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Epidemiology and production effects of leptospirosis in New Zealand sheep

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Abstract

Leptospirosis causes clinical disease in sheep and is an important occupational disease in New Zealand. Contact with sheep has been shown to be a significant risk factor for human infection, particularly in meat workers. Up to 97% of New Zealand sheep flocks are seropositive to *Leptospira borgpetersenii* serovar Hardjo (Hardjo) and/or *Leptospira interrogans* serovar Pomona (Pomona), yet vaccination is rare.

The work presented in this thesis investigates the epidemiology and effects on sheep growth and reproduction of Hardjo and Pomona, as well as the effectiveness and the effects on sheep production of a commercial bivalent Hardjo and Pomona vaccine.

A split-herd vaccination trial involved a bivalent Hardjo and Pomona vaccination programme for one third of 2260 ewe lambs on 8 farms starting at one month of age. Repeated blood samples were taken over one (6 farms, mated as hoggets) or two (2 farms, mated as 2-tooths) years for microscopic agglutination testing to assess exposure to Hardjo and Pomona in the unvaccinated group. Weight and pregnancy, docking and weaning data were recorded and compared between vaccinated and unvaccinated, as well as between seropositive and seronegative within the unvaccinated group. Urine samples were collected from a random subsample of both vaccinated and unvaccinated sheep on each farm one to two years after the beginning of the study and the samples were analysed by real-time PCR.

The Hardjo exposure pattern was consistent across seven out of eight farms, with exposure occurring at around 10-15 months. On one farm Hardjo exposure started before weaning. Three farms became positive for Pomona at around 8-15 months. The description of the serological patterns identified a period at risk for sheep exposure to leptospirosis, and also possibly at risk for humans handling sheep.

The overall vaccine effectiveness was 86.3% [63.6-94.8], with the lowest farm level effectiveness 76% [29-92], in spite of a vaccination schedule differing from the manufacturer's recommendations on some farms. Vaccination timing seemed to be crucial in achieving optimum reduction in shedding in urine of vaccinated sheep. These results can be used to inform vaccination best practice guidelines and recommendations.

Comparison of growth performance between sheep seropositive for Hardjo and/or Pomona and seronegative did not allow for definitive conclusions as the results varied between farms and periods in magnitude and direction of difference. The results showed a significant effect of recent Hardjo infection in hoggets on reducing lamb survival from docking to weaning. No other statistically significant difference in reproductive rates was observed for either serovar. No difference in growth or reproduction was observed

between vaccinated and unvaccinated sheep. Hence, vaccination appears unlikely to be cost-effective on most New Zealand sheep farms where exposure patterns would be similar to those observed in this study. However, more data is needed to understand the variability in the results observed between the different study farms. This conclusion also does not account for the possible cost of human infection. Furthermore, the Pomona exposure was possibly not high enough to identify any production effect associated with this serovar, so more data on the effects of Pomona would be needed for robust conclusions.

This likely absence of production effects contrasts with what has been observed in New Zealand farmed deer, where vaccination was shown to improve growth rates and weaning rates.

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List of Publications

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Heuer C*, Sanhueza J, Vallée E, Wilson P, Benschop J, Collins-Emerson JM. Can vaccination of animals protect humans against Leptospirosis?. *9th scientific meeting of the International Leptospirosis Society*, Semarang, Indonesia, 2015

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Chapter 1. Introduction

This introduction presents context and background information about leptospirosis in livestock (cattle, sheep, goats and farmed deer) and its impact on human health. A specific focus is given to the situation in New Zealand. It also provides an introduction to the aims and structure of the thesis.

1.1. Etiologic agent: *Leptospira* species

1.1.1. Characteristics

Leptospirosis is a ubiquitous disease caused by spirochaete bacteria of the *Leptospira* genus (Levett 2001). Leptospirens have a width of around 0.1 μm , with hooked ends. They are flexible and mobile as a result of axial filaments, endoflagella and a fluid outer envelope (Baranton and Old 1995). They are aerobic and use β -oxidation of long-chain fatty acid as a source of energy. The LPS (lipopolysaccharide) layer supports the main antigens and has a structure close to gram negative bacteria, although this staining is not possible due to low affinity for the Gram stain (Turner 1976; Baranton and Old 1995; Faine *et al.* 1999).

Genetic material of *L. interrogans* is composed of two circular chromosomes of around 5Mb and 350 kb. The exact size varies depending on the strain, but it is larger than that of other spirochaetes (Saint Girons *et al.* 1992; Baranton and Old 1995). Ribosomal DNA is scattered in the genome and not organised in an operon, as is usually the case for other bacteria (Saint Girons *et al.* 1992; Baranton and Old 1995). The ribosomal genetic material is present in only one or two copies (Baranton and Old 1995), indicating a relatively slow growth compared with most bacteria, as well as a slower reaction to a change of environmental conditions. Low growth rate can however be a selective advantage in a poor environment and avoid spoilage of resources (Klappenbach *et al.* 2000).

1.1.2. Classification

Historically, the first classification divided the leptospirens into saprophytic (*L. biflexa*) and pathogenic (*L. interrogans sensus lato*) organisms. Nowadays, two classification systems currently coexist. The serological classification is based on antigenic properties of *Leptospira* and the serological response of the host (discussed in section 1.3.6.), especially the production of antibodies directed against specific epitopes of LPS antigens. It defines serogroups and serovars and is still the most widely used due to its epidemiological and ecological significance. The identification at the serovar level is by a cross agglutination absorption test with a reference antigen. The reference serological test, the microscopic agglutination test (MAT, presented in section 1.3.7.), provides information on the infecting serogroup. Currently more than 260 serovars comprising 24 serogroups have been identified (Adler and de la Pena Moctezuma 2010), but new serovars are regularly identified.

