Barley & straw
	C	6.31	4.98	-1.32	-0.14	Barley & dry pasture
	D	6.97	6.18	-0.79	-0.08	Barley & dry pasture
Pregnancy diagnosis	A	11.16	17.42	+6.26	0.25	Barley & dry pasture
	B	8.56	8.54	-0.02	0.00	Barley & straw
	C	7.07	5.63	-1.44	-0.15	Pasture only
	D	7.81	7.18	-0.64	-0.05	
Lambing (C)	A East[1]	22.44	26.78	+4.34	0.15	Pasture only
	A West[1]	22.44	23.97	+1.53	0.08	
	B[1]	17.20	16.73	-0.47	-0.05	
	C[2]	14.21	11.46	-3.90	-0.39	
	D[2]	15.71	13.96	-3.01	-0.31	
Lambing (V)	A East	22.44	26.78	+4.34	0.15	Pasture only
	A West	22.44	23.97	+1.53	0.08	
	B	17.20	16.29	-0.92	-0.10	
	C	14.21	9.38	-5.97	-0.60	
	D	15.71	13.96	-3.01	-0.31	

[1]Feed on offer assessed at day 10 lambing; [2]feed on offer assessed at day 28 lambing

3.2.3 Worm egg counts

No overt clinical signs of gastrointestinal nematodiasis were observed on any farm at any stage of the trial.

All ewes were treated with anthelmintic at mating due to elevated WECs, except on Farm B (Table 13). Worm egg counts decreased from mating to pregnancy diagnosis, following the administration of anthelmintic to all trial ewes on Farms A, C and D (Table 13). A moderate peri-parturient rise in egg output contributed to the increase in WEC from pregnancy diagnosis to lamb marking. This was most marked on Farms B and C.

At lamb marking, the WEC of the vaccinated ewes in the western paddocks on Farm A was notably higher than the WEC of the control ewes in the western paddocks, and both vaccinated and control ewes in the eastern paddocks (Table 13). There was a small increase in WEC in the vaccinated ewes on Farms B, C and D compared to the control ewes at lamb marking.

Table 13 Bulk worm egg count (WEC) in eggs per gram (epg) from control and vaccinated ewes on each trial farm at three time points (mating, mid-gestational pregnancy diagnosis and lamb marking).

Farm	Group	Bulk worm egg count (eggs per gram faeces)			
		Mating	Pregnancy diagnosis	Lamb marking	
A	Control	966[1]	75	25[5]	30[6]
	Vaccinated	1475[1]	175	1340[5]	35[6]
B	Control	0[2]	0	70	
	Vaccinated	0[2]	75	235	
C	Control	185[3]	50	100	
	Vaccinated	315[3]	15	320	
D	Control	430[4]	100	35	
	Vaccinated	565[4]	85	300	

[1]Farm A treated with an anthelmintic 8 weeks prior to mating

[2]Farm B treated with an anthelmintic 6 weeks prior to mating;

[3]Farm C treated 9 months prior to mating;

[4]Farm D treated 4 months prior to the mating WEC;

[5]Farm A ewes lambed down in two sets of paddocks: this WEC is from the western pair of paddocks;

[6]Farm A ewes from the eastern pair of paddocks.

3.3 Reproduction traits

3.3.1 Pregnancy proportions and conception rates

The proportion of ewes pregnant, calculated as the number of pregnant ewes divided by the total number of ewes present at pregnancy diagnosis, ranged from 66% on Farm C to 98% on Farm A. There was no statistically significant difference in the proportion of ewes that were pregnant between the vaccinated and control groups on any individual farm (Table 14).

Conception rate, calculated as the number of foetuses conceived divided by the number of ewes present at pregnancy diagnosis, ranged from 67% on Farm C to 117% on Farm A (Table 15). In the univariable Poisson regression analysis of conception rate in control and vaccinated groups, there was no significant difference on any farm (Table 15).

Effect of condition score on conception rate

On Farms A and B, there was no association between CS at mating and conception rate (A: $P = 0.80$; B: $P = 0.74$). However, there was a significant association between CS at mating and conception rate on Farms C ($P < 0.01$) and D ($P < 0.05$). Change in condition score over the first 90 days of pregnancy had no effect on conception rate on individual farms (A: $P = 0.96$; B: $P = 0.51$; C: $P = 0.41$; D: $P = 0.98$).

Table 14 Proportion of control (C) and vaccinated (V) ewes detected as pregnant at mid-gestational pregnancy diagnosis on each farm (P-values compare proportions between groups within farm).

Farm	**Treatment group**	**Proportion pregnant** (lower and upper 95% CI)	**P-value**
A	C	98% (95%, 99%)	0.12
	V	95% (92%, 98%)	
B	C	95% (92%, 98%)	0.60
	V	96% (93%, 98%)	
C	C	66% (60%, 72%)	0.44
	V	69% (63%, 75%)	
D	C	86% (80%, 90%)	0.67
	V	84% (78%, 89%)	

Table 15 Conception rates of control (C) and vaccinated (V) groups, and incidence rate ratios (IRR) of conception in vaccinate compared to control ewes, from univariable Poisson regressions on each farm.

Farm	Treatment group	Conception rate		
		Percent foetuses: ewes (lower and upper 95% CI)	**IRR**	**P-value**
A	C	117% (104%, 132%)	0.98	0.84
	V	115% (102%, 130%)		
B	C	108% (95%, 123%)	1.00	0.99
	V	108% (95%, 123%)		
C	C	67% (61%, 73%)	1.06	0.59
	V	71% (65%, 77%)		
D	C	90% (77%, 104%)	1.02	0.86
	V	91% (79%, 106%)		

Multivariable analyses of conception, condition score and treatment

A multivariable logistic regression model was used to test differences in the proportion of pregnant ewes between farms, including treatment, CS at mating, change in condition score from mating to pregnancy diagnosis, and farm as fixed effects. Vaccination had no significant effect on the proportion of pregnant ewes across all farms ($P = 0.27$). In this model, CS at mating had a significant effect on pregnancy proportion ($P < 0.001$), as did the change in CS from mating to pregnancy diagnosis ($P < 0.001$). The proportion of pregnant ewes on Farm A did not differ from those on Farm B (OR 1.13, $P = 0.75$), but did differ significantly from Farm C (OR 0.37, $P < 0.01$) and D (OR 0.49, $P < 0.05$). The pregnancy proportion on Farm B differed significantly from Farm C ($P < 0.001$) and Farm D ($P < 0.01$), but Farm C and D did not differ significantly from one another ($P = 0.13$).

Multivariable Poisson regressions were also used to compare conception rate (number of foetuses divided by number of ewes at pregnancy diagnosis) between treatment groups, accounting for CS and including farm as a fixed or random effect. There was no effect of vaccination on conception rate in the fixed-effect ($P = 0.83$) or random-effect ($P = 0.89$) models. In the multivariable Poisson regression model that included conception rate, CS and farm as a fixed term, conception rates were significantly lower on Farm C than on any other farm (Farm A to C: $P < 0.01$, Farm B to C: $P < 0.01$, Farm C to D: $P < 0.05$; Figure 8). There were no significant differences in conception rate between Farms A, B and D.

As the effect of treatment was not significant, it was dropped from the model ($P = 0.90$). Additionally, the interaction between CS at mating and CS change to pregnancy diagnosis had no effect on conception rate and was also dropped from the model ($P = 0.69$). The final random effects model revealed that condition score at mating, and to a lesser degree, the change in condition score between mating and pregnancy diagnosis, had the greatest effect on conception rates on individual farms. Overall, conception rates were higher in ewes that were in heavier condition at mating (IRR 2.06, $P < 0.001$), and in ewes whose CS increased from mating to pregnancy diagnosis (IRR 1.53, $P < 0.01$).

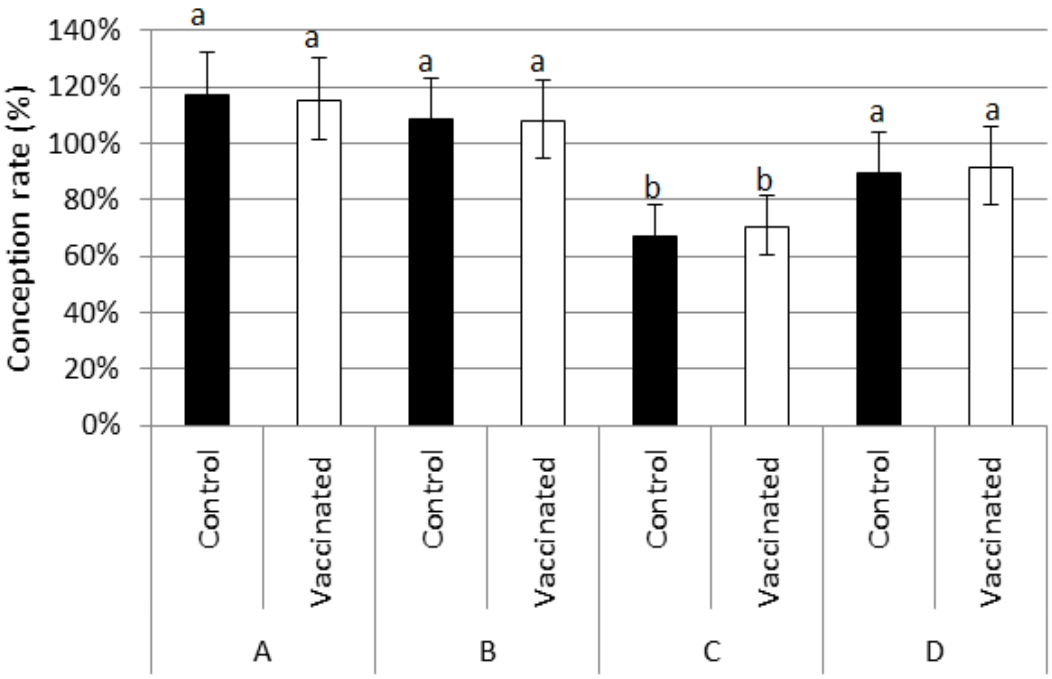

Figure 8 Conception rate (number of foetuses conceived divided by the number of ewes at pregnancy diagnosis) in control (black columns) and vaccinated (white columns) groups (error bars show 95% confidence intervals). Columns with the same letter label are statistically similar according to the results of a fixed effect multivariable Poisson regression model ($P > 0.05$).

3.2.2 Neonatal lamb mortality: cross-sectional study of cause of death

Across all four farms, necropsy examinations were conducted on 50 lambs born to control ewes and 26 lambs born to vaccinated ewes. In total, 42 ram lambs and 32 ewe lambs were necropsied. The sex of two lambs, one in each treatment group, was unable to be determined. The proportion of ram to ewe lambs was the same in the control (57% male: 43% female) and vaccinated (56% male: 44% female) groups.

Independent of farm, dystocia was the most commonly diagnosed cause of death across both treatment groups (Table 16). Dystocia was responsible for a greater proportion of the deaths in the necropsied lambs that were born to vaccinated ewes (11 of 26, 42%) than in lambs born to control ewes (14 of 50, 28%; OR 1.89; P = 0.30). Starvation-mismothering-exposure was the cause of death in 15% of lambs born to vaccinated ewes and 26% of necropsied lambs born to control ewes (OR 0.52; P = 0.39).

Predation was the second most commonly diagnosed cause of death in the lambs necropsied from vaccinated ewes (35%), and the third most commonly diagnosed cause of death in the control lambs (18%; OR 2.41; 0.70, 8.15; P = 0.15). Predation was more commonly diagnosed on Farm B than on any other farm (53% of necropsied lambs born to vaccinated ewes, 26% of necropsied lambs born to control ewes; Table 17).

Campylobacter infection was confirmed in necropsied lambs born to control ewes on Farm A only (Table 16 and Table 17). *Campylobacter fetus fetus* was cultured. Culture was most successful from aspirated abomasal contents. *Campylobacter*-like organisms were also detected on stained smears of abomasal fluid (3 of 5 and 4 of 5 suspected cases, respectively). The lambs in these five cases were born alive, although four had only partial lung inflation. Three were meconium-stained, none had walked, fed or metabolised brown-adipose-tissue. Two had moderate and two had mild hepatomegaly, with rounded liver margins, and one had subcutaneous oedema but no obvious hepatomegaly (Figure 9). No lesions were visible on the surface or cut-surface of any livers. Serosanguinous fluid was present in the pleural and peritoneal cavities.

Microbiological culture of abomasal contents and liver swabs from seven lambs with unknown causes of death from either the vaccinated or control group, or from those where an infectious agent was suspected, were negative for *Campylobacter* spp. in all other cases.

Table 16 Results of cross-sectional study of cause of neonatal lamb mortality in lambs born to control (n = 50) and vaccinated (n = 26) ewes on trial farms.

Cause of death	**Proportion (95% CI) of necropsies from each treatment group [*n*]**	
	Lambs born to control ewes	Lambs born to vaccinated ewes
Dystocia[1]	**28%** (16-41%) [14]	**42%** (23-61%) [11]
Starvation-mismothering-exposure	**26%** (14-38%) [13]	**15%** (1-29%) [4]
Predation	**18%** (7-29%) [9]	**35%** (17-53%) [9]
Infectious[2]	**10%** (-16-36%) [5]	0%
Unknown	**14%** (-12-40%) [7]	**8%** (-29-45%) [2]
Congenital and premature	**4%** (-23-31%) [2]	0%

[1] This is an underestimate. The producer on Farm A did not submit four lambs from the vaccinated ewes that were obviously the result of dystocia. These lambs were not included in the results.

[2] Curved, gram negative rods were detected on smears from the abomasal contents and/or cut surface of the liver from four lambs born to control ewes on Farm A. Culture of abomasal contents confirmed *C. fetus fetus* in three of these lambs. An infectious agent was suspected in one other lamb from the control group on Farm A, but was not detected on smears or culture

Table 17 Cause of death of necropsied lambs born to either control (C) or vaccinated (V) ewes on each of the four farms (A to D). Farm D was only able to submit two lambs for necropsy.