The genotypic classification is based on DNA-DNA hybridization studies and increasingly by sequencing methods and currently distinguishes nine pathogenic genomospecies: *L. alexanderi*, *L. alstonii*, *L. borgpetersenii*, *L. interrogans*, *L. kirschneri*, *L. noguchi*, *L. santarosai*, *L. kmetyi* and *L. weilii*, five species for which pathogenicity has not been demonstrated experimentally: *L. wolffii*, *L. inadai*, *L. licerasiae*, *L. fainei* and *L. broomii* as well as at least six saprophytic genomospecies (Adler and de la Pena Moctezuma 2010; Picardeau 2013). However, little or no correlation exists between the two classification systems and serovars can belong to different genomospecies, and a genomospecies can include both pathogenic and saprophytic serovars (Brenner *et al.* 1999; Levett 2001). The serological and molecular characteristics of the circulating strains can vary slightly, and be region-specific in some remote parts of the world, especially on islands (Bourhy *et al.* 2012).

1.2. Epidemiology and ecology

Leptospirosis is a ubiquitous disease and it thought to be the most prevalent zoonosis worldwide (Hartskeerl *et al.* 2011). Estimations of the global burden of disease showed that around 58,900 deaths are caused by leptospirosis each year, with more than 1 million cases assumed to happen (Costa *et al.* 2015). Traditionally, the epidemiology of leptospirosis involves one or several maintenance host species, accidental hosts and the environment. Animals and humans get infected by direct or indirect contact with urine of shedding animals.

In New Zealand, six serovars have been isolated from animals (Table 1-1). *L. interrogans* serovars Canicola and Australis have also been isolated from human patients but not from animals (Marshall and Manktelow 2002).

Table 1-1: Species, serovars and serogroups isolated from animals in New Zealand and traditionally recognized reservoir species (Hathaway and Marshall 1980; Levett 2001; Marshall and Manktelow 2002)

Serovar	Serogroup	Known reservoir host
<i>L. borgpetersenii</i> Hardjo (type Hardjo-bovis)	Sejroe	Cattle Sheep Deer?
<i>L. interrogans</i> Pomona	Pomona	Pigs
<i>L. borgpetersenii</i> Ballum	Ballum	Rodents
<i>L. borgpetersenii</i> Tarassovi	Tarassovi	Pigs
<i>L. interrogans</i> Copenhageni	Icterohaemorrhagiae	Rodents
<i>L. borgpetersenii</i> Balcanica	Sejroe	Possums

Serovars are associated with one or a group of maintenance species, showing strong nidality. These were defined by Hathaway (1981) as species that are able to:

- Become infected with a low dose of leptospire
- Host the leptospire in their kidneys for a “long” time
- Infect one another within the species

Marshall and Manktelow (2002) added that the considered serovar should not kill the maintenance host, and that renal carriage could last for up to a lifetime. Asymptomatic and seronegative carriage can happen in maintenance hosts (Ellis *et al.* 1981). Genital carriage has also been reported in maintenance species and the venereal route could play a role in the transmission within maintenance species (Ellis 2015). For example, cattle, the recognised maintenance host for serovar Hardjo, were shown to shed leptospire for at least 18 months after experimental infection (Thiermann 1982). Leptospiuria, measured by culture, was observed for 10 months in heifers naturally infected with *L. interrogans* Hardjo (Leonard *et al.* 1992). Dogs and Canicola, swine and Pomona or rats and Icterohaemorrhagiae are other common maintenance host/serovar associations.

Infection of accidental (i.e. non-maintenance) hosts is the most noticeable as may cause clinical manifestations of the disease. According to Hathaway’s (1981) ecological classification, accidental hosts are not expected to be able to establish renal carriage and require a higher infectious dose than maintenance hosts. However, the distinction maintenance/accidental host is a simplification and does not allow representation of the complexity and the dynamics of the epidemiology of leptospirosis. For example, sheep and cattle are considered incidental hosts for Pomona and possibly present clinical manifestations (Vermunt *et al.* 1994; Bruere 2013). However, 74% of sheep flocks and 72% of beef cattle farms show evidence of exposure to Pomona, along with 14% of sheep and 25% of cattle respectively (Dreyfus *et al.* 2011). Furthermore, these species can shed Pomona in urine (Morse *et al.* 1957; Hodges 1974; Carter *et al.* 1982; Kingscote and Wilson 1986). This suggests that while they are still susceptible to clinical manifestations at the individual level, sheep and cattle could be a reservoir for Pomona at the population level in New Zealand.

Leptospire are able to survive outside hosts, in the environment. Their survival is promoted in humid places such as surface fresh water. Canicola is able to survive for up to 110 days in distilled water (Trueba *et al.* 2004). Pomona can survive for at least 42 days in soil in simulated New Zealand winter conditions (Hellstrom and Marshall 1978) with their ideal temperature range is between 10 and 34°C and their ideal pH is between 6.0 and 8.4 (Okazaki and Ringen 1957). Khairani-Bejo *et al.* (2004) showed that *L. interrogans* Hardjo can survive for six hours in cattle urine in the shade, three days in urine diluted with water and up to six days in loam soil with acidic pH. Survival is variable between strains, with some more adapted to environmental survival while others are suspected to lose this ability (Bulach *et al.* 2006). In endemic situations, cattle

can be re-exposed to serovar Hardjo from environment and surface water (Martins *et al.* 2010).

The host-serovar relationship is dynamic and can change in time and place. For example, sheep were previously not considered to be a maintenance host for *L. borgpetersenii* serovar Hardjo type Hardjo-bovis in New Zealand (Blackmore *et al.* 1982; Marshall and Manktelow 2002), while this role was suggested in other countries (Cousins *et al.* 1989; Gerritsen *et al.* 1994b). The role of sheep in the epidemiology of Hardjo-bovis in New Zealand then evolved to now match the criteria for being maintenance hosts, including high seroprevalence with few or no clinical signs reported (Dreyfus 2013) and renal carriage (Dorjee *et al.* 2008). Black rats are the usual maintenance host for Ballum in New Zealand, but brown rats can play this role in the situation where there is a high population density and where black rats are absent (Hathaway and Blackmore 1981).

1.3. Leptospira and leptospirosis in pastoral livestock

1.3.1. Situation in New Zealand

Leptospiral infection, especially due to serovar Hardjo-bovis and Pomona, is endemic in New Zealand. The animal level seroprevalence and farm-levels seroprevalence are presented in Table 1-2.

Within-flock/herd seroprevalence in Fang *et al.* (2014a) ranged from 39-95 % for Hardjo-bovis and/or Pomona in sheep and 69-83 % in cattle. Dreyfus *et al.* (2011) found a median within-farm seroprevalence of 44% for Hardjo-bovis and 10% for Pomona in sheep, 51% for Hardjo-bovis and 15% for Pomona in beef cattle and 5% for Hardjo-bovis and zero for Pomona in deer. Heuer (2007) found a within-herd prevalence of 54% for Hardjo-bovis and 46% for Pomona in beef cattle herds. Ayanegui-Alcerreca *et al.* (2010) reported a within-herd seroprevalence ranging from 0 to 100% for both Hardjo-bovis and Pomona in deer.

Table 1-2: Summary of published cross-sectional studies of leptospirosis seroprevalence in New Zealand livestock since 2007, including origin and age of the animals, serovars tested, MAT cut-off used, number of animals tested, observed animal-level seroprevalence, number of farms tested and farm-level seroprevalence.

Reference	Origin of animals	Species	Age of animals	Serovar	MAT cut-off	Animal level		Farm level	
						Number tested	Seroprevalence (%)	Number tested	Seroprevalence (%)*
(Heuer 2007)	Beef cattle farms	Beef cattle	≥ 12 months old, mainly 12-18 months old	Hardjo-bovis Pomona	NA	1,265	34 12	85	62 26
(Subharat <i>et al.</i> 2007)	Farms in the Manawatu and Hawkes Bay	Sheep	≥ 12 months old	Hardjo-bovis	1:48	791	31	14	71
				Pomona	1:48		0		0
				Dual Hardjo-bovis -Pomona	1:48		1		0
		Beef cattle	≥ 12 months old	Hardjo -bovis	1:48	273	55	15	80
				Pomona	1:48		2		0
				Dual Hardjo-bovis -Pomona	1:48		4		13
Deer	≥ 12 months old	Hardjo-bovis	1:48	391	17	20	45		
		Pomona	1:48		8		5		
		Dual Hardjo-bovis -Pomona	1:48		3		20		
(Dorjee <i>et al.</i> 2008)	Sheep-only abattoir in the Manawatu	Sheep	Slaughter lambs <1 year old	Hardjo-bovis	1:48/1:50	2,758	5	95 (lines)	33
				Pomona	1:48/1:50		1		4
				Dual Hardjo-bovis -Pomona	1:48/1:50		0		7
(Ayanegui-Alcerreca <i>et al.</i> 2010)	Farms from North and South Islands and deer abattoir	Deer	Predominantly 9-30 months old	Hardjo-bovis	1:24	2,016	54	111	62
				Pomona	1:48		2		4
				Dual Hardjo-bovis -Pomona	1:48		7		16
				Copenhageni	1:48		1		0
(Dreyfus <i>et al.</i> 2011)	Farms from North and South Islands	Sheep	Mixed age	Hardjo-bovis	1:48	3,361	43	162	91
				Pomona	1:48		14		74
		Beef cattle	Mixed age	Hardjo-bovis	1:48	2,308	50	116	92
				Pomona	1:48		25		72

	Deer	Yearlings (1-2 years old)	Hardjo-bovis Pomona	1:48 1:48	1,992	26 15	99	60 49	
(Fang <i>et al.</i> 2014a)	Abattoir in the Waikato region	Sheep	78% lambs (<1 year old), 12% hoggets (1-2 years old), 11% mixed age	Hardjo-bovis Pomona Dual Hardjo-bovis -Pomona	1:48 1:48 1:48	399	21 20 15	6	NA
		Beef cattle	80 % young stock (\leq 18 months old), 20% mixed age	Hardjo-bovis Pomona Dual Hardjo-bovis -Pomona	1:48 1:48 1:48	146	47 14 11	3	NA

*Definition of farm seropositivity:

- Heuer (2007), Dorjee *et al.* (2008), Dreyfus *et al.* (2011) at least one animal seropositive
- Subharat *et al.* (2007) at least 3/20 animals with MAT titres \geq 48 for cattle and deer, at least 5/60 for sheep
- Ayanegui-Alcerreca *et al.* (2010) at least 3 animals with MAT titres \geq 24 for Hardjo or \geq 48 for Pomona and Copenhageni

1.3.2. Risk factors for seropositivity

The risk factors for seropositivity to *Leptospira* spp. at the animal and farm level vary depending on the considered species, serovar and country. The majority of the published work comes from Latin America and may not be always relevant to the situation in New Zealand. Most of these Latin American studies were conducted in a similar way, looking at farm status and using similar questionnaires, with similar data analysis methods, hence it is difficult to discuss the reasons for observed discrepancy. Risk factors can be classified into three groups: contact with other species, environmental factors and management factors. Most studies at the animal level did not account for clustering of sampled animals within-herd, hence artificially increasing the power of those studies. The risk factors were most often reported for infection by any serovar (seropositivity against *Leptospira* spp.), and a few studies looked at the risk factors for Hardjo infection. Both Hardjo-bovis and Hardjo-prajitno are known to be present in Latin America (Oliveira *et al.* 2010) but the studies do not always specify which one was tested for.