	Farm A		**Farm B**		**Farm C**		**Farm D**	
Treatment group	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**
Number necropsied (*n*)	11	4	27	17	11	4	1	1
Starvation-mismothering-exposure (SME)	18%		26%	12%	27%	25%	100%	100%
Dystocia	18%	75%	30%	29%	36%	75%		
Primary predation	9%		26%	53%	9%			
Infectious	45%[1]							
Unknown	9%	25%	15%	6%	18%			
Congenital			4%					
Premature					9%			

[1]*Campylobacter fetus fetus* cultured from 3 of 5 suspicious cases on Farm A, and curved rods detected on gram stain from 4 of 5 suspicious cases

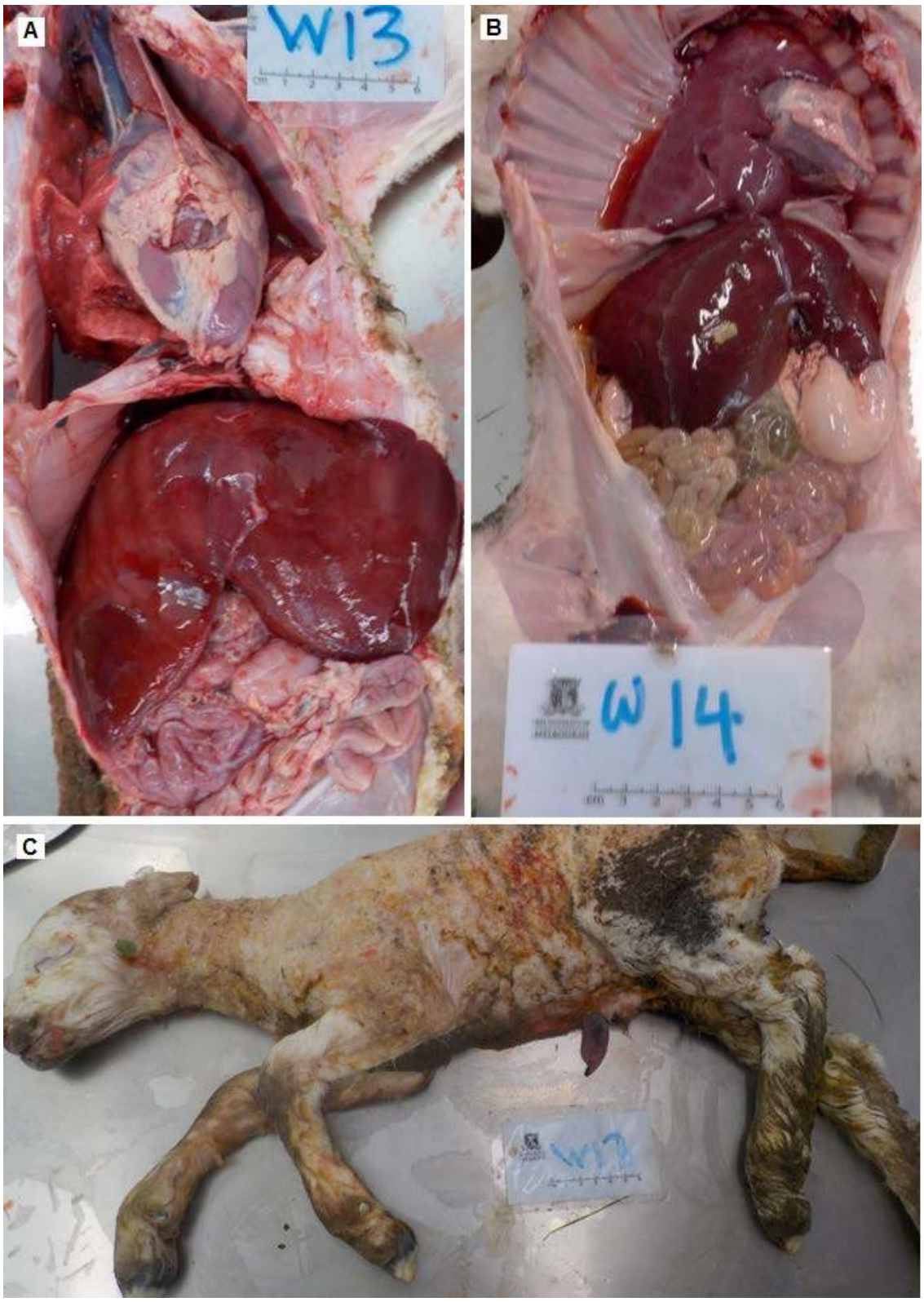

Figure 9 Two of the three lambs from which *C. fetus fetus* was cultured. Internally, moderately enlarged livers and serosanguinous pleural fluid was appreciable (A & B). Externally, other than meconium staining, there were no obvious distinguishing features of these lambs (C). Without investigation, they could have been mistaken for uncomplicated stillbirth or dystocia cases.

3.3.1.1 Farm D: neonatal lamb mortality as determined by the producer

In addition to the lambs necropsied by the investigator, the producer on Farm D picked up dead lambs on 16 days over the peak of lambing. In consultation with the investigator, a most likely cause of death was recorded for 30 lambs from control ewes and 32 lambs from vaccinated ewes (Table 18). The pattern of the differences in cause of death in lambs born to control ewes compared with lambs born to vaccinated ewes was consistent with those detected by the investigator on Farms A and C (Table 17). Starvation-mismothering-exposure was the most commonly reported cause of death in lambs born to both control and vaccinated ewes. The proportion of lambs born to the vaccinated ewes reported as having died due to SME (31%) was almost half that of lambs born to control ewes (57%; OR 0.35; P = 0.07). Dystocia was more often recorded as a cause of death in lambs born to the vaccinated ewes (16%) compared to the control ewes (3%; OR 5.37; P = 0.20). There were no differences between vaccinate and control ewes in proportions of lambs dying from predation (OR 0.93; P = 1.00) or stillbirth (OR 0.93; P = 1.00).

Table 18 Cause of death of neonatal lambs, as recorded by the producer on Farm D, born to control (n = 30) and vaccinated (n = 32) ewes, as a proportion of the total lambs sampled in each group.

Cause of death	**Proportion (95% CI) of necropsies from treatment group [*n*]**	
	Lambs born to control ewes (30)	Lambs born to vaccinated ewes (32)
Starvation-mismothering-exposure	**57%** (39-73%)	**31%** (15-49%)
Dystocia	**3%** (-3-10%)	**16%** (3-28%)
Predation	**10%** (-1-21%)	**9**% (-1-19%)
Stillborn	**17%** (3-30%)	**16%** (3-28%)
Unknown	**13%** (1-25%)	**28%** (13-44%)

3.3.1.2 Necropsied lambs: body-weight

Overall, there was no difference in the mean weights of necropsied lambs born to vaccinated or control ewes (4.49 ± 0.20 kg and 4.35 ± 0.12 kg, respectively; P = 0.55). Within farms, on Farm A, necropsied lambs from control ewes were marginally significantly lighter than the necropsied lambs born to the vaccinated ewes (P = 0.06; Figure 10). There was no significant difference in the weights of lambs necropsied from control ewes compared to vaccinated ewes on Farm B (P = 0.79) or Farm C (P = 0.64; Figure 10). On Farm D, the weights of lambs picked up by the producer were not recorded and insufficient samples were submitted for necropsy for analysis within-farm (Table 17).

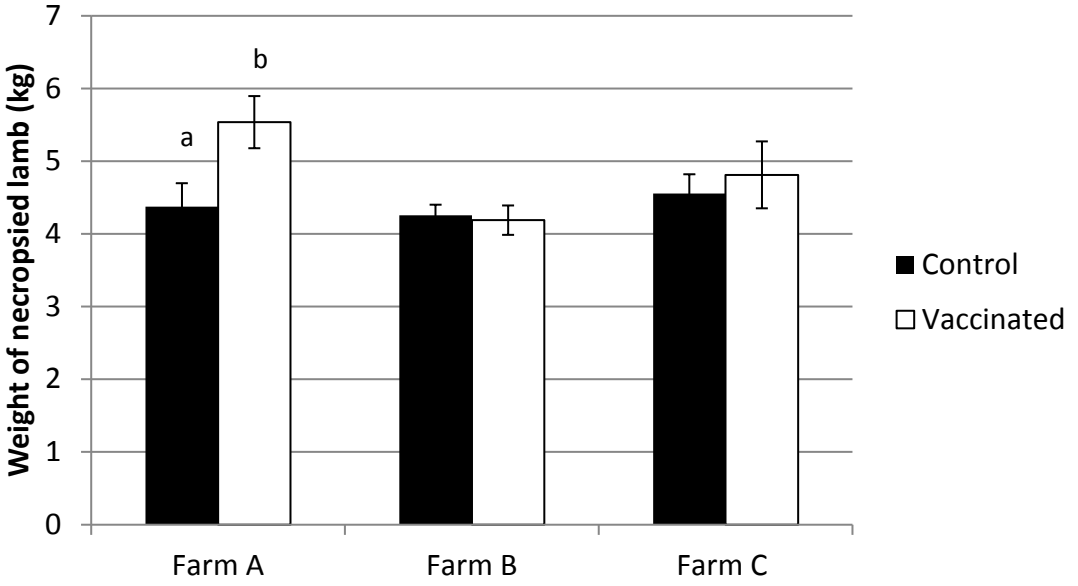

Figure 10 Body-weight (mean ± SEM) of necropsied lambs born to control ewes (black columns) and vaccinated ewes (white columns) on three of the four farms in the trial (A, B and C). Letters signify a marginally significant difference between-groups within-farm (P = 0.06).

3.3.2 Lamb marking rates

Lamb marking rates, calculated as number of lambs present at marking divided by the number of ewes present at marking to account for mis-mustering, ranged from 63% on Farm B to 100% on Farm A (Table 19). There was no significant difference in the percentage of lambs marked from vaccinated compared to control ewes when farms were analysed individually in separate models (within-farm IRR, Table 19).

Differences between farms were examined in a fixed-effects multivariable Poisson regression model. The percent of lambs marked differed significantly between all farms except for Farms B and C (Figure 11). Accounting for the effect of farm, in the fixed-effects model there was no overall effect of treatment on lamb marking rate (P = 0.88).

Multivariable Poisson regression analyses were performed including farm as either a fixed or random effect. The random effects model including terms for treatment, average ewe CS at marking and the interaction of these two terms was the best-fitting, biologically plausible model. In this model, lamb marking rate was marginally statistically associated with treatment, CS at lamb marking and the interaction of these terms (P = 0.06; Table 20). Vaccinated ewes in lighter CS at lamb marking marked more lambs than control ewes, but vaccinated ewes in heavier CS marked fewer lambs than control ewes (Table 20).

Accounting for farm effects, CS at lamb marking overall had a significant main-effect association with lamb marking rates. Ewes one CS heavier marked 2.4 (95% CI 0.89-6.3) to 7.6 (95% CI: 1.4-42) times more lambs than lighter ewes in vaccinated and control groups, respectively.

Table 19 Lamb marking rates (number lambs marked compared to number of ewes present at lamb marking) in control (C) and vaccinated (V) ewes on trial farms.

Farm	Group	Raw numbers		Average CS at lamb marking (Figure 5)	Lamb marking % (95% CI)	Within Farm incidence rate ratio (IRR) (95% CI)	P-value[1] (from farm specific models)
		Ewes	Lambs				
A	C	216	211	2.86	98% (85%, 112%)	1.02 (0.84, 1.24)	0.85
	V	208	207	2.83	100% (86%, 114%)		
B	C	202	141	2.78	70% (59%, 82%)	0.90 (0.70, 1.15)	0.39
	V	210	132	2.81	63% (53%, 75%)		
C	C	154	100	2.61	65% (53%, 79%)	1.01 (0.76, 1.34)	0.95
	V	165	108	2.42	65% (54%, 79%)		
D	C	173	140	2.82	81% (68%, 95%)	1.03 (0.81, 1.32)	0.79
	V	153	128	2.79	84% (70%, 99%)		

[1] There was no difference in lamb marking rate in control and vaccinated groups on any farm.

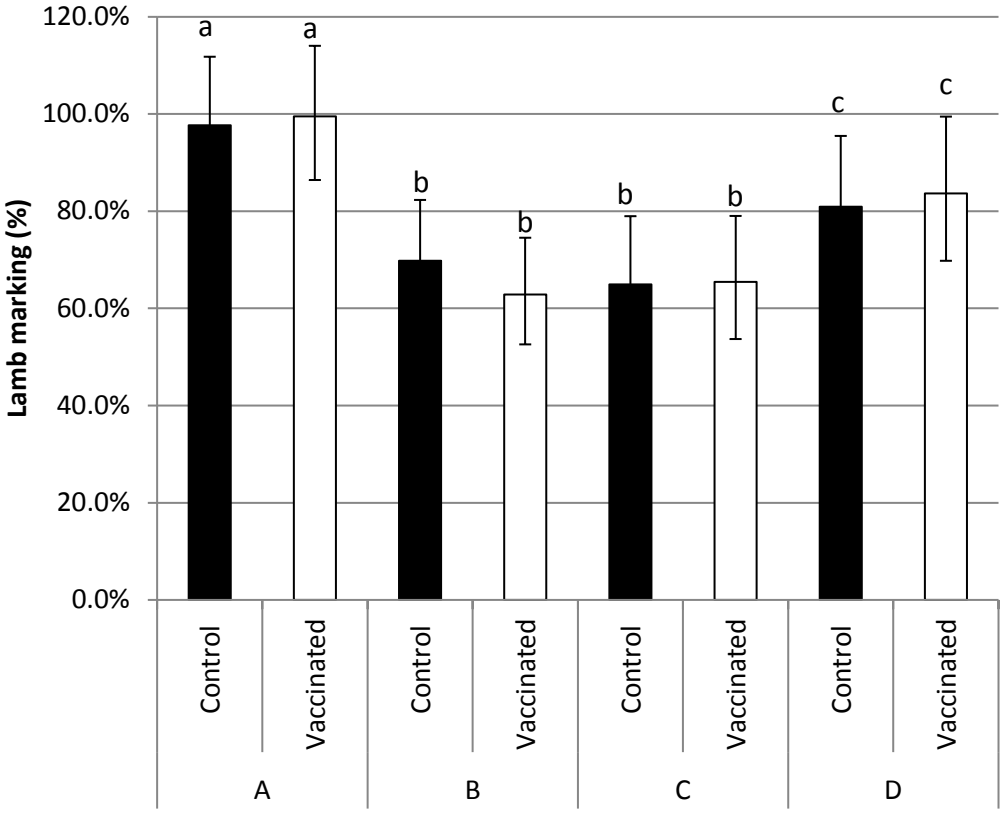

Figure 11 Lamb marking rate (number of lambs divided by the number of ewes present at lamb marking) in both control (black columns) and vaccinated (white columns) treatment groups on each farm (bars show upper and lower 95% CI) with significant differences between farms denoted by letters above the columns ($P < 0.05$).

Table 20 Estimated ratio of lamb marking rates of vaccinated ewes compared to control ewes of different condition scores from the best-fitting random-effects multivariable Poisson regression model, containing terms for *treatment*, *ewe CS at marking* and (*treatment*)*(*ewe CS at marking*).

Condition score at lamb marking	***n***	**Marking rate ratio of vaccinated compared to control ewes** (95% CI)	**P-value**
2.0	12	2.5 (0.95-6.7)	0.07
2.25	84	1.9 (0.96-3.7)	0.07
2.5	366	1.41 (0.96-2.06)	0.078
2.75	574	1.05 (0.92-1.20)	0.47
3.0	288	0.78 (0.59-1.04)	0.09
3.25	98	0.59 (0.33-1.04)	0.07

3.3.3 Foetal and lamb survival from mid-gestation to lamb marking

Lamb survival, calculated as the proportion of lambs expected based on mid-gestational pregnancy diagnosis that were present at lamb marking, ranged from 55% (49%, 61%) on Farm B to 81% (76%, 85%) on Farm A (Figure 12). The proportion of lambs surviving did not differ between the vaccinated and control groups on any farm (Farm A: P = 0.85; Farm B: P = 0.27; Farm C: P = 0.94; Farm D: P = 0.24).