1.3.2.1. Contact with other species

Contact with wildlife, mainly capybaras (Marques *et al.* 2010; Silva *et al.* 2012) or cervids (Oliveira *et al.* 2010), was reported to be a risk factor for seropositivity of cattle herds in Latin America. However, a number of studies looking at contact with wildlife in general did not identify it as a significant risk factor for seropositivity of both herds and animals against *Leptospira* spp. (Carvajal-de la Fuente *et al.* 2012; Hashimoto *et al.* 2012; Salgado *et al.* 2014). The role of contact with wildlife was also unclear for small ruminants in Latin America. Higinio *et al.* (2013) found a significant positive association with seropositivity of dairy goat herds and Salaberry *et al.* (2011) with seropositivity of sheep. However, Topazio *et al.* (2015) and Genovez *et al.* (2011) did not report an association of the presence of wildlife and seropositivity in goats and in sheep flocks, respectively. Mazeri *et al.* (2013) did not find contact with wildlife a risk factor for seropositivity of cattle in Cameroon. Studies reporting significant association and studies reporting no association with the presence of wildlife identified similar serovars on the study farms.

The presence of pigs was not associated with seropositivity in sheep or goats flocks and goats at the individual level in Brazil (Araujo Neto *et al.* 2010; Higinio *et al.* 2013; de Carvalho *et al.* 2014) or cattle herds in Tanzania (Schoonman and Swai 2010). Castro *et al.* (2009) and Oliveira *et al.* (2010) found a significant positive association between the presence of pigs and seropositivity against Hardjo (mainly Hardjo-prajitno) on Brazilian cattle farms. However, no such association was found in other studies on cattle farms in the same area with similar serovar profiles (Figueiredo *et al.* 2009; Marques *et al.* 2010; Hashimoto *et al.* 2012; Silva *et al.* 2012).

Horses have also been identified as a risk factor for seropositivity against Hardjo in South American cattle herds (Hashimoto *et al.* 2012; Silva *et al.* 2012) but other

studies did not identify the presence of horses as a risk factor (Marques *et al.* 2010; Oliveira *et al.* 2010; Oliveira *et al.* 2013; Salgado *et al.* 2014). It was also not a risk factor for goats seropositive to Autumnalis and Whitcombi (Higino *et al.* 2013).

Co-grazing or presence of other ruminant livestock on the farm was commonly reported as a risk factor for Hardjo seropositivity of cattle herds (Castro *et al.* 2009; Marques *et al.* 2010 in Latin America; Hardjo-prajitno in Tanzania, Schoonman and Swai 2010), of deer herds (Hardjo-bovis, Subharat *et al.* 2012b) and in small ruminants flocks (dos Santos *et al.* 2012; Topazio *et al.* 2015). However, this finding was not observed in some cattle herds (Oliveira *et al.* 2010; Hashimoto *et al.* 2012; Silva *et al.* 2012; Pimenta *et al.* 2014) or in goats and goat flocks (Araujo Neto *et al.* 2010; Higino *et al.* 2013).

The presence of cats or dogs, when studied, was always reported as not being a risk factor in the above studies.

1.3.2.2.Environmental factors

The presence of ponds or waterholes was a risk factor for seropositivity of Brazilian sheep and goats and of small ruminants flocks (Cortizo *et al.* 2014; de Carvalho *et al.* 2014), but the source of water was not a significant risk factor for seropositivity of cattle in Tanzania (Schoonman and Swai 2010) or Mexico (Carvajal-de la Fuente *et al.* 2012). The presence of flooded pasture was reported as not being a risk factor for Hardjo (both Hardjo-bovis and Hardjo-prajitno) seropositivity of cattle herds in Ireland (Ryan *et al.* 2012) or for cattle, cattle herds and goats exposed to various serovars in Brazil (Langoni *et al.* 2008; Figueiredo *et al.* 2009; Oliveira *et al.* 2010; Silva *et al.* 2012; Higino *et al.* 2013; Oliveira *et al.* 2013). However, Pimenta *et al.* (2014) reported an increased risk of a Brazilian cattle herd being seropositive for Hardjo, Icterohaemorrhagiae or Australis when the presence of flooded pasture was recorded.

Climate or seasonality were reported as possible risk factors but the conclusions varied according to place and serovars. Guitián *et al.* (2001) reported a higher risk for Grippotyphosa seroconversion in spring than in winter, but not for Bratislava, in Spanish dairy cattle. Lilenbaum *et al.* (2008) reported a higher risk of seropositivity of Brazilian goats in tropical than in temperate regions but Lilenbaum and Souza (2003) did not find any association between location and seropositivity of cattle. Mineiro *et al.* (2007) found a significant correlation between within-herd prevalence in Brazilian dairy cattle and pluviometry but not with temperature.

1.3.2.3.Management factors

Most of the previously cited studies reported herd size as a risk factor for seropositivity, which is unsurprising as studies are usually done at the herd level, and

larger herds may be classified as infected simply because they have a larger population at risk.

Open cattle herds (herds purchasing animals or lending males for breeding) were more at risk of seropositivity than closed herds in several studies (Castro *et al.* 2009; Marques *et al.* 2010; Oliveira *et al.* 2010; Hashimoto *et al.* 2012). On the other hand, other studies showed that closed dairy cattle herds in Brazil (Mineiro *et al.* 2007) or closed deer herds (Subharat *et al.* 2012b) in New Zealand were more at risk of seropositivity, particularly for Hardjo-bovis. Other studies showed no association between open herd and seropositivity of the herd in cattle (Segura-Correa *et al.* 2003; Figueiredo *et al.* 2009; Ryan *et al.* 2012; Silva *et al.* 2012; Mazeri *et al.* 2013; Pimenta *et al.* 2014) or goats (Araujo Neto *et al.* 2010; Higino *et al.* 2013).

Renting or sharing pastures was regularly reported as a risk factor for seropositivity in cattle herds in Latin America (Castro *et al.* 2009; Marques *et al.* 2010; Oliveira *et al.* 2010; Hashimoto *et al.* 2012) and Africa (Mazeri *et al.* 2013). However, this association was not observed in some other studies (Schoonman and Swai 2010; Silva *et al.* 2012; Pimenta *et al.* 2014), and was not observed for goats (Higino *et al.* 2013).

1.3.3. Clinical signs and lesions

Acute clinical manifestations have been reported in livestock after infection with different serovars, usually after infection of incidental hosts. They are usually associated with short-term urinary excretion (Ellis 2015). The clinical signs are very similar across species (Table 1-3).

Table 1-3: Major clinical signs and associated serovar in cattle, sheep and deer

Species	Serogroup	Clinical signs	References
Cattle	Pomona	Anorexia	(Ellis <i>et al.</i> 1985b; Thompson 1986; Ellis 2015)
	Icterohaemorrhagiae	Depression	
	Grippotyphosa	Hemoglobinaemia	
	Hardjo (abortion)	Hemoglobinuria	
		Jaundice	
		Meningitis	
		Abortion	
	Death		
Deer	Pomona	Hematuria	(Ayanegui-Alcerreca <i>et al.</i> 2007; Ellis 2015)
	Icterohaemorrhagiae	Hemoglobinuria	
		Anemia	
		Jaundice	
		Death	
Sheep	Pomona	Hematuria	(Ellis <i>et al.</i> 1983; McKeown and
	Sejroe	Hemoglobinuria	

Icterohaemorrhagiae	Jaundice	Ellis 1986;
Grippotyphosa	Death	Vermunt <i>et al.</i>
	Abortion	1994)

Kidney and renal lesions also frequently occur following infection with *Leptospira* spp. (Thompson 1986): enlarged, red and friable liver, spleen and kidney. White foci in the renal cortex, 1-5mm degenerative and necrotic lesions in the epithelial cells of the tubules and loss of microvilli are also reported. This suggests no evidence of intracellular effect and that the effect of *Leptospira* infection could be toxin mediated (Marshall 1974).

1.3.4. Subclinical effects

Subclinical effects are seen mainly as economic losses in production species. This is developed in chapter 2.

1.3.5. Pathogenesis

Leptospire enter via mucous membranes like the mouth, nose or genital membrane. They can also enter through cut or abraded skin, and vertical transmission can occur. The bacteria circulate in the blood stream and reach target organs like the kidney, where they locate in proximal tubules. In hamsters experimentally infected with *L. kirshneri*, leptospire reached renal tubules in 10 days (Barnett *et al.* 1999). During natural exposure, circulating agglutinating antibodies, detectable by the MAT, are detectable after 10-14, which marks the end of the leptospiremic phase (Ellis 2015).

Pathogenic leptospire are also able to enter the cytoplasm of cells, cross cell layers through intercellular junction and adhere to endothelial cells thanks to some outer membrane proteins (Thomas and Higbie 1990).

The highly conserved LipL32, which is absent from saprophytic leptospire and accounts for 75% of the outer membrane proteins, was first assumed to have an important role in the pathogenesis of leptospirosis. However, recent findings indicate that this protein is not necessary for infection and acute or chronic disease with renal colonization (Murray *et al.* 2009), although it is highly expressed during infection and renal colonization (Haake *et al.* 2000). Its precise role needs to be elucidated. Other outer membrane proteins such as the porine OmpL1 and LipL41 are implicated in renal tubule colonization (Barnett *et al.* 1999).

In chronic carriers, leptospire usually localize in the proximal renal tubules which allows urinary excretion (Hathaway 1981) or in the genital tract (Ellis 1987; Arent *et al.* 2013).

1.3.6. Immunity

Humoral immunity is based on the production of antibodies directed against protein antigens found on the outer membrane of leptospire (Nunes-Edwards *et al.* 1985). Agglutinating antibodies directed against these antigens are protective but serovar-specific, preventing cross-protection between most serovars. The antibodies act by agglutinating leptospire, reducing their adherence potential or by opsonizing leptospire and enhancing phagocytosis (Wang *et al.* 1984; Thomas and Higbie 1990).

In calves experimentally infected with serovar Pomona, antibodies identified by microscopic agglutination test appeared after 9-11 days (Thompson 1986). However, in cattle, the role of humoral immunity against *L. interrogans* Hardjo appears limited, since some vaccines which induce a small and temporary rise in agglutinating antibodies can protect animals against challenge even after the antibodies are not detected any more (Marshall *et al.* 1979). The presence of agglutinating antibodies indicates past exposure but does not always indicate protection (Bolin *et al.* 1989b).

During successful cattle *L. borgpetersenii* Hardjo vaccine experiments, the Th1 path was stimulated, with production of interferon γ by CD4+ and CD8+ lymphocytes and proliferation of $\gamma\delta$ T cells (Naiman *et al.* 2001; Brown *et al.* 2003; Moffat 2007; Zuerner *et al.* 2011), which suggests that the cellular immunity is more important in cattle, as opposed to humans where humoral immunity plays a major role (Levett 2001).

Passively transferred maternal immunity disappears at around three months in calves (Palit *et al.* 1991) and one month in lambs (Andreani *et al.* 1983). However the presence of antibodies does not appear to be incompatible with efficient vaccination, with vaccine trials reported successful in calves as young as 4 to 6 weeks (Palit *et al.* 1991).

1.3.7. Diagnosis

1.3.7.1. Detection of Leptospira

Leptospire can be directly observed by dark-field microscopy in urine or blood of infected animals. However this method is has a low specificity and can detect leptospirosis only when the concentration is at least 10⁴ organisms/mL of blood (Picardeau 2013).

The bacteria can also be isolated (Faine *et al.* 1999) from several organs or fluids like kidneys, blood, urine, aqueous or fetal tissues. Specific culture media were formulated, with the most common, the Ellinghausen, McCullough, Johnson and Harris medium (EMJH), being a semi-solid medium suitable for *Leptospira* which are strictly aerobic. Antibiotics to which *Leptospira* are resistant, such as 5-fluorouracil, are often used to avoid growth of unwanted contaminant organisms in culture (Levett 2001). Isolation from tissues requires incubation at 30°C. Cultures should be checked weekly for three months before declaring a sample culture-negative (Adler and de la Pena Moctezuma 2010), which limits the use of culture as a routine diagnostic test. However, isolating the organism was necessary for the cross-agglutination-absorption test which

was required for identification up to the serovar level (Faine *et al.* 1999) but this method has now largely been superseded by molecular techniques (Adler and de la Pena Moctezuma 2010).