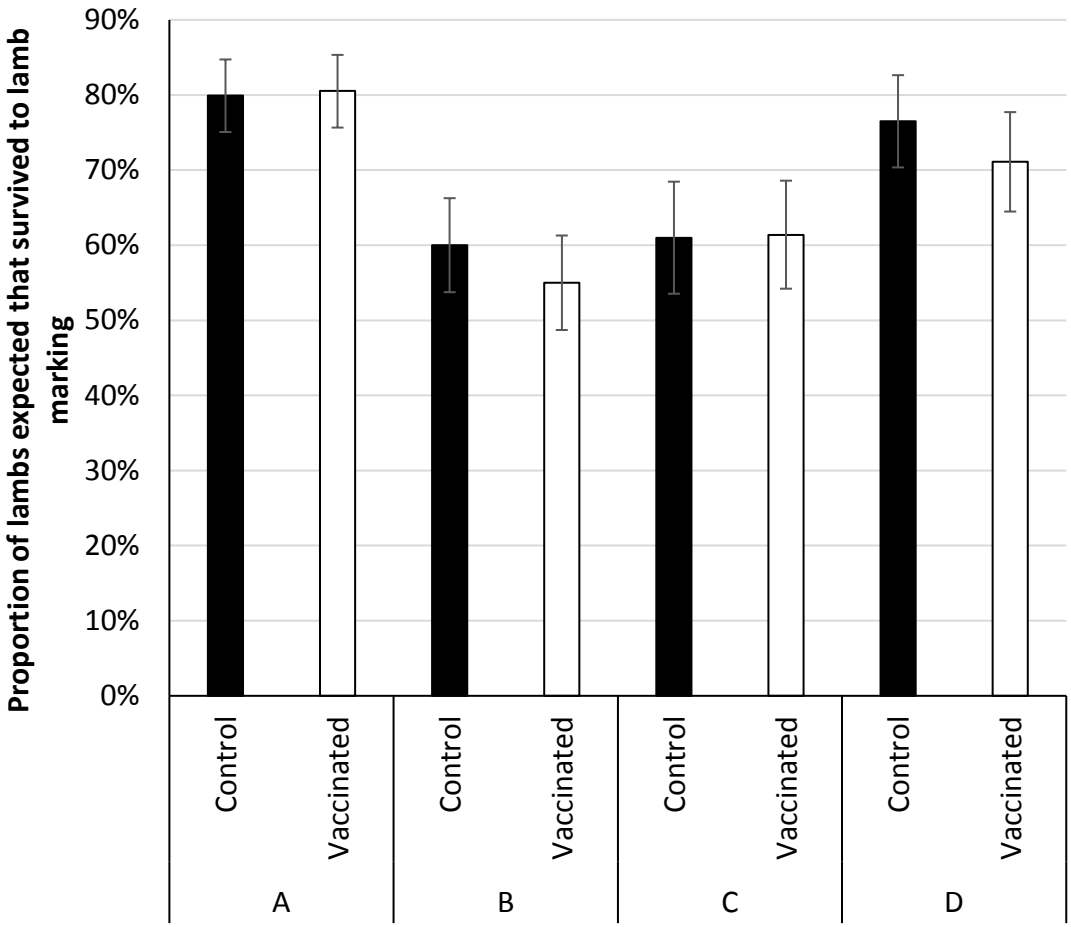

Figure 12 Lamb survival (the proportion of lambs expected based on pregnancy diagnosis that survived through to lamb marking) in the control (black columns) and vaccinated (white columns) groups on each of the four farms (bars show upper and lower 95% confidence intervals).

3.3.4 Proportion of wet and dry ewes at lamb marking

The proportion of ewes detected as pregnant and found to have an udder at lamb marking ('wet') was significantly greater in the vaccinated (0.91) compared to the control (0.81) group on Farm A ($P < 0.01$; Figure 13). On Farm A, vaccinated ewes were 1.14 (1.05, 1.22) times more likely to have been 'wet' at marking than control ewes. On Farms B, C and D, there was no significant difference in the proportion of 'wet' ewes in the vaccinated ewes compared to controls (B: $P = 0.67$; C: $P = 0.69$; D: $P = 0.93$).

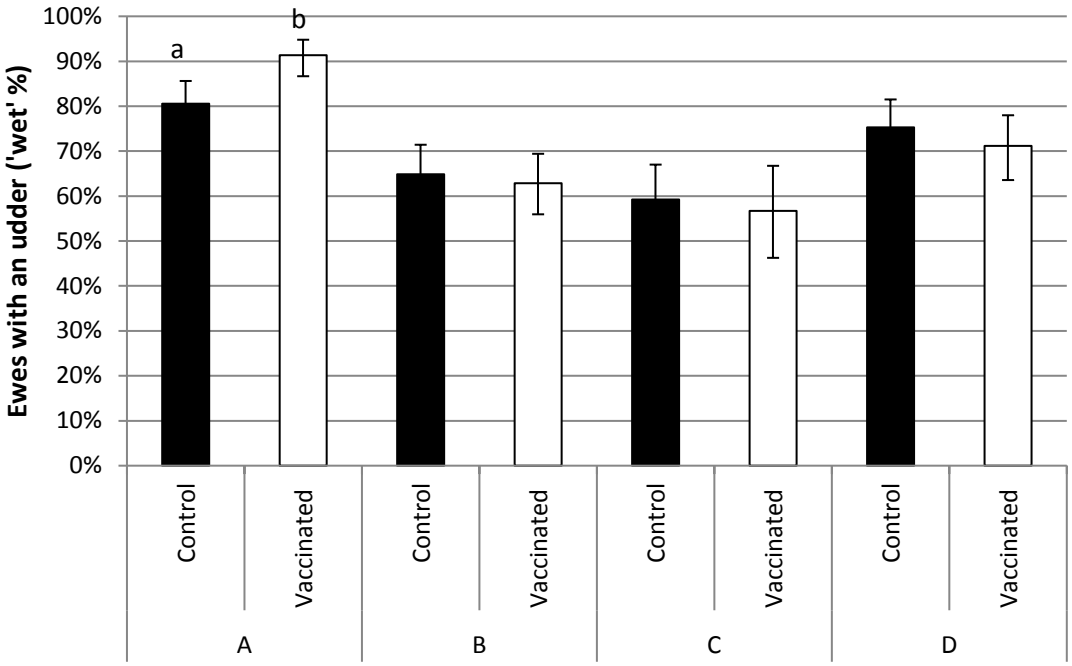

Figure 13 Percent of ewes detected to have an udder ('wet') in control (black columns) and vaccinated (white columns) groups at lamb marking (denominator: total number of ewes present and detected as pregnant at lamb marking). A significant difference between-groups within-farm is signified by the lettering above the columns. Error bars represent the upper and lower 95% confidence intervals.

3.4 Ewe Serology

3.4.1 *Campylobacter fetus fetus*

Prior to vaccination, Farms A and B had serological evidence of infection with *C. fetus fetus*. Most ewes on Farm A had either 'high' or 'moderate' titres (Figure 14). All ewes on Farm B had 'moderate' titres (Figure 15). Titres were low in all ewes bled on both Farm C (Figure 16) and D (Figure 17). The proportion of ewes with 'high', 'medium' or 'low' titres did not differ significantly between control and vaccinated groups prior to vaccination at mating on all farms (P = 0.32–1.0; Figures 14-17).

Three months following the first dose of vaccine, at the pregnancy diagnosis visit, significantly more ewes in the vaccinated group had 'high' titres compared to ewes in the control group, who predominantly had 'low' titres at this visit (all farms: difference in proportions between treatment groups at pregnancy diagnosis, P < 0.001). 'High' titres in vaccinated ewes persisted until lamb marking and control ewes continued to have mainly 'low' antibody titres (all farms: P < 0.001; Figures 14-17).

Changes in the proportions of ewes with antibody titres in different categories were examined over time in each treatment group of the four farms. In the control ewes on Farms A and B, there was a significant change in the proportion of ewes with 'low', 'medium' and 'high' titres from the mating visit to lamb marking (P < 0.001; Figures 14 and 15). On Farms C and D, the change in proportions over time was not statistically significant, as most ewes had 'low' antibody titres throughout the trial and only several seroconverted (Farm C: P = 0.22; Farm D: P = 0.58; Figures 16 and 17). Although not statistically significant on all farms, the number of control ewes with 'high' *C. fetus fetus* titres increased throughout the trial, suggesting some level of *C. fetus fetus* exposure occurred during gestation on all farms. In the vaccinated ewes on all farms, the proportion of ewes in each serostatus category changed significantly following vaccination, with the majority increasing to the 'high' bracket (all farms: P < 0.001; Figures 14-17).

There was no statistically significant difference in the proportion of non-pregnant ewes with 'moderate' or 'high' *C. fetus fetus* titres compared to pregnant ewes at pregnancy diagnosis on any farm (Farm A: P = 0.37, Farms B, C and D: P = 1.00; Table 21).

Exposure to *C. fetus fetus* over the entirety of gestation was greatest in ewes from Farm A. On Farm A, 55% of sampled ewes that were detected as 'dry' at lamb marking had 'high' *C. fetus fetus* titres (Table 21). None of the 'wet' ewes sampled on Farm A had 'high' titres ($P < 0.05$). On all other farms, the proportion of exposed 'dry' ewes did not differ significantly from exposed 'wet' ewes (Farms B, C and D: $P = 1.00$).

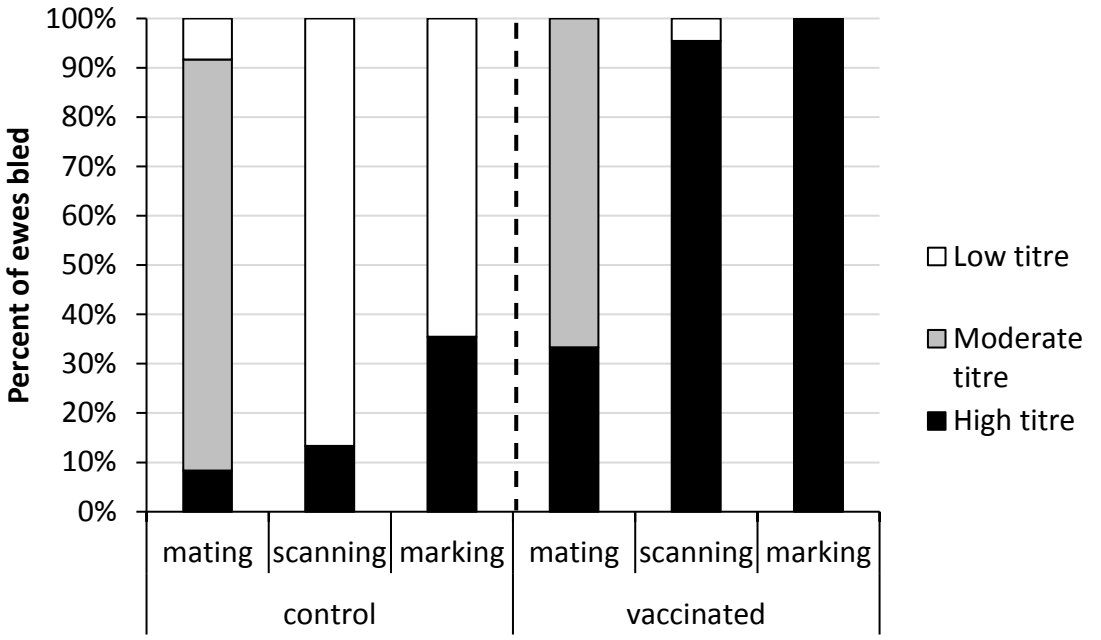

Figure 14 Proportions of vaccinated and control ewes with 'high' (≥1:80; black columns), 'moderate' (1:10-1:60; grey columns) or 'low' (<1:10; white columns) *Campylobacter fetus fetus* titres at mating, mid-pregnancy ('scanning') and lamb-marking on Farm A.

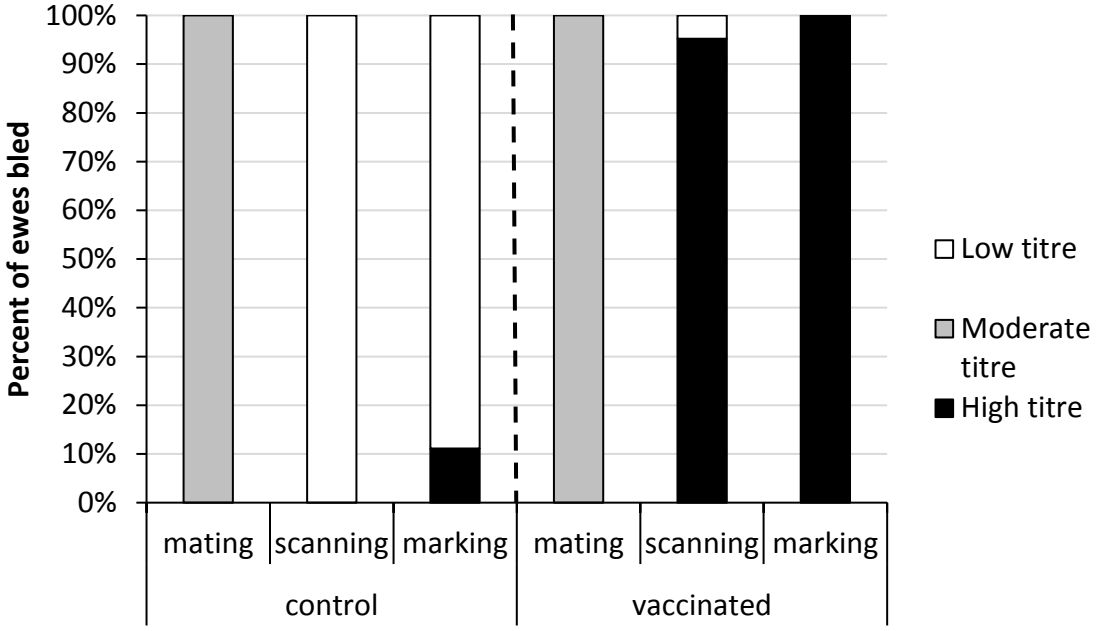

Figure 15 Proportions of vaccinated and control ewes with 'high' (≥1:80; black columns), 'moderate' (1:10-1:60; grey columns) or 'low' (<1:10; white columns) *Campylobacter fetus fetus* titres at mating, mid-pregnancy ('scanning') and lamb-marking on Farm B.

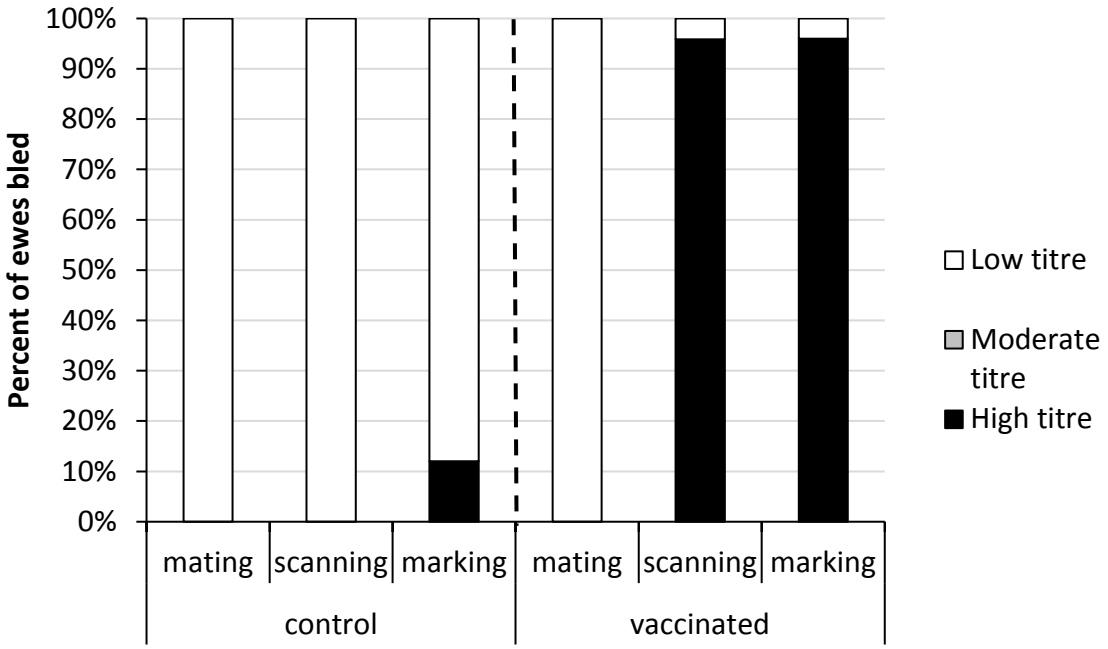

Figure 16 Proportions of vaccinated and control ewes with 'high' (≥1:80; black columns), 'moderate' (1:10-1:60; grey columns) or 'low' (<1:10; white columns) *Campylobacter fetus fetus* titres at mating, mid-pregnancy ('scanning') and lamb-marking on Farm C.

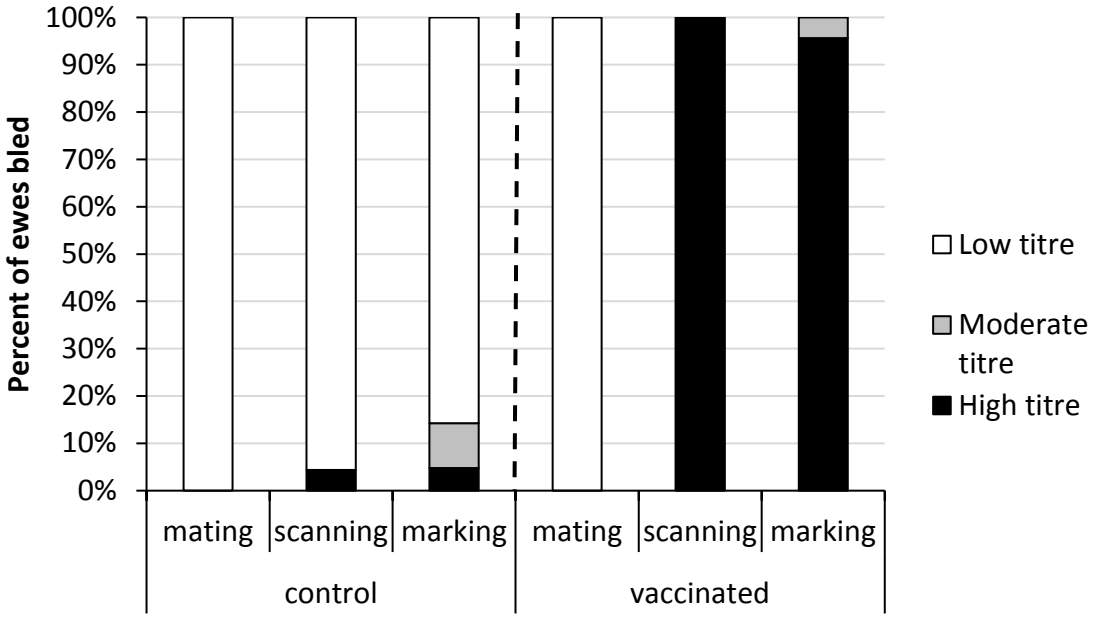

Figure 17 Proportions of vaccinated and control ewes with 'high' (≥1:80; black columns), 'moderate' (1:10-1:60; grey columns) or 'low' (<1:10; white columns) *Campylobacter fetus fetus* titres at mating, mid-pregnancy ('scanning') and lamb-marking on Farm D.

Table 21 Proportion of control ewes with 'high' (≥1:80), 'moderate' (1:10 - 1:60) or 'low' (<1:10) titres to *Campylobacter fetus fetus* that were pregnant or non-pregnant (pregnancy diagnosis), and 'wet' or 'dry' (lamb marking). Superscript and bold type identifies a significant difference in the proportion of exposed ewes to unexposed ewes within farm ($P < 0.05$).

Farm	**Ewe status at pregnancy scanning** (non-pregnant /pregnant) **or lamb marking** ('wet'/'dry')	**Pregnancy diagnosis**			**Lamb marking**		
		'High' titres	'Moderate' titres	'Low' titres	'High' titres	'Moderate' titres	'Low' titres
A	non-pregnant/ 'dry'	33%	0%	67%	**55%**	0%	**45%**[a]
	pregnant/'wet'	8%	0%	92%	**0%**	0%	**100%**[b]
B	non-pregnant/ 'dry'	0%	0%	100%	8%	0%	92%
	pregnant/ 'wet'	0%	0%	100%	14%	0%	86%
C	non-pregnant/ 'dry'	0%	0%	100%	9%	0%	91%
	pregnant/'wet'	0%	0%	100%	15%	0%	85%
D	non-pregnant/ 'dry'	0%	0%	100%	0%	13%	87%
	pregnant/'wet'	8%	0%	92%	10%	10%	80%

[a,b]The proportion of 'dry' ewes with exposure to *C. fetus fetus* (ewes with high and moderate titres) differed significantly from exposure in the control ewes on Farm A only.

3.4.2 *Campylobacter jejuni*

Most ewes on all farms had 'high' *C. jejuni* antibody titres prior to vaccination, with no significant difference between the treatment groups in the proportion of ewes falling into 'low', 'moderate' or 'high' antibody categories prior to vaccination (Farm A: P = 0.48; Farm B: all in one category; Farm C: P = 1.00; Farm D: all in one category; Figures 18-21).

Three months after the first vaccine dose, significantly more vaccinated ewes had 'low' or 'moderate' titres compared to control ewes (Farms A and D: P < 0.001; Farms B and C: P < 0.05). This difference persisted at lamb marking on Farms A and B, where vaccinated ewes had predominantly 'low' titres and control ewes predominantly 'moderate' titres (Farms A and B: P < 0.001; Figures 18 and 19). However, on Farms C and D, at lamb marking there was a marginally significant or non-significant difference between the control and vaccinated groups in the proportion of ewes falling into each antibody category, with ewes in both groups having mostly 'moderate' titres (Farm C: P = 0.05; Farm D: P = 0.48; Figures 20 and 21).

In the control groups on all farms, ewe antibody titre to *C. jejuni* dropped progressively over gestation from predominantly 'high' at mating to predominantly 'moderate' at lamb marking (difference in proportions within control ewes over time on all farms: P < 0.001). In the vaccinated groups on all farms, *C. jejuni* antibody titres also dropped significantly over the course of gestation to be 'moderate' or 'low' at lamb marking (difference in proportions within vaccinated ewes over time on all farms: P < 0.001; Figures 18-21).

The difference in proportions of pregnant ewes with exposure ('high' or 'moderate' *C. jejuni* titres) to *C. jejuni* did not differ significantly from the proportion of non-pregnant ewes with exposure to *C. jejuni* at mid-gestational pregnancy diagnosis (Farm A: identical proportions; Farm B: P = 0.46; Farms C and D: P = 1.00; Table 22). There was also no statistically significant difference between the proportion of *C. jejuni* exposed 'dry' ewes and the proportion of *C. jejuni* exposed 'wet' ewes at lamb marking (Farm A: P = 0.28; Farm B: P = 1.00; Farm C: P =0.58; Farm D: P = 0.18; Table 22).

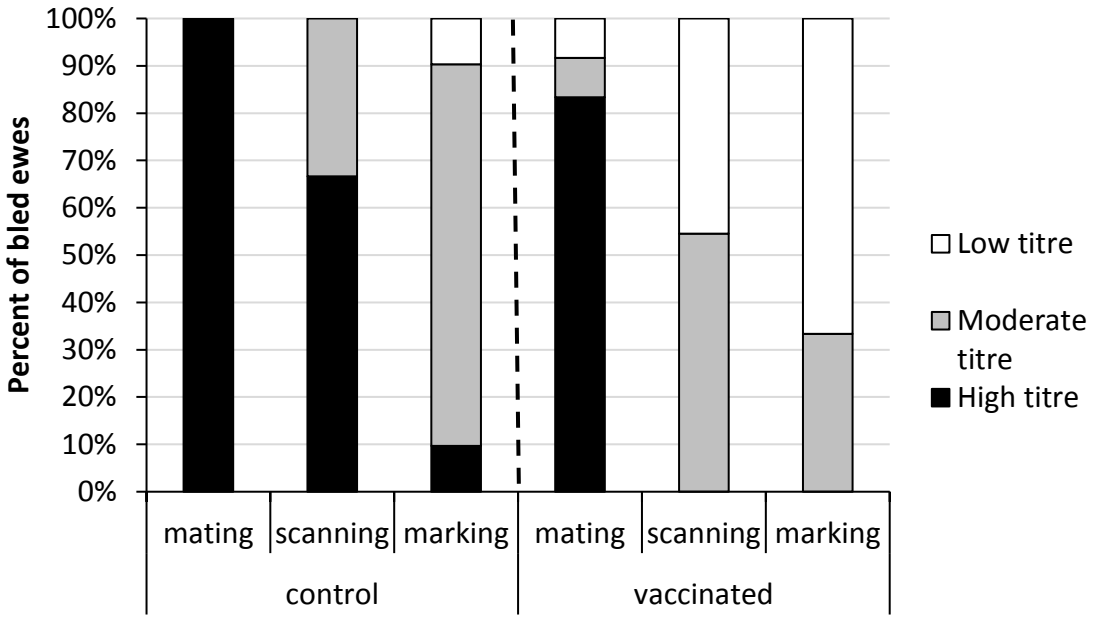

Figure 18 Proportions of vaccinated and control ewes with 'high' (≥1:80; black columns), 'moderate' (1:10-1:60; grey columns) or 'low' (<1:10; white columns) *Campylobacter jejuni* titres at mating, mid-pregnancy ('scanning') and lamb-marking on Farm A.

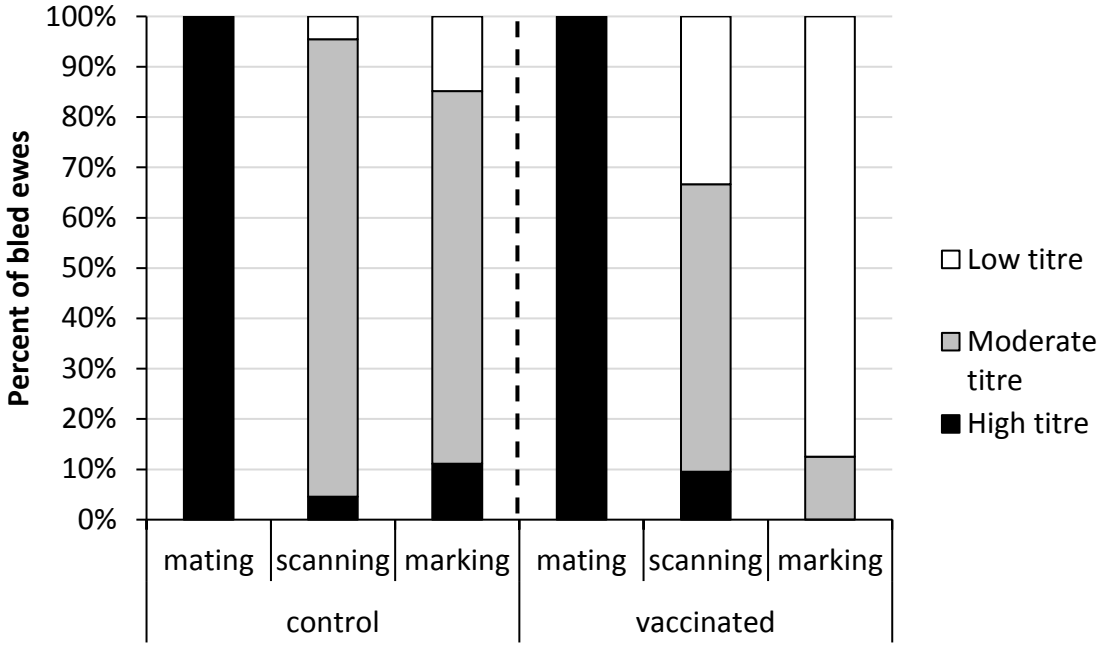

Figure 19 Proportions of vaccinated and control ewes with 'high' (≥1:80; black columns), 'moderate' (1:10-1:60; grey columns) or 'low' (<1:10; white columns) *Campylobacter jejuni* titres at mating, mid-pregnancy ('scanning') and lamb-marking on Farm B.

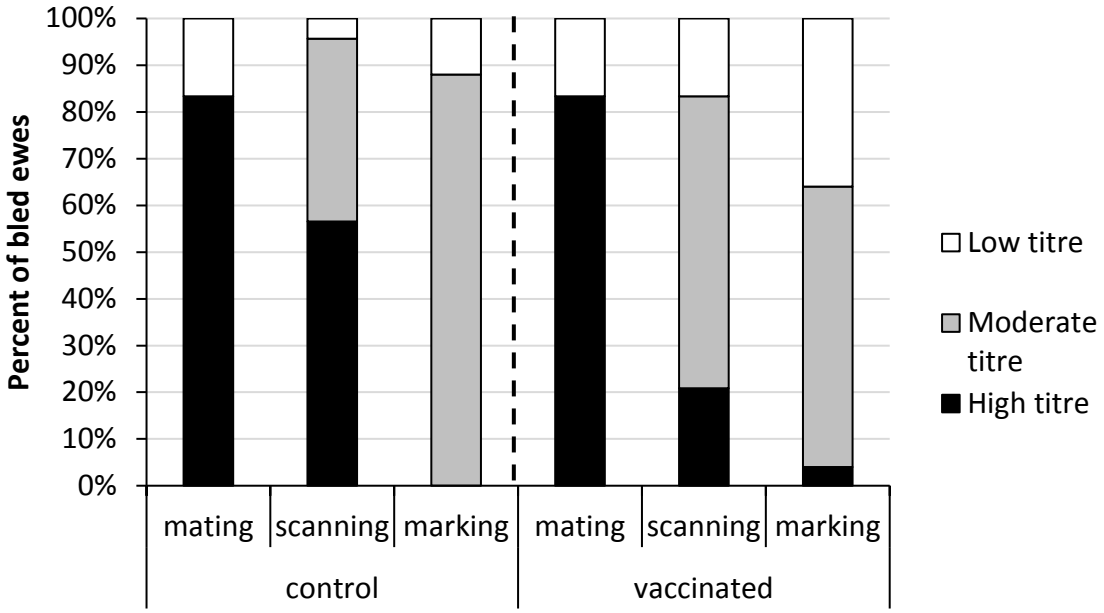

Figure 20 Proportions of vaccinated and control ewes with 'high' (≥1:80; black columns), 'moderate' (1:10-1:60; grey columns) or 'low' (<1:10; white columns) *Campylobacter jejuni* titres at mating, mid-pregnancy ('scanning') and lamb-marking on Farm C.

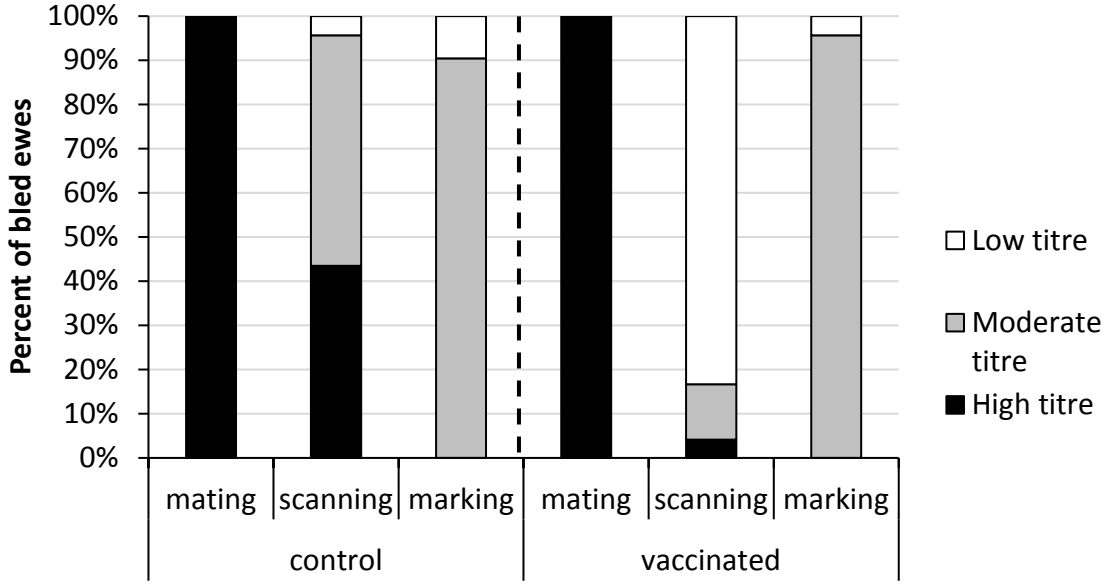

Figure 21 Proportions of vaccinated and control ewes with 'high' (≥1:80; black columns), 'moderate' (1:10-1:60; grey columns) or 'low' (<1:10; white columns) *Campylobacter jejuni* titres at mating, mid-pregnancy ('scanning') and lamb-marking on Farm D.