1.3.7.2. Serological diagnosis

The microscopic agglutination test (MAT) is the standard serological test for the diagnostics of leptospirosis, for the diagnosis of individual animal disease or herd exposure status (OIE 2014). It is based on the detection of agglutinating IgG and IgM (Morris and Hussaini 1974). Agglutinating antibodies were detectable within one week after experimental infection of sheep with Hardjo-bovis (Hathaway and Marshall 1979) or after 9-11 days after experimental Pomona infection in calves (Thompson 1986). Ellis (2015) reported that agglutinating antibodies could be detected 10-14 days after natural exposure. MAT was shown to be positive (titre ≥ 10 , ≥ 100 and ≥ 25 respectively) for at least four months in sheep after infection with Hardjo or Pomona (Hodges and Ris 1974; Andreani *et al.* 1983; Gerritsen *et al.* 1994b) while in cattle Hardjo-bovis MAT titres can be positive (≥ 12) for two years after infection (Hellstrom 1978). Hence, the MAT allows for detection of past or current exposure. Using the MAT on paired samples is often recommended for diagnosis of current infection, as opposed to interpreting a single sample with a given cut-off (Musso and La Scola 2013).

Although it is logistically demanding and requires the maintenance of live cultures of all the tested serovars, ideally those present in the area where the serum comes from (OIE 2014), the MAT allows for the identification of the infecting serogroup, which is necessary in veterinary medicine to provide flock- or herd-level control measures such as vaccination which is serovar specific (Picardeau 2013).

The MAT titre was also shown to be associated with renal carriage of leptospire. In a cross-sectional abattoir study on slaughter lambs (Dorjee *et al.* 2008), animals with a MAT titre of 1:50 or above were 21.7 times more likely to have culture-positive kidneys than seronegative sheep. In another abattoir study (Fang *et al.* 2014a) Hardjo-bovis seropositive sheep were 12.5 times more likely to shed leptospire in urine than seronegative animals. Fang *et al.* (2014b) when looking at paired samples from sheep and cattle showed a fair agreement ($\kappa = 0.33$ and $\kappa = 0.32$) between MAT and PCR on kidneys and urine, respectively, and calculated that animals with titres for Hardjo-bovis and/or Pomona were 9.1 times (95% CI = 3.7-22.9) more likely to be urine PCR positive than seronegative animals. However, the isolation of Hardjo-bovis from the kidneys of seronegative animals is also possible (Blackmore *et al.* 1982; Dorjee *et al.* 2008).

Hence, the use of a cut-off to define seropositivity and infer infection from the MAT at an individual level is challenging. Blackmore *et al.* (1982) suggested a cut-off of 1:48 and considered reactions at 1:24 to be non-specific detecting previous exposure. The geometric mean titre (GMT) of flocks was also used and can bring meaningful information about the flock leptospirosis status in association with seroprevalence. The

World Animal Health Organization (OIE 2014) recommends the use of paired serum samples, taken in the acute and convalescent phases, to diagnose the disease. The observation of a four-fold rise in titre is considered the threshold for diagnosis.

The MAT is also subject to intra- and inter-laboratory variability, partly due to the subjectivity in reading the test (Picardeau 2013), at least for some serovars. Fang *et al.* (2014b) found an almost perfect agreement for Hardjo-bovis ($\kappa=0.93$) between two laboratories in the same town in New Zealand, but a moderate agreement only (0.53) for Pomona.

Other serological tests like complement fixation and ELISA are available and can be specific for a serogroup. Serological tests targeting IgM such as ELISA allow early detection, including during the first week after the symptoms appeared, when MAT titres are not always present (Levett 2001). IgG antibodies have been detected for 11 months after *L. interrogans* Hardjo infection in sheep using ELISA (Cousins *et al.* 1989). Adler *et al.*'s (1981) ELISA, able to detect IgG and IgM, used on sheep experimentally infected with *L. interrogans* Hardjo showed a good agreement with the MAT in detection of the presence of antibodies. However, that the peak titre for ELISA occurred at a different time than the MAT peak titre and the poor correlation between titres showed that the two tests measured different types of antibody.

1.3.7.3. Molecular diagnosis

1.3.7.3.1. PCR

The PCR is based on the detection of leptospiral DNA in the animal's tissues, including blood and urine (Bomfim *et al.* 2008). Urine and kidneys from the same sheep tested by qPCR gave an almost perfect agreement (Fang *et al.* 2014b), suggesting that these two samples can be used interchangeably.

Different protocols have been published, using different sequences of primers and targeting different parts of the leptospiral genome. For example, Bezerra Da Silva *et al.* (2011) published a method that targets the *rfb* operon coding for the LPS biosynthesis while Levett *et al.* (2005) targeted part of the gene coding for the LipL32 to identify pathogenic leptospires. Smythe *et al.* (2002) used primers directed at a sequence of ribosomal DNA to detect pathogenic leptospires and this method was shown to have almost perfect agreement with another protocol targeting the *gyrB* gene (Fang *et al.* 2014b), also aiming at detecting pathogenic leptospires (Slack *et al.* 2006).

However, currently available PCR protocols are not able to distinguish between different serovars (Bezerra Da Silva *et al.* 2011; Picardeau 2013) without further sequencing (Perez and Goarant 2010). The PCR is a useful tool to complement the MAT to identify currently infected animals that are carriers and/or shedders (Lilenbaum *et al.* 2009; Otaka *et al.* 2012; Hamond *et al.* 2014).