Table 22 The proportion of control ewes with 'high' (≥1:80), 'moderate' (1:10 - 1:60) or 'low' (<1:10) titres to *C. jejuni* that were either pregnant or not-pregnant (mid-gestation pregnancy diagnosis) and 'wet' or 'dry' (lamb marking).

Farm	**Ewe status at pregnancy scanning** (non-pregnant /pregnant) **or lamb marking** ('wet'/'dry')	**Pregnancy diagnosis**			**Lamb marking**		
		'High' titres	'Moderate' titres	'Low' titres	'High' titres	'Moderate' titres	'Low' titres
A	non-pregnant or 'dry'	67%	33%	0%	5%	90%	5%
	pregnant or 'wet'	67%	33%	0%	18%	64%	18%
B	non-pregnant or 'dry'	10%	80%	10%	0%	85%	15%
	pregnant or 'wet'	0%	100%	0%	21%	64%	14%
C	non-pregnant or 'dry'	67%	25%	8%	0%	82%	18%
	pregnant or 'wet'	45%	55%	0%	0%	92%	8%
D	non-pregnant or 'dry'	60%	40%	0%	0%	75%	25%
	pregnant or 'wet'	31%	62%	8%	0%	100%	0%

4 – Discussion

4.1 General Introduction

Reproductive wastage costs the Australian sheep industry more than AUD$541 million Australian dollars per year (Lane et al., 2015). It is a significant source of frustration for producers, who are routinely faced with a 20-40% discrepancy between the number of lambs expected, based on pregnancy diagnosis, and the number of lambs alive at lamb marking (Kilgour, 1992; Hinch and Brien, 2014). Most of this wastage occurs in the perinatal period, predominantly around birth and the first 48-72 hours of life (Alexander, 1984). Despite attempts to minimise these losses via the implementation of evidence-based strategies designed to reduce neonatal lamb mortality, many producers still report dissatisfaction with lamb survival.

Infection with *Campylobacter* spp. may contribute significantly to reproductive loss, reducing lamb marking rates by 6-10% even in the absence of abortion outbreaks. This has been demonstrated repeatedly in New Zealand by comparing the reproductive output of ewes vaccinated against *Campylobacter* spp. with that of un-protected ewes (Quinlivan and Jopp, 1982; Anderson, 2001; West, 2002). A serological study of the prevalence of *C. fetus fetus* positive flocks in New Zealand revealed a similar level of exposure as the one published Australian study, with 89% of 298 New Zealand flocks and 78% of 218 Australian flocks antibody positive (Dempster et al., 2011; Walsh, 2016). The Australian study is ongoing, and the most recent serology results suggest 62% of 346 farms tested were positive or exposed, however this data is not yet published (J. Walsh, personal communication, December 2017). Whilst extrapolating between disease and exposure is difficult, associated reproductive loss may be similarly common in Australian and New Zealand flocks. A bivalent vaccine against *C. fetus fetus* and *C. jejuni,* Ovilis Campyvax® has been available commercially in Australia since 2013. The current study is the first detailed, independent multi-farm trial of the vaccine conducted in Victoria, Australia.

Vaccination with Ovilis Campyvax® had no significant main effect on the reproductive output of maiden ewes on any of the four farms involved in this trial. On individual farms, and across all farms, there was no difference in either the proportion of ewes pregnant or conception rate between vaccinated and control groups of ewes. Importantly, this confirms that Ovilis Campyvax® may be administered over a standard 5 to 6 week mating

period without decreasing the likelihood of a ewe conceiving or maintaining a pregnancy through the first trimester.

Vaccination did not increase the survival of lambs from mid-gestation, when pregnancy diagnosis was performed, to lamb marking, nor did it increase lamb marking rates. This was despite evidence of some exposure to *C. fetus fetus* during gestation on all farms. Exposure was most marked on Farm A. However, environmental factors may have reduced lamb survival in vaccinated groups on two farms, potentially masking any positive effect of vaccination. In contrast to *C. fetus fetus*, there was no evidence for gestational exposure to *C. jejuni* on the trial farms, and antibody titres decreased over the course of the trial in control ewes.

No outbreaks of abortion occurred in either the control or vaccinated ewes on any farm. However, *Campylobacter* infection was confirmed via necropsy of neonatal lambs born to control ewes on Farm A. Despite *Campylobacter*-associated neonatal lamb mortality on Farm A, vaccination did not increase lamb marking rates on this farm. Anecdotally, more vaccinated ewes had dystocia than control ewes on Farm A. A similar observation was made on Farm D. Necropsy results supported these anecdotal reports, and whilst the proportions were not significantly different, starvation-mismothering-exposure (SME) was recorded more often in necropsied lambs born to control ewes and dystocia was recorded more often in necropsied lambs born to vaccinated ewes.

A multivariable random effects model investigating the effect of the interaction between ewe CS at lamb marking and treatment on lamb marking rate was marginally significant. The results of this analysis suggest that vaccinated ewes in heavier CS had lower lamb marking rates, but vaccinated ewes in lower CS had higher lamb marking rates. This statistical interaction is consistent with the producers reports and the necropsy results described above, all of which could be explained by a positive effect of *Campylobacter* vaccination on lamb birth-weight. The hypothesis that the causes of neonatal lamb mortality differ in vaccinated compared to control ewes needs to be tested in intensive and appropriately powered trials (see Section 6.3).

The topics described in the preceding introductory paragraphs are discussed in greater detail throughout the following sections.

4.2 Ewe *Campylobacter* serology

4.2.1 *Campylobacter fetus fetus*

Ewe antibody titres from the initial visit showed ewes had had previous exposure to *C. fetus fetus* on two of the four farms (Farm A and B). The presence of 'high' and 'moderate' titres in ewes on Farm A was consistent with likely recent exposure and/or endemicity (Anonymous, 2015). The 'moderate' titres in ewes on Farm B were consistent with *C. fetus fetus* being endemic on that farm, with a low level of exposure. Neither Farm C or D had any serological evidence of prior exposure to *C. fetus fetus,* consistent with those mobs being naïve. The relationship between *C. fetus fetus* antibody titres and protection against infection has not been quantified (Dempster et al., 2011). Thus, although antibodies are known to be key to agglutinating the surface antigens of *C. fetus fetus,* the extent of protection provided by the initial titres present in ewes on Farms A and B cannot be estimated (McCoy et al., 1976; Blaser et al., 1988; Dempster et al., 2011).

The absence of any serological evidence of previous exposure on Farm C is interesting. Farm C had previously experienced *Campylobacter*-abortions in ewes, diagnosed by an independent veterinary service and reported to the investigator by the producer. Subsequent independent sero-surveillance conducted on maiden ewes on the property 18 months before this trial found *C. fetus fetus* titres consistent with endemicity (Appendix 4). Farm C is a self-replacing enterprise, with maiden ewes selected from lambs born and reared on the property. Based on the history of Farm C, the assumption could have been made that these maiden ewes would have been exposed to *C. fetus fetus* prior to mating. However, this was not the case. This study's sample size of 24 for AGID serology prior to vaccination would have detected a seroprevalence of 15% (Sergeant, 2017). Therefore, the 2016 maiden ewes used in this trial were either truly naïve, only exposed at a very low level, or their titres had waned between exposure and testing at mating in 2016. There is insufficient published data describing longitudinal monitoring of *Campylobacter* spp. serology in sheep following natural exposure for conclusions to be made on the duration of antibody persistence. This is an avenue for future research (see Section 6.2).

It is unclear whether 'moderate' to 'high' titres protect ewes against subsequent reproductive loss. The titres detected at mating in ewes on Farm A were consistent with recent exposure or endemicity. Eight of the initial 12 control ewes randomly selected for serological monitoring on Farm A remained in the mob to lamb marking. Two of these eight seroconverted from initial 'low' or 'moderate' titres to 'high' titres at lamb marking.

Despite being detected as pregnant, neither of these ewes reared a lamb, based on udder status. The other six ewes had 'moderate' titres at mating that fell to 'low' titres by lamb marking, consistent with no re-exposure to *C. fetus fetus.* All six of these ewes reared a lamb. This suggests that a 'moderate' titre pre-pregnancy is not necessarily protective against reproductive loss. However, further research is required to better understand the extent of protection provided by different antibody titres.

On endemic farms, rearing replacement ewes with the main flock is thought to result in exposure to the *Campylobacter* present on a farm, resulting in subsequent immunity (Frank et al., 1959; Frank et al., 1965; Meinershagen et al., 1969). Anecdotally, this strategy is still used by many sheep producers to provide protection from *Campylobacter*-abortion. However, naturally acquired exposure may be unreliable. This was apparent on Farm C, where there was no evidence of pre-mating exposure of 2016 maiden ewes to *C. fetus fetus* despite a history of previous infections on the farm. Introducing naïve ewes to an endemic flock is also a risk factor for infection, especially when opportunities for exposure prior to pregnancy are limited. Farm A purchases replacement ewes prior to mating each year. Because the AGID does not differentiate between IgM and IgG (A. Vanderfeen, personal communication, November 2017), it was not possible to determine whether the 2016 maiden ewes were exposed on their property of origin or on Farm A immediately prior to mating.

The findings on Farm A and C highlight the risk of using historical *C. fetus fetus* antibodies as evidence of protective natural immunity. As discussed above, the extent of protection afforded by a certain titre has not been described and likely varies with the level of challenge (Dempster et al., 2011). These relationships warrant further research.

The titres of most ewes on all four farms fell to 'low' by pregnancy diagnosis, suggesting minimal exposure to the bacteria over the first three months of pregnancy. No association between *C. fetus fetus* exposure and pregnancy status was found on any farm, although the minimal exposure in the first half of pregnancy meant the effect of vaccination on early pregnancy loss could not be evaluated.

By lamb marking, some exposure to *C. fetus fetus* had occurred on all farms, with 11% to 35% of control ewes having elevated titres at lamb marking. These titres were predominantly 'high' (≥1:80), a level likely associated with reproductive loss (Anonymous, 2015). However, exposure was only definitively associated with

reproductive loss on Farm A, where *C. fetus fetus* was cultured from necropsied lambs born to control ewes but not from necropsied lambs born to vaccinated ewes. Additionally, a larger proportion of 'dry' control ewes had 'high' *C. fetus fetus* antibody titres (55% of sampled 'dry' ewes) but no 'wet' ewes had elevated titres. However, on Farms B, C and D, the proportion of control ewes with exposure to *C. fetus fetus* did not differ between ewes that were 'wet' and ewes that were 'dry' at lamb marking. This finding is clinically relevant for veterinarians investigating lower than expected lamb marking performance. Sampling both 'wet' and 'dry' ewes is important for the interpretation of serology results. This is discussed in greater detail in Section 4.2.3.

Despite the serological evidence of gestational exposure to *C. fetus fetus*, lamb survival from pregnancy diagnosis to lamb marking was no lower in control ewes than vaccinated ewes on any farm. Potential explanations for this finding include:

1. Differences in the causes of neonatal lamb mortality between vaccinated and control groups, masking the direct effect of vaccination. The differences in cause of death may have been exacerbated by farm-specific environmental factors (see Section 4.4.3.2)
2. *C. fetus fetus* exposure was insufficient to result in a statistically detectable increase in lamb marking rate or lamb survival in vaccinated compared to control groups
3. Natural immunity decreased the severity of the reproductive loss associated with exposure. This could explain the findings on Farms A and B, where ewes had 'moderate' or 'high' *C. fetus fetus* titres at mating. However, this is not a valid explanation for Farms C and D, where ewes were naïve at the start of the trial
4. Co-grazing of vaccinated and control groups throughout most of pregnancy may have resulted in some level of herd immunity provided by the vaccinated cohort. This could have reduced the extent of challenge faced by the control ewes and any subsequent lamb mortality. The decision to co-graze the two treatment groups through mating and pregnancy was made to minimise potentially confounding environmental or management effects on conception, foetal growth and gestational exposure to *Campylobacter*

On all farms, vaccination with Ovilis Campyvax® resulted in significantly elevated *C. fetus fetus* antibody titres by pregnancy diagnosis (up to 90 days after the first dose and 45 days after the second dose). These titres remained elevated until lamb marking in most

tested ewes. There was no suspicion that *Campylobacter* contributed to neonatal lamb losses in vaccinated ewes, and *C. fetus fetus* was not cultured from lambs born to vaccinated ewes. The occasional 'low' titre in vaccinated ewes could be explained by a ewe that did not receive the vaccine dose as intended, or an inaccuracy in the serological testing procedure. The AGID test used by the contracted commercial laboratory has only moderate sensitivity, but high specificity (A. Vanderfeen, personal communication, 2017). Consequently, false negatives are possible.

4.2.2 *Campylobacter jejuni*

Ewe antibody titres to *C. jejuni* were predominantly 'high' at the first visit and subsequently fell to 'moderate' or 'low' levels over the course of the trial. These initial 'high' titres were potentially due to increased faecal-oral transmission of *C. jejuni* over the preceding summer, from trail feeding of grain onto paddocks with little ground cover and feeding of ewes in containment lots at high stocking density. These are purported risk factors for both infection with *Campylobacter* spp. and for increased shedding of *Campylobacter* spp. by carrier sheep (Frank et al., 1965; Quinlivan and Jopp, 1982; Clough, 2003). The pattern of high titres at the initial visit followed by a progressive decline in titres at subsequent visits, suggests that peak exposure occurred prior to the first visit on all farms. The pattern of antibody titres is consistent with minimal subsequent exposure to *C. jejuni.*

There was no association between reproductive status and *C. jejuni* serology on any farm, at either mid-gestation pregnancy diagnosis or at lamb marking. Additionally, *C. jejuni* was not retrieved from any of the neonatal lamb carcasses. There was therefore no evidence of reproductive loss associated with *C. jejuni* exposure. This again reinforces the importance of examining serology in both 'wet' and 'dry' ewes before using ewe titre at lamb marking to infer the contribution of *C. jejuni* to reproductive loss, as discussed in Section 4.2.3.

Campylobacter jejuni titres in vaccinated ewes decreased after vaccination to levels below those of control ewes. Two potential explanations for this finding are low immunogenicity of the vaccine or an inability of the diagnostic test to reliably and accurately detect antibodies to *C. jejuni.* The AGID test used in this research apparently has moderate sensitivity, but no published test sensitivity exists for this test due to complications obtaining truly seronegative sheep, as *C. jejuni* is a ubiquitous commensal

organism (A. Vanderfeen, personal communication, November 2017). Thus, calculations to adjust for insensitivity cannot be performed. It can only be understood that a test with moderate sensitivity will result in more false negatives than one with higher sensitivity.