1.3.7.3.2. Other molecular techniques

Other molecular techniques such as pulse-field gel electrophoresis (PFGE) restriction patterns allow for identification of the genomospecies. The building of physical maps, which use the variability in the genome of *Leptospira*, allows the distinction between some serovars like *L. interrogans* serovars Icterohaemorrhagiae and Pomona (Saint Girons *et al.* 1992). Genetic variability also exists between strains from different geographic origin, which can be evidenced by molecular methods such as restriction fragment length polymorphism (RFLP), for example, within *L. borgpetersenii* serovar Hardjo type hardjo-bovis (Zuerner *et al.* 1993). It is unclear if this variability leads to a difference in pathogenicity between strains or clones but this should be kept in mind when extrapolating results from one part of the world to another.

Interspecies transmission can also be explored using molecular techniques. The multilocus sequence typing method (MLST) is based on the different profiles or combination of alleles of several housekeeping genes. However, this method requires isolation of the organism from the host. This method used in a preliminary study revealed that one strain of Pomona present in New Zealand is closely related to a strain of serovar Kennewicki from USA (Fang 2014).

1.3.8. Treatment

In the face of an outbreak of leptospirosis in livestock, the usual approach involves the use of antibiotics. Gerritsen *et al.* (1994a) observed the end of shedding of Hardjo type Hardjo-bovis in PCR positive dairy cows within one week after treatment with dihydrostreptomycin at 25 mg/kg. A single dose given to every animal in the herd was sufficient to stop the spread of infection, as supported by a previous controlled trial on cows experimentally infected with *L. borgpetersenii* serovar Hardjo (Gerritsen *et al.* 1993) but in contradiction with another uncontrolled trial (Ellis *et al.* 1985a) where the same treatment did not clear renal or genital carriage in seven of ten heifers treated after *L. interrogans* serovar Hardjo challenge. Alt *et al.* (2001), also using a randomized controlled trial on cattle artificially infected with *L. borgpetersenii* serovar Hardjo, demonstrated the efficacy of oxytetracycline 20 mg/kg, tilmicosin 10 mg/kg and a combination dihydrostreptomycin-penicillin G 25 mg/kg using a single injection, but had to use longer treatments with ceftiofur 2.2 or 5 mg/kg (daily for five days) to eliminate shedding in urine. Tylosin at 18 mg/kg for five days, oxytetracycline at lower dose and ceftiofur during a shorter period were not efficient.

During an outbreak in goats seropositive for Icterohaemorrhagiae, Hardjo and Bratislava (Martins *et al.* 2012), dihydrostreptomycin treatment at the same posology decreased seropositivity by 91% and positivity by PCR in urine by 90% one year after treatment of the whole herd followed by vaccination. No information on correspondence between serology and PCR results was given. This suggests that the animals that were positive a year later were probably carriers at the time of vaccination. The reproductive performance of the herd also substantially improved, although the effect of treatment and vaccination could not be differentiated from possible natural clearance.

1.3.9. Prevention and vaccination

In addition to common biosecurity measures such as testing and/or treating purchased animals or protection from contact with wildlife, inactivated vaccines against *Leptospira* infection have been developed and are commercially available. Since generally little or no cross-protection exists, vaccines should include all the serovars present in the area (Adler and de la Pena Moctezuma 2010).

Vaccines that can protect cattle against *Leptospira* infection should produce a type 1 immune response in the host, and this may not be induced by multivalent vaccines (Brown *et al.* 2003). This would explain earlier finding where multivalent vaccines could not protect against Hardjo shedding (Bolin *et al.* 1989a). However, a recent trial (Rinehart *et al.* 2012) showed 100% efficacy against urinary shedding of a pentavalent vaccine containing *L. interrogans* serovar Hardjo, including cross-protection against *L. borgpetersenii* serovar Hardjo, suggesting that current multivalent vaccines do confer protection.

Vaccines also have a long term efficacy, either in face of natural exposure or experimental challenge. Hancock *et al.* (1984) showed an efficacy of a commercial bivalent *L. interrogans* serovars Hardjo and Pomona vaccine in reduction of shedding in urine, measured by dark field microscopy and culture, of 95%, when vaccinated by 9-10 months of age and then naturally exposed to *L. interrogans* serovar Hardjo for up to 55 weeks. Vaccination of adults already exposed was not efficacious. On the other hand, Zimmerman *et al.* (2013) studying a multivalent vaccine including valence for *L. borgpetersenii* serovar Hardjo given to 4-week-old heifers with a booster four weeks later and subsequently experimentally infected as heifers 12 months later found an efficacy of 78% in reducing urinary shedding measured by urine culture during the following eight weeks. However, the vaccinated animals appeared to be shedding a lower quantity and/or less often, and the efficacy measured on kidney and genital tract culture eight weeks after challenge was 100%, showing that vaccination prevented renal colonization and chronic carriage but transient shedding may occur in vaccinated animals. The study animals had been fed colostrum from vaccinated dams as calves but had MAT titres <1:100, showing that calves in herds vaccinating against leptospirosis can be vaccinated at an early age if they are seronegative at this cut-off without reduction in efficacy.

In New Zealand farmed deer naturally exposed to Hardjo-bovis (Subharat *et al.* 2012a), a vaccine efficacy of 100% on shedding in urine, measured by qPCR, was observed eight months after vaccination. In another on-farm vaccination trial of deer (Ayanegui-Alcerreca 2006), vaccine efficacy measured by kidney culture five to 12 months after initial vaccination was 55%, with only Hardjo-bovis and/or Pomona isolated from the study farms. The trial was conducted on farms already exposed to Hardjo-bovis and/or Pomona with seroprevalence for Hardjo-bovis ranging 10 to 58% and for Pomona 0 to 95% at the beginning of the study. Only seronegative animals or animals with titres attributable to maternal antibodies were included in the vaccinated

group. The estimated duration of shedding, measured by a combination of dark field microscopy, urine and kidney culture was significantly ($p < 0.001$) reduced from an average of 47 days (median 7 days) in the control group to an average of 19 days (median 0 days) in the vaccinated group.

Fewer data are available on vaccination of sheep. Chapman and Clough (2014) showed an efficacy, measured by urine and kidney culture, of 100% in sheep vaccinated with a commercial vaccine combining clostridial, *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona valence, and experimentally challenged with *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona.