In contrast to the *C. fetus fetus* AGID, interpreting *C. jejuni* AGID results is complicated by the lack of a gold standard microbiological test, the frequent presence of 'background' reactions and the fact that the test has not been properly validated for *C. jejuni* (A. Vanderfeen, personal communication, November 2017). A preferable test would be a more sensitive, potentially immunoglobulin specific Enzyme-Linked Immunosorbent Assay (ELISA). However, attempts to develop a reliable commercial ovine *C. jejuni* antibody-ELISA have so far been unsuccessful (A. Vanderfeen, personal communication, November 2017). Due to the difficulties interpreting the results of the *C. jejuni* AGID, the lack of a profound immune response to vaccination is not interpreted as evidence of absence of immunogenicity of the vaccine. Additionally, where there are suspicions of *Campylobacter*-associated reproductive loss, obtaining the appropriate samples for microbiological culture is recommended rather than relying solely on serology.

4.2.3 Use of *Campylobacter* serology in reproductive loss investigations

If conducted at all, investigations into unsatisfactory ewe reproductive performance are often conducted retrospectively, when the expected number of lambs is compared to the actual number of lambs present at lamb marking. At lamb marking, ewes that were detected as pregnant but have suffered a perinatal loss can be identified by the presence of breech staining and careful examination of the udder. If the udder is small and dirty or non-existent, the ewe is recorded as 'dry'. In addition to a detailed history and brief clinical examination of ewes, blood samples - usually only from 'dry' ewes - may be taken to assess the ewes antibody titres to common abortigenic agents, including *Campylobacter* spp..

The findings of this research show that both 'dry' and 'wet' ewes should be examined to avoid misinterpretation of serological results. At lamb marking on Farms B, C and D, similar proportions of 'wet' and 'dry' ewes had evidence of exposure to *C. fetus fetus.* This may have occurred because elevated titres only demonstrate prior exposure, which may have occurred before gestation. Ideally, demonstrating causation requires diagnosing cases of campylobacteriosis in perinatal lambs with necropsy and microbiology. However, in many scenarios this is impractical because a problem is not identified until

weeks after lambing, at lamb marking. In these cases, it is still possible to sample 'wet' ewes, as well as 'dry' ones, to more accurately evaluate likely causes of reproductive loss.

4.3 Production variables: condition score, nutrition and WECs

Maiden ewes on three of the four farms were in good condition for mating, with a mob average around CS 3.0, and a narrow spread across the mob (Figure 6). Maiden ewes on Farm C were managed to a lower CS than other farms due to different management goals. The potential effect of differences in CS between farms was accounted for by including CS as an independent variable in multivariable models, and by including farm as a random effect.

The effect of CS on ewe fertility, fecundity and lamb survival meant that the most important criteria for this trial was that treatment groups were comparable within farm (Kenyon et al., 2014). At no time was there a difference between the groups on Farms A and B. However, at the start of mating there was a small difference in average CS between treatment groups on Farms C and D, with vaccinated ewes 0.08 and 0.03 CS heavier than control ewes, respectively. These differences would not be appreciable to an operator. Using the convention of one CS equating to 9.2 kilograms live-weight, the 0.08 CS difference amounts to a 736 gram difference in live-weight and the 0.03 CS difference to a 276 gram difference in live-weight (van Burgel et al., 2011). Despite being statistically significant, such small differences in live-weight are unlikely to be biologically significant.

To ensure both groups received identical nutrition, vaccinated and control ewes were grazed together for the majority of pregnancy. The feed available differed between farms (Table 12), but within farm there was no difference in the CS of control and vaccinated ewes at pregnancy diagnosis (Figure 5). The feed budget at pregnancy diagnosis on Farm A revealed ewes had access to 56% more feed than required. Maternal nutrition from day 30 to 90 has significant consequences for placental development (Kelly, 1992b; Redmer et al., 2004). In turn, there is a positive association between placental mass and the foetal size at birth (Reynolds and Redmer, 1995). Thus, early maternal overnutrition on Farm A may have contributed to the anecdotally heavy lambs born on this farm.

The degree of CS change between mating and pregnancy diagnosis differed between the treatment groups. Vaccinated ewes lost slightly more, or failed to gain as much, condition

as control ewes on three of the four farms. However, the difference was minimal (~0.05 CS, equating to 460 grams) and was unlikely to be biologically significant with no apparent consequence for ewe conception. There was no difference in the proportion of ewes pregnant, or in the conception rate, of control and vaccinated ewes.

The trial design required that ewes be drafted into treatment groups and allocated to paired paddocks 2 to 3 weeks prior to lambing. This design is consistent with previous *Campylobacter* vaccine trials in both Australia and New Zealand (Quinlivan and Jopp, 1982; Anonymous, 2011). The selected lambing paddocks had similar pasture composition and the same initial feed availability. By lamb marking, vaccinated ewes were in the same condition as control ewes of the same reproductive status ('wet' or 'dry') on three of four farms (A, B and D). On these farms, vaccinated ewes that were lactating lost as much condition as control ewes that were lactating over the months from pregnancy diagnosis to lamb marking (Figure 7), and this was consistent with feed availability. A small estimated difference in feed availability on Farm B between the two treatment groups did not result in differences in ewe CS between the two groups at lamb marking. Thus, on three of the four farms, there was no evidence that differences in ewe nutritional status between groups contributed to lamb marking or lamb survival.

Despite efforts to minimise paddock effects, environmental circumstances constrained feed availability more severely in the vaccinated paddock than the control paddock on Farm C. Accordingly, vaccinated 'wet' ewes lost more condition than control 'wet' ewes and vaccinated 'dry' ewes lost more condition than control 'dry' ewes (Figure 7). The vaccinated ewes were in a paddock that was severely affected by flooding, reducing both feed on offer and the dry ground available (see Section 4.5). Consequently, the energy intake of vaccinated ewes was estimated to be only 61% of that required by ewes at day 28 lactation (Table 12; Ferguson et al., 2007). By chance, the paddock containing the control ewes was less severely affected by flooding, and available pasture provided an estimated 75% of ewe requirements during lambing. The paddock conditions combined with the demands of late pregnancy and lactation reduced the average condition score of vaccinated 'wet' ewes to only 2.3. No alternative paddock was available for these ewes, and flooding meant the paddock was inaccessible to vehicles for most of lambing.

Despite the paddock differences on Farm C, lamb survival from mid-gestation pregnancy diagnosis to lamb marking was no lower in the vaccinated group than the controls. These environmental circumstances and the resulting decrease in ewe CS would be expected to

reduce lamb survival (Kleemann and Walker, 2005a). Vaccination could have indirectly increased the survival of lambs born to vaccinated ewes compared to controls exposed to *C. fetus fetus*, by increasing resilience to starvation-mismothering-exposure (see Section 4.4.3.2). If this were the case, vaccination may have masked any effect of the environmental conditions on lamb survival. A low level of *C. fetus fetus* exposure did occur on Farm C, and an infectious agent was suspected to have contributed to the death of three of the 11 lambs necropsied from the control ewes. Nonetheless, investigations were inconclusive and there was no serological evidence for reproductive loss associated with exposure.

In contrast to the nutritional deficiency on Farm C, ewes on Farm A had excess feed available from late pregnancy throughout lambing. At the farmer's discretion, ewes on Farm A were allocated to two sets of paired-paddocks. In the eastern pair of paddocks, vaccinates and controls had very high quality feed on offer in late gestation (day 140 gestation estimate: 20 MJ ME DM per day per ewe based on 2500 kg DM/ha 75% digestibility rye grass and clover). This is 40% greater than the 14 MJ ME DM per day per ewe requirement. By day 10 of lactation, the vaccinated and control ewes in the eastern paddocks still had 19% more feed than required. The feed available in the western paddocks to both vaccinates and controls more closely matched the nutritional requirements of the ewes, with availability by day 10 of lactation only exceeding requirements by 7%. As most foetal growth occurs in the last trimester, excess feed availability in late gestation can increase the risk of dystocia, especially for single-bearing ewes (Sargison, 2009). The producer on Farm A remarked that the number of ewes requiring birth assistance appeared to be higher in one of the eastern paddocks compared to all other paddocks. The implications of this observation are discussed in greater detail in section 4.4.3.2.

The multivariable models that evaluated the effect of vaccination on conception rate found that accounting for the effect of farm, CS at mating had the biggest single effect on conception rate. The positive relationship between CS at mating and conception rate is consistent with previous Australian studies (Kleemann and Walker, 2005a).

Worm egg counts

Bulk worm egg counts of each mob were used to guide management of gastrointestinal nematodes. Ewes on Farm A had the greatest WECs at the initial visit, but the worm

species present were not identified. It is possible that the high WECs on Farm A may have been due to the presence of *Haemonchus contortus*, as the size of the eggs in the sample from mating were consistent with *H. contortus* (D. Rees, personal communication, June 2016). However, at all other visits and on all other farms the egg population was dominated by eggs of sizes typical for diarrhoea-causing nematodes such as *Trichostrongylus*.

Overall, at lamb marking, vaccinated ewes had moderately greater bulk WECs than controls. However, the WEC of the vaccinates was still unlikely to be great enough to cause production loss, apart from in one group on Farm A. More detailed investigations, such as more frequent, individual-animal monitoring, are needed to evaluate any potential associations between *Campylobacter* vaccination and gastrointestinal parasitism in ewes.

Vaccinated ewes grazing one of the western paddocks on Farm A recorded a higher WEC at lamb marking than ewes in each of the three other paddocks used for this trial. This could be explained by shorter pasture availability in the western paddocks increasing larval pickup compared to the diluting effect of the longer feed in the eastern paddocks (Table 12) and different grazing histories of each paddock resulting in different degrees of larval contamination. A WEC of 1340 epg dominated by scour worms could be associated with production loss (Love and Hutchinson, 2007). However, the vaccinated ewes in the western paddock were no more affected by scouring than any other trial mob on Farm A. Approximately 10 percent of ewes on Farm A had soft faeces at lamb marking, likely due to the abundant rye grass and clover rather than a clinical effect of gastrointestinal parasitism. Additionally, the CS of vaccinated ewes on Farm A was no lower than that of control ewes. Thus, in this case, it is considered unlikely that there was an association between gastrointestinal parasite burden and ewe condition.

At lamb marking, the WEC of vaccinated ewes on Farm D was ten-fold greater than that of the control ewes. Most ewes on Farm D were administered a long-acting drench capsule prior to lambing. However, some ewes were left untreated at the producers' discretion but were not correspondingly identified. It is possible that ewes that did not receive a capsule were inadvertently sampled from the vaccinated ewes. The eggs shed from these ewes may explain the WEC increase from pregnancy diagnosis to lamb marking in the vaccinated ewes compared to the decrease in the control ewes. Overall, there appeared to be no clinically important association between worm status and vaccination.

4.4 Reproduction variables

4.4.1 Pregnancy proportion and conception rate

Administration of Ovilis Campyvax® during mating had no effect on either the proportion of maiden ewes pregnant, or the conception rate of maiden ewes on any of the four farms in this trial. Ewe conception varied between farms, as was expected for flocks with different geographic locations, management styles and enterprise types (Fowler, 2007; Suter, 2016). Despite differences between farms, there was no difference in the effect of treatment - vaccination did not change conception rates. Water in oil vaccine adjuvants can induce granulomas, abscesses and fevers (Aucouturier et al., 2001). Independent to this trial, sheep producers have raised concerns about administering Ovilis Campyvax® over mating following anecdotal reports of ewes failing to stand for rams and showing discomfort and inappetance after vaccination. On the farms involved in this trial, there was no evidence of any detrimental impact of vaccination on ewe conception as recorded at pregnancy diagnosis, allaying producers' concerns.

Interestingly, each of the participating producers reported that one group of ewes started lambing, and reached peak lambing, later than the other group. Producers were blind to paddock allocation, but reported mob ear-tag colour when observations were made. The ewes that appeared to reach peak lambing later were the vaccinated group. Although anecdotal, this observation could be explained by a shift in the mating pattern of the ewes in the vaccinated group associated with a short period of discomfort following the initial dose of the vaccine. Any ewe whose first oestrus of the mating period occurred immediately after vaccination may not have stood for the ram. However, under routine management, mating occurs over 5 to 6 weeks. Hence ewes typically have two or three opportunities to conceive. If the first oestrus was immediately after vaccination, there would be two further cycles in which ewes could be successfully mated. Consequently, there was no enduring effect of administering the vaccine during mating on conception rates. Producers using a shorter mating period could avoid any potential effects by administering the vaccine prior to the mating start date.

Campylobacter spp. are rarely implicated in early reproductive loss, with gestational infection most commonly resulting in third-trimester abortions (West et al., 2009). However, for completeness, serology was conducted at pregnancy diagnosis to assess whether any exposure to *Campylobacter* spp. had occurred over the first 45 to 90 days of pregnancy (up to pregnancy diagnosis). There appeared to be no association between

antibody titres and whether or not a ewe conceived, although no significant exposure to *Campylobacter* spp. occurred between mating and pregnancy diagnosis. Thus, firm conclusions cannot be drawn on whether vaccination could increase mid-gestation pregnancy diagnosis results compared to control ewes if the mob is challenged by *Campylobacter* infection early in gestation.

4.4.2 Lamb marking rate

Lamb marking rates did not differ between vaccinated and control groups on any farm. Nor was there any effect of vaccination on marking rate when the effect of farm was accounted for in multivariable Poisson regression models. Lamb marking rates were calculated as the number of lambs present at lamb marking divided by the number of ewes present at lamb marking, whereas the number of ewes mated would normally be used as the denominator. This deviation from convention was required due to a mis-muster of vaccinated ewes and their lambs that occurred during lambing on Farm D (Appendix 3). If the denominator had not been adjusted to the number of ewes present at lamb marking, lamb marking rates would have been underestimated in one group on Farm D and the results could not have been fairly included in the multivariable analyses.

Lamb marking rates differed significantly between farms, ranging from 100% on Farm A to 63% on Farm B (Figure 11). Due to the use of a different denominator in the calculation, these rates cannot be directly compared with those of similar enterprises in Victoria. However, differences between farms are expected due to the effect of ewe, environmental and management factors on lamb survival (Alexander, 1984; Hinch and Brien, 2014; Blackshaw and Ough, 2016; Refshauge et al., 2016). For example, Farm A produces prime lambs from Merino-Border Leicester ewes mated to South Down rams whilst Farms B, C and D run Merino ewes mated to Merino rams. Thus, farm was included as a latent term in random effects multivariable Poisson regression models to account for these differences between farms.

Differences in lamb marking rates between vaccinated and un-vaccinated cohorts of ewes have previously been used to assess the effect of vaccines against *Campylobacter* spp. on ewe reproductive output (see Section 1.5; Quinlivan and Jopp, 1982; Anonymous, 2011). Three important pieces of information are required in addition to the lamb marking rate, if differences between groups are to be attributed to vaccination. These are i) the conception rate of each treatment group, ii) the extent of *Campylobacter* spp. exposure

over gestation and iii) any potential paddock differences. Earlier studies, including the extensive study conducted by Quinlivan and Jopp (1982), report neither conception rates nor ewe serological status. Pregnancy diagnosis was conducted in the Australian vaccine study by the Glenthompson BestWool BestLamb group, but the results were not included in that report (Anonymous, 2011). The serological status of the unvaccinated ewes was not reported, and any potential paddock effect was unclear. In the current study, there was no difference in the conception rate of control and vaccinated ewes, and some exposure to *C. fetus fetus* occurred over gestation. Reporting by each participating producer in addition to neonatal lamb necropsies aided the interpretation of results. Without this information, a fair comparison of lamb marking rates between groups, and hence a complete understanding of the effect of vaccination on reproductive output would not have been possible. Evaluating lamb survival relative to expected numbers of lambs based on pregnancy diagnosis is an alternative means of assessing the effect of the vaccine on reproductive output (see Section 4.4.3). This also requires that pregnancy diagnosis be conducted and reported.

Despite some level of exposure to *C. fetus fetus* occurring between pregnancy diagnosis and lamb marking on all farms, lamb marking rates were not increased by vaccination. The level of exposure to *Campylobacter*, the environmental circumstances in the vaccinated paddocks on Farms B and C over lambing, and a possible shift in the pattern of causes of neonatal lamb mortality may explain the lack of effect (see Section 4.4.3 and 4.5 for further detail). A similar lack of difference in lamb marking rates between vaccinated and control ewes was reported on one farm in each of the two more recently published vaccine trials conducted in Australia, and in small-scale farm trials. The Coopers, MSD Animal Health trials of Ovilis Campyvax® found a 0% to 31% increase in lamb marking rates in vaccinated ewes compared to unvaccinated ewes on four farms (Walsh, 2016). An independent producer-led trial of the monovalent *C. fetus fetus* vaccine Guardian® (Coopers, MSD Animal Health, NSW, Australia) found vaccination increased lamb marking rates by 0% to 11.1% compared to unvaccinated ewes in the five ewe mobs in that trial (Anonymous, 2011). Additionally, anecdotal reports of unpublished on-farm trials of Ovilis Campyvax® conducted by Victorian sheep producers suggest variable results of vaccination, ranging from increased to decreased lamb marking rates in vaccinated ewes compared to control ewes. As the results of this current study emphasise, many factors contribute to lamb survival, and influence lamb marking rates (Alexander, 1984; Hinch and Brien, 2014). Thus, in the absence of more detailed information, the interpretation of simple field trials is difficult. However, the results of such trials are often

discussed amongst producers and in producer groups. This emphasises the importance of larger, well-structured field trials, such as the current study, to more fully evaluate the effects of vaccination in commercial flocks.

4.4.3 Lamb survival and causes of neonatal lamb mortality

4.4.3.1 Lamb survival in the context of Australian sheep production systems

As with lamb marking rate, there was no difference in lamb survival from mid-gestation to lamb marking between treatment groups on any farm. Lamb survival ranged from 80% on Farm A to 57% on Farm B, with between-farm differences most likely due to different ewe, environment and management factors. These figures represent a 20% to 43% discrepancy between the number of foetuses detected at pregnancy diagnosis and the number of lambs that survived through to lamb marking. This is consistent with published figures and discussions with sheep producers (Kilgour, 1992; Fowler, 2007; Hinch and Brien, 2014; Jacobs, 2015). The scale of the discrepancy on the Merino enterprises (Farms B, C and D) compared to the prime lamb enterprise (Farm A) mirrors that reported by the Sentinel Flock Project run from 2009-2012 by Agriculture Victoria (Suter, 2016).

4.4.3.2 Causes of neonatal lamb mortality

Across all farms and between treatment groups, dystocia was the most commonly diagnosed cause of neonatal lamb mortality. The starvation-mismothering-exposure complex (SME) and predation were also in the top three causes of death, with a difference in rank between treatment groups. Other extensive studies investigating neonatal lamb mortality have similarly found dystocia to be the dominant cause of neonatal lamb mortality (Hughes et al., 1964; Holst et al., 2002). This differed from a recent large-scale investigation into neonatal lamb mortality in Victoria, Australia (Suter, 2016). That study reported the results of 2,262 lambs necropsied from 18 flocks followed from 2009-2012. The major causes of death were the SME complex (48%), followed by prematurity (20%) and dystocia (14%). Primary predation was responsible for 6% of mortality and infection 4%. In the present study, prematurity was only recorded as a cause of death when there were clear signs of musculoskeletal dysmaturity. However, in the Suter (2016) study, the criteria for prematurity were a small lamb that had not breathed or walked, nor metabolised brown adipose tissue, and had no signs of dystocia or meconium staining (R. Suter, personal communication, August 2016). Lambs that met some of these diagnostic criteria but had no obvious cause of death were recorded in the ‘unknown’ category in

the present study. Starvation and exposure have also been reported as the dominant causes of mortality in other large scale Australian studies (Dennis, 1974; Hinch and Brien, 2014).

Participating producers were asked to check periparturient and lambing ewes regularly and the investigator was in regular contact with each of the producers throughout lambing. This allowed the investigator to document any observations producers made over lambing, and to respond to any veterinary questions producers had. In addition to the observed differences in the timing of peak lambing (see Section 4.4.1), producers reported that more ewes in one paddock required birth assistance compared to ewes in the other paddock. The paddock where increased assistance was required contained the vaccinated ewes. This observation alludes to an increased incidence of dystocia, although the actual number of ewes assisted and/or observed to have dystocia in each of the paddocks was not always reliably recorded or were not recorded, so these results could not be included.

In parallel with producer observations, necropsy results suggested different patterns in the causes of death of lambs born to vaccinated ewes compared to those born to control ewes, which may have masked a positive effect of vaccination on lamb survival. However, the neonatal mortality component of this trial was resource limited, and interpretation of the results is limited by small sample size. There was never a statistically significant difference in the proportion of necropsied lambs that died from each of the major causes of death between treatment groups. The small sample size, 50 control group lambs and 26 vaccinated group lambs, limited the ability to detect significant differences in proportions of the magnitude reported in this study. For example, 65 neonatal lambs from each group would be required to detect a significant difference of the magnitude reported for dystocia (42% in the vaccinated group; 28% in the control group) with 80% power and 95% confidence (Sergeant, 2017). To detect a statistically significant difference of the magnitude reported for SME (11% difference in proportions from 26% to 15%), 229 lambs per group would be required for 80% power and 95% confidence (Sergeant, 2017). Hence, more intensive investigations are required to measure the differences in proportions of different causes of neonatal lamb mortality in lambs born to vaccinated and unvaccinated ewes.

If the observed differences in the present study were a true effect, one explanation could involve the pathogenesis of *Campylobacter* infection altering the susceptibility of lambs to the major causes of neonatal mortality. Exposure of the late-pregnant ewe to *Campylobacter* spp. may cause placental insufficiency and increase the risk of neonatal

lamb mortality due to other causes, even in the absence of overt *Campylobacter*-associated abortions (Skirrow, 1994). In this study, the SME complex was more frequently recorded as a cause of death in lambs born to control ewes than in lambs born to vaccinated ewes (see Section 3.2.2). Additionally, on the one farm where *Campylobacter* infection was confirmed by culture, necropsied lambs from control ewes were lighter than the necropsied lambs from the vaccinates (Figure 10).

Placentitis is central to the pathogenesis of ovine reproductive campylobacteriosis (Blaser et al., 2008; Sanad et al., 2014; Sahin et al., 2017), and may explains the link between non-lethal *Campylobacter* infection of the foetus and death due to other causes, such as SME. A compromised placenta is less able to transfer sufficient energy and oxygen to the foetus, restricting foetal growth and potentially reducing birth-weight (Kelly, 1992b). In turn, low birth-weight lambs have higher surface area to volume ratio and are more susceptible to hypothermia and death from exposure (Hinch and Brien, 2014). Additionally, low birth-weight lambs tend to have lower vigour, are slower to stand and suck less frequently than higher birth-weight lambs (Hinch et al., 1985a; Dwyer et al., 2003). Placental restrictions also impact lamb behaviour (Dwyer et al., 2005). These behaviours increase the risk of mismothering. Hence, lambs born to control ewes exposed to even a low level of *C. fetus fetus* or *C. jejuni* infection may be at greater risk of dying due to the SME complex. The corollary of this would be that lambs born to vaccinated ewes, protected against the placental insufficiency induced by *Campylobacter* infection, may be heavier with greater vigour and thus lower susceptibility to SME. Starvation-mismothering-exposure is often cited as the most common cause of neonatal lamb mortality in studies on Victorian sheep flocks (Suter, 2016). Thus, decreasing any infection-associated susceptibility to SME by vaccinating against *Campylobacter* could have a substantial impact on neonatal lamb survival, if other causes of neonatal lamb mortality can be managed.

While SME was less frequently recorded in lambs born to vaccinated ewes, dystocia was more frequently recorded (Table 16). This pattern was consistent on three of the four farms (A, C and D; Table 17). Farm A did not submit four lamb carcasses picked up from the vaccinated ewes prior to the first collection because the producer believed dystocia to be the clear cause of death. Unfortunately, these lambs could not be retrieved for confirmation of cause of death and were thus not included in the overall necropsy results. Hence the proportion of neonatal lamb deaths due to dystocia in the vaccinated ewes is likely an underestimate. Although the difference in proportions was not statistically

significant, possibly due to small sample size, the necropsy findings were still consistent with anecdotal reports of more ewes requiring lambing assistance in the vaccinated paddocks compared to the controls.

Furthermore, the decrease in SME deaths but increase in deaths due to dystocia in the lambs born to vaccinated ewes could be associated with a positive effect of vaccination on lamb birthweight. In this study, foeto-pelvic disproportion was the most common cause of dystocia, based on lamb weight and the presence of localised oedema of the head and neck (Wilsmore, 1989). Factors that contribute to foeto-pelvic disproportion include ewe age, lamb sex and lamb birth-weight, influenced in part by ewe nutrition and sire (Smith, 1977). Lambs that died due to dystocia were more likely to be male than female, compared with lambs that died from other causes of death (Appendix 5). However, the proportion of ram-lambs to ewe-lambs necropsied did not differ between the treatment groups. Treatment groups were mixed for mating, ensuring sires were homogenously distributed, and dam age was uniform across all farms. Hence these factors are unlikely to have contributed to an increase in dystocia in the vaccinated groups.

Ewe nutrition in late pregnancy is vital for foetal growth, with 75% of foetal growth occurring in the last six weeks of pregnancy (Stevenson, 2014). Consequently, maternal under- or over- nutrition in the last six weeks of gestation can affect lamb survival by increasing susceptibility to hypothermia or dystocia respectively (Sargison, 2009). Late gestational maternal over-nutrition is suspected to have affected both treatment groups on Farm A, likely increasing the risk of dystocia (Table 12). In comparison, the under-nutrition due to flooding on Farm C differentially affected the vaccinated paddock but this occurred after lambing had commenced and was observed at day 28 lactation (Table 12). Over-nutrition may more severely affect single-bearing ewes and under-nutrition multiple-bearing ewes. The risk of mortality and the susceptibility to different causes of death change for single or multiple-born lambs (Hinch and Brien, 2014). Whilst it was not possible to determine whether lambs were single- or multiple-born in this study, there was no difference in the number of multiple pregnancies between treatment groups and single pregnancies were more common than multiples on each farm. Thus, it is likely that overnutrition of the predominantly single-bearing ewes on Farm A could have increased the risk of dystocia on this farm.

Vaccination may have protected late-pregnant ewes against placental compromise due to *Campylobacter* infection, increasing foetal growth rate and lamb birth-weight compared

to unvaccinated, infected ewes. For ewes in the target lambing condition score of 3.0 on an appropriate plane of nutrition, the resulting higher lamb birth-weight lamb could have decreased risk of SME compared to a lighter lamb born to a *Campylobacter*-exposed ewe. However, for ewes in higher CS grazing excess feed, higher birth-weight lambs could be at greater risk of dystocia. This pattern seemed to occur on Farm A, in the absence of potential confounding factors. For example, lambs that died of dystocia were heavier than those that died due to any other cause in both vaccinates and controls (Appendix 5). Similarly, both treatment groups were co-grazed until late gestation and vaccinated ewes were in equal or lighter CS than controls, with similar or even lower pasture availability on the farms where this pattern was observed.

These observations suggest that *Campylobacter* infection, and the effects of vaccination, have more subtle effects on ewe reproduction. These may be mediated through lamb birth-weight, leading to different patterns of lamb mortality between unvaccinated and vaccinated ewes. This could explain situations where *Campylobacter* exposure occurs but there is no significant effect of vaccination on lamb survival or lamb marking rates. The hypothesised effect would be greatest in mobs where singleton pregnancies are more common. The potential interaction between treatment group and CS at lamb marking on lamb marking rate is also consistent with this hypothesis: at lower CS, vaccinated ewes marked more lambs than controls, whereas the opposite occurred in heavier ewes. This may mean that producers can reduce the amount of feed allocated to vaccinated ewes on *Campylobacter* endemic farms and achieve improved reproductive outcomes.

It should be noted that the multivariable models were very sensitive to the inclusion or exclusion of different binomial and continuous variables. Thus, the multivariable analyses reported contribute to the hypothesis but more research, in a large-scale study with sufficient power is required. A brief discussion of this future research is contained within Section 6.3.

4.4.4 Recommendations for clinical investigations of neonatal lamb mortality associated with infectious agents

Infectious agents were suspected to have contributed to death in five lambs necropsied from the control ewes on Farm A and three lambs from Farm C, based on their low birth-weight, meconium staining and/or mildly enlarged livers. *Campylobacter fetus fetus* was only successfully cultured from samples on Farm A. Where culture was unsuccessful on

Farm A, comma shaped, gram-negative bacteria resembling *Campylobacter* spp. were observed on stained, air-dried smears of abomasal contents. *Campylobacter* spp. was not isolated from the lambs on Farm C, and so the cause of death for these lambs was recorded as 'unknown' or SME, depending on other findings. Importantly, *Campylobacter* diagnosis in neonatal lambs was not always associated with obvious gross post-mortem lesions that might raise suspicion of infection such as markedly enlarged livers and an increased volume of serosanguinous peritoneal or pleural fluid. No 'classic' necrotic liver lesions were observed. This shows the importance of considering the involvement of *Campylobacter* spp. as a cause of neonatal lamb death in situations where classic post-mortem lesions are absent, especially in cases of stillbirth of a term lamb with no other explanation for cause of death, and diagnoses of the SME complex in relatively mild weather.

An aspirate of lamb abomasal contents for smear and microbiological culture was the most useful sample for diagnosis of *Campylobacter* infection in this study. In situations where long travel times were required, sample delivery was protracted or necropsies were conducted on lambs that had been dead for 24-48 hours, conducting a smear of abomasal contents was simple, cost-effective and clinically useful. In these cases, *Campylobacter*-shaped organisms were seen following staining when there was a strong suspicion of infection, but culture was unsuccessful.

4.5 Further comments on the trial

Despite attempts to minimise potential confounding effects of paddock on lamb marking rate and lamb survival, unexpected environmental effects occurred on three of the four participating farms. Details of unforeseen farm or paddock specific factors documented over the course of the trial are reported in detail in Appendix 3.

Extreme rainfall, exceeding historical 90th percentile figures, coincided with peak lambing on Farms C and D (Figure 4). This, combined with poor paddock drainage, resulted in severe flooding. On Farm C, the paddock containing the vaccinated ewes was most severely affected. No alternative paddocks were available for the vaccinates and the paddock was inaccessible when the flooding was at its peak. There was a 14% difference in feed available in this paddock compared to the control paddock at day 28 of lambing, and vaccinated ewes lost significantly more condition over lambing and were in lower CS than control ewes of the same 'wet'/'dry' status at the subsequent lamb marking visit.

This might have been expected to reduce lamb survival in this paddock. However, lamb survival and lamb marking rates from the affected paddock were similar to the controls. Thus, it is possible vaccination mitigated against any effects of weather on lamb survival. Farm D was also affected by this severe weather event. However, both vaccinated and control paddocks were reported to have been affected to a similar degree on Farm D.

The excessive rainfall on Farms C and D also meant only one lamb necropsy visit was conducted. Producers on Farms C and D were sent sampling kits to collect swabs and record details of neonatal lamb mortality in each paddock, following the investigator's instructions (Appendix 2), but this was only performed on Farm D. On Farm C, dead lambs were still collected over a full 72 hours prior to the necropsy visit.

Across all farms, predation was diagnosed more often in the necropsied lambs born to vaccinated ewes than in those born to control ewes. This result was likely mainly due to outcomes on Farm B, where predation was about four times more common than on the other farms. It is likely that predators were more active in a single paddock, consistent with local observations, which coincidentally held vaccinated ewes during the trial. Despite the prevalence of predation in the vaccinated lambs on Farm B, lamb survival was not significantly lower in the vaccinated paddock. Any positive effect of vaccination on lamb survival may have been masked by the increased mortality from predation.

The results and analysis of ewe 'wet' or 'dry' status at lamb marking have been included in the results section for completeness (see Section 3.3.4). These results are not discussed in detail, because the practicalities of the lamb-marking visit meant that ewes were inconsistently assessed across farms, due to the speed of evaluation and experience of different operators. Ewes that were included in the serology monitoring component of the project were assessed carefully by the investigator.

So as to be representative of the Victorian sheep enterprises, the trial included one prime lamb and three Merino enterprises. Potential differences between these farms include geographic location, genetics and management. However, any potential confounding effects of these differences were addressed by including farm as a random effect in multivariable analyses.

5 – Conclusions and implications for producers

On the trial farms, there was no detrimental effect of vaccination with Ovilis Campyvax® on

1. Ewe condition score, with no significant difference between the vaccinated and control group at pregnancy diagnosis (accept the null hypothesis)
2. Pregnancy proportion, with no significant difference in the proportion of ewes conceiving between vaccinated and control groups (accept the null hypothesis)
3. Conception rates, with no significant difference in the number of foetuses relative to ewes present at pregnancy diagnosis in vaccinated and control groups (accept the null hypothesis)

These findings show that Ovilis Campyvax® can be used at the start and end of a standard 5 to 6 week mating period without detrimental effect.

No significant increase in lamb marking rate or lamb survival from mid-gestation to lamb marking was detected in the vaccinated group compared to the control group on any farm, despite evidence for exposure to *C. fetus fetus* during gestation and the diagnosis of *Campylobacter*-associated reproductive loss on one farm. There are three main explanations for the lack of difference between the treatment groups:

1. Exposure to *Campylobacter* spp. was insufficient to cause significant reproductive loss in the control ewes compared to vaccinates
2. Benefits of vaccination were masked by unpredictable and uncontrollable paddock-level confounding effects that differentially affected the vaccinate paddocks compared to the controls, especially on Farms B (predation) and C (flooding)
3. Benefits of vaccination were masked by differences in the patterns of neonatal lamb mortality in vaccinates compared to controls, especially on Farm A

The results emphasise that the effect of vaccination against *Campylobacter* spp. on reproductive output is complex and multifactorial. Even where *Campylobacter* is diagnosed as a cause of neonatal lamb mortality, other causes of death may mask the benefits of vaccination. The risk of the other causes of death may be influenced by vaccination directly, or may be the result of 'external' factors, such as predation and weather. Therefore, sheep producers concerned about a perceived ineffectiveness of

vaccination against *Campylobacter* spp. in one year should interpret lamb marking results in light of the conditions faced by ewes throughout gestation and lambing. Prospective monitoring of vaccinated ewes may help more clearly describe the potential effects of vaccination in specific circumstances, especially where *Campylobacter* spp. is known to be endemic on farm.

The findings of this research again highlight the importance of appropriate ewe nutritional management throughout pregnancy for optimal reproductive output. As in any situation, producers should avoid overnutrition of ewes carrying single pregnancies. Vaccination may require producers to re-evaluate ewe nutritional management, and may lead to more economically efficient feed management practices by further reducing the feed requirements of vaccinated ewes. This needs to be examined in future research.

6 – Future directions

6.1 Introduction

Several important questions have been raised by the findings presented in this thesis. These could be addressed in the future by either continuing to monitor the existing trial flocks or by initiating new investigations, specifically designed to test the hypotheses developed as a result of this work. These include conducting longitudinal serological monitoring of both naturally exposed and vaccinated ewes, and determining the effect of vaccinating against *Campylobacter* spp. on the progeny of vaccinated ewes.

6.2 Longitudinal serological monitoring

There is no published information describing how antibody titres change with time in ewes either naturally challenged with *C. fetus fetus,* or in those vaccinated with Ovilis Campyvax® considering background exposure to this organism. Antibody titres are often measured at lamb marking as part of investigating reproductive loss. However, their interpretation is limited by this lack of information, which longitudinal serological monitoring could overcome. Continuing to monitor the serological status of both the vaccinated and control ewes, including those that seroconverted during gestation in 2016, would build on the serological observations in this study. The results would demonstrate the longevity of raised antibody titres in vaccinated ewes in light of the exposure level on farm, as indicated by the serology results from control ewes. Continuing to follow the control ewes which seroconverted in 2016, and those that did not, would be informative for veterinarians needing to interpret the results of targeted mob serological surveillance.

6.3 The effect of Ovilis Campyvax® on the progeny of vaccinated ewes

There are two avenues that warrant further attention concerning the possible benefits of vaccinating against *Campylobacter* for the progeny of vaccinated ewes.

6.3.1 Differences in the causes of neonatal lamb mortality between groups

Although only a modest number of lambs were necropsied, there was a suggestion of different causes of neonatal lamb mortality in the vaccinate and control groups. The results indicated that lambs born to control ewes may be more susceptible to SME than those born to vaccinated ewes, who may be more susceptible to dystocia. However, the

current study did not have the resources to investigate this in more detail. The results presented are essentially a pilot study that have resulted in the formation of a specific hypothesis - that the distribution of causes of neonatal lamb mortality differ between lambs born to vaccinated ewes and those born to control ewes. A larger, more intensive, appropriately powered study is required to test this hypothesis.

In addition to increasing the number of necropsies and the frequency of sampling, comparing the weights of dead neonatal lambs between the groups would be an important component of the future research. The results from the current research suggest that on the farm where *C. fetus fetus* was confirmed, lambs born to control ewes were lighter than those born to vaccinated ewes. As described, *Campylobacter* induced placentitis could reduce lamb birth-weight, increasing neonatal susceptibility to SME. The possible increased dystocia in vaccinated ewes could result from a combined effect of overnutrition in late pregnancy and the benefits of vaccinating, i.e. in the absence of *Campylobacter*, overnutrition in late pregnancy may increase the risk of dystocia.

The risk of neonatal mortality, and the susceptibility to different causes of mortality changes with the number of foetuses conceived (Hinch and Brien, 2014). If any difference in causes of neonatal lamb mortality was mediated by the effect of *Campylobacter* on lamb birth-weight, the consequence may differ for single and multiple-born lambs. For example, increased birth-weights may be detrimental for single-born lambs but beneficial for multiples. Hence, the hypothesis should be tested in single- and multiple-bearing ewes managed separately. This would also remove any confounding effect of litter size.

6.3.2 Benefits of vaccination for the progeny of vaccinated ewes

The pathogenic mechanisms discussed as potentially contributing to differences in neonatal lamb mortality could also result in differences in the live-weight of lambs from different treatment groups. This could be investigated by testing whether there are any differences in the weights of lambs born to vaccinated and control ewes, and the weight of these lambs at both lamb-marking and weaning. The investigator would need to account for ewe CS, feed availability and *Campylobacter spp.* challenge. However, if *Campylobacter* infection did influence lamb birth-weight, and this difference persisted until lamb marking and weaning, there could be further positive economic effects of vaccination for the producer.

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8 Appendices

Appendix 1 Visit schedule

Visit	Date	Purpose / objective	Samples or data
Rams in	**Autumn 2016**	**1) Random allocation** of maiden ewes to vaccinates and controls, RFID and/or coloured ear tag placed accordingly **2) First vaccine** given to all vaccinates	**1) 12 bloods** from each group **2) Condition score** each enrolled ewe **3) Feed budget** **4) 10 faecal samples** from each group for bulk WEC **5) Record of RFID vaccinate and control**
Throughout mating	Autumn	Trial ewes run as one mob for the duration of joining (5-6 weeks) and managed identically	
Rams out	Autumn	**1) Second vaccination** to all ewes in vaccination group	**1) Condition score 50 random ewes** **2) Feed on offer** assessment
Early-mid pregnancy	**Run maiden ewes as <u>one mob</u>**: routine management procedures		
Pregnancy diagnosis	Winter	**1) Record results of scanning** **2) Condition scoreewes** **3) Draft off dry ewes** **4) Determine serostatus** of pregnant and dry ewes	**1) Scanning** results (0,1,2 against each ewe) **2) Condition score** each ewe against EID **3) Feed on offer** assessment **4) Lambing paddock** assessment **5) Bleed pregnant and dry ewes from each group** (max 12) and submit samples to ACElabs for serostatus 6) Confirm lambing paddocks 7) Bulk WEC
Late-pregnancy (2-4 wks pre lambing)	Winter	**Pre-lambing** clostridial ± pre-lambing drench, according to regular farm management	****in consultation with investigators** **1) FOO assessment** **2) Allocate to paired paddocks** for lambing **3) Watch for abortions** – quiet drive around ewes twice a week, call Mackinnon veterinarian if required

Visit	Date	Purpose / objective	Samples or data
Lambing	Spring	**1) Lamb pick up** on two days for necropsy of cause of death (day 8 and day 15). 2) Otherwise follow normal husbandry on each farm	**1) Routine supervision of lambing ewes with contact made when manager concerned about lamb or ewe loss** **2) Farms A & B:** two lamb pick ups (estimate 10/farm; 5/paddock) **3) Farms C & D:** farmer assess cause of death and swab as necessary; one visit only for necropsy due to weather
Lamb marking	Spring-summer	**1) Determine marking percentage** **2) Post-lambing serostatus** of ewes **3) Ewe condition score**	**1) Number of lambs** marked **2) Number of dry ewes** in vax versus control **3) Condition score ewes** **4) Feed on offer** **5) Bleed** 12 dry and 12 wet ewes from vaccinated & controls **6)** Bulk WEC for each mob

Appendix 2 Example of lamb pick up recording sheet for producers on Farms C and D; one set of sheets for the ear tag colour that coincided with each treatment group.

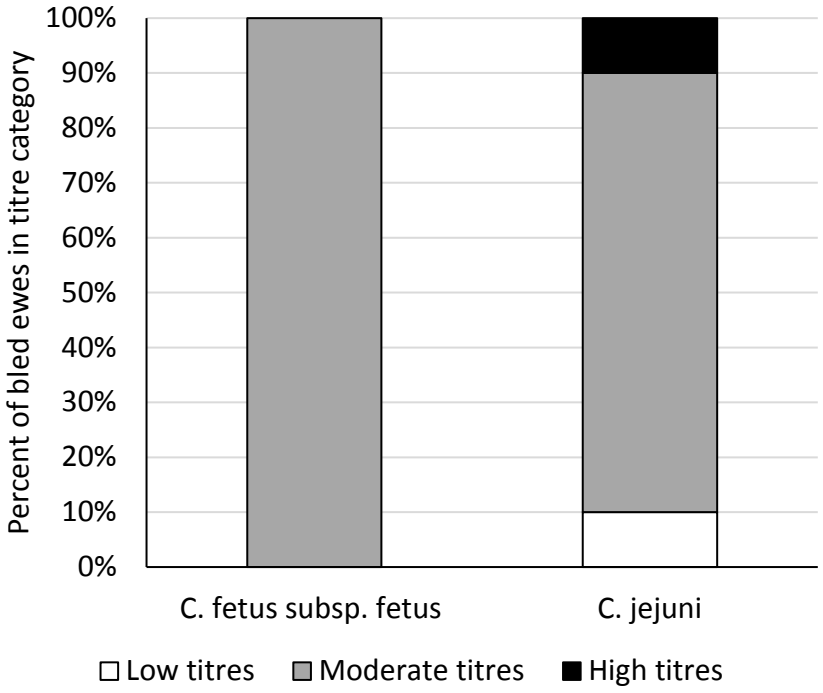
Date	**Ear tag group**	**Lambs**						**Ewes**
		No obv cause (note if small)	**Stillborn:** didn't walk (slippers on feet); note if meconium	**Dystocia** (slow birth, got stuck: swollen head or hindlimbs)	**Exposure**	**Major predation**	**Total # picked up**	Ear tag # of any that die

Appendix 3 Events recorded over the duration of the trial that could have influenced the outcome of the trial (Farms B, C and D)

Farm	Issue	Explanation and consequence
B	**Predation:** one paddock found to have higher predator activity	To match paddocks for size and feed availability, one paddock was adjacent to a road, and one adjacent to a creek line. Greater predation was observed in one of the paddocks. The producer was blind to the paddock allocation. During lamb necropsies, care was taken to determine whether predation was pre- or post-mortem and that the extent of the predator damage was consistent with it being a primary cause of death
C	**Ewe live-weight**	A proportion of ewes were lighter than ideal for joining, resulting in a smaller cohort of pregnant ewes in each treatment group
	Flooding: one of the two lambing paddocks more severely affected	Above average rainfall resulted in ground water accumulating in the lower paddock over lambing, decreasing feed availability for ewes and potentially increasing the risk of neonatal lamb loss. These paddocks were inaccessible to vehicles throughout much of lambing. No higher ground was available.
D	**Disease management practices**	Ewes removed from maiden ewe flock prior to joining, decreasing sample size to 220 per group
	Mis-muster	**1) Pregnancy scanning**: a number of ewes were absent at scanning but later reappeared. These ewes were excluded from the trial from this visit onward to avoid unfair assumptions about pregnancy status **2) Lamb marking:** a number of ewes were absent from the vaccinated cohort especially at lamb marking. These ewes were not recorded as deceased by the farmer, and it was considered likely that their lambs and them had pushed through a fence into an adjacent paddock.
	Flooding: across both lambing paddocks	Increased surface water, and saturated paddocks resulted in an increase in disease associated with macerated skin including strawberry footrot, and death of 7-10 day old lambs associated with septicaemia extending to neurological signs (one case was investigated and *E.coli* cultured)

Producer observation	Mortality of young lambs during the time of greatest water accumulation on paddocks in late lambing. The producer observed more lambs from the vaccinated ewes were born later, and felt that these lambs were more affected by the flooding than lambs from the control ewes that were born earlier in lambing and were thus older when the flooding was worse

Appendix 4 *Campylobacter fetus fetus* and *C. jejuni* serology results from n = 10 maiden ewes sampled by an independent veterinarian in October 2014, 18 months prior to the start date of the reported trial. The dominance of 'moderate' titres (1:10-1:60) to *C. fetus fetus* is suggestive of endemicity and low-level flock exposure.



Appendix 5 Mean weight and male to female proportion of lambs necropsied born to control and vaccinated ewes that died as a result of dystocia or any other cause of death.

Cause of death	Treatment group	Male : female %	Mean lamb weight (kg)	*n*	SEM
Dystocia	Control	71%	5.1[a]	14	0.17
	Vaccination	73%	5.3[a]	10	0.23
Other	Control	53%	4.0[b]	35	0.12
	Vaccinated	43%	3.9[b]	14	0.18

[a,b]Superscript denotes the presence of a significant difference between mean lamb weights ($P < 0.05$)