No information on the effectiveness or field efficacy of vaccination in commercial sheep farming conditions in New Zealand is available so far. A preliminary study conducted in New Zealand dairy cattle (Parramore *et al.* 2011) and recent cases reported in dairy workers (McLean *et al.* 2014) showed that timing of vaccination in particular seems to be crucial in insuring a good effectiveness of the vaccine. In the dairy cattle study (Parramore *et al.* 2011), 30% of herds that had been vaccinating had animals that were positive by PCR and/or dark field microscopy, and the proportion of positive animals in positive herds was 13%. One common factor between positive herds was that vaccination of calves was not completed before 6 months of age.

In New Zealand vaccination against Hardjo and Pomona is wide spread in dairy cattle (Marshall 1987) but is still rarely implemented by deer, sheep and beef farmers (Wilson *et al.* 2008; Dreyfus *et al.* 2011).

1.4. **Leptospirosis as a zoonosis**

Humans are incidental hosts for *Leptospira* and become infected by contact with urine of infected animals, either directly or via the environment. The disease can present in a variety of ways, from an asymptomatic infection to multiple organ failure. In 80-90% of cases, the disease presents as asymptomatic to flu-like symptoms, with fever being a common feature (Fraga *et al.* 2011). The most severe presentation, known as Weil's disease, combines icterus with pulmonary haemorrhage, renal, hepatic and vascular failure (Bharti *et al.* 2003) and occurs in 10-15% of cases. Complications such as uveitis can also happen. The mildness of most of the cases, along with issues with diagnostic testing, healthcare seeking behaviour and awareness among clinicians leads to under-reporting of the disease (Thornley *et al.* 2002), and the actual burden of leptospirosis is often underestimated.

Infection can follow three possible epidemiological patterns: climate associated in tropical regions; recreational; or occupational (Levett 2001). In New Zealand the majority of the cases are occupation-related, and leptospirosis tops the list of occupationally-acquired diseases in this country (Thornley *et al.* 2002). Workers in abattoirs, on farm or in the forestry industry are the most at risk, and while the incidence

of cases due to Hardjo-bovis and Pomona have been decreasing during the end of last century, serovar Ballum has been emerging as a new risk (Thornley *et al.* 2002; The Institute of Environmental Science and Research Ltd. 2015).

Sheep present a risk for abattoir workers, with an incidence of Hardjo-bovis and/or Pomona in sheep abattoirs of 11.9% with an initial seroprevalence of 14%, an incidence of 0% in deer abattoirs with an initial seroprevalence of 22%, and an incidence of 1.2% in cattle abattoirs with an initial seroprevalence of 7.6%, despite the use of protective personal equipment (Dreyfus *et al.* 2015). Dorjee *et al.* (2011) estimated that workers in one North Island sheep abattoir workers handled between three and 18 infected carcasses a day, and up to 54 a day in high risk periods such as after floods.

Cases related to dairy farming are still regularly reported (Thornley *et al.* 2002; McLean *et al.* 2014) in spite of a large uptake of vaccination of dairy cattle along with a country-wide awareness campaign that reduced the incidence of Hardjo-bovis and Pomona infection by half between 1981 and 1987 (Marshall 1987). On the other hand, veterinarians had a prevalence of 5.1% for any of the serovars known to be present in New Zealand recorded during a 2012 cross-sectional study (Sanhueza *et al.* 2015). No livestock species could be identified as being a risk for infection of veterinarians, and veterinarians working at 50-75% with dogs and cats were more at risk of being seropositive (OR=6.3 , 95% CI 1.0-38.7) .

A meta-analysis conducted by Charan *et al.* (2013) concluded that there is still not enough evidence to recommend antibiotics as being efficient in routine treatment of human leptospirosis. Furthermore, human vaccination (Haake and Levett 2015) is usually restricted to populations at risk, has sometimes been associated with side effects and is not available in New Zealand. Like animal vaccination it lacks cross-protection between serovars (Adler and de la Pena Moctezuma 2010). The current vaccines also present some side-effect risks and a short term protection (Bharti *et al.* 2003). Hence, in New Zealand, an appropriate strategy should reduce contact with infected animals and environment, and when possible, vaccination of the reservoir species with which contact is frequent.

1.5. Aims of this thesis

The general goal of this thesis was to elucidate the epidemiology of Hardjo-bovis and Pomona infection in New Zealand sheep, to identify possible associated economic losses, quantify them if they exist, and assess the cost-efficiency of vaccination related to the production losses. The study allowed a description of infection patterns in eight commercial farms. It also provided the opportunity to evaluate the efficacy of a commercial bivalent vaccine in reducing urine shedding.

The data used in the thesis come from six commercial sheep and beef farms in the North Island and two sheep, beef and deer farms in the South Island and were collected between September 2011 and January 2014. On each farm, ewe lambs were recruited and approximately one third of them were vaccinated. Unlike the animals used for a PhD study with similar aims in farmed deer (Subharat 2010), the lambs were not treated with antibiotics at the beginning of the study and the researchers had no control on whether or when the trial animals were mixed with other animals on the farm.

Chapter 2 is a review of the published literature on the effect of leptospirosis on production of pastoral livestock in the world, identifying knowledge gaps, especially in sheep, and is intended for publication in a refereed journal.

To estimate the production effect on farms it was necessary to understand the dynamic of infection. Serological data for Hardjo and Pomona from the control sheep were used in chapter 3 to describe the seroconversion pattern and estimate agglutinating antibodies half-life. Shedding data in the control group was also described in this chapter. This chapter has been published in the New Zealand Veterinary Journal.

Investigating if the vaccine did protect the animals against infection was also required. The results of the vaccine effectiveness study, with shedding in urine as the outcome, are presented in chapter 4. This chapter has been submitted to the journal “Vaccine”.

Growth and reproduction effects are presented in chapters 5 and 6, respectively. The production effects were estimated in two ways: by comparing performance of vaccinated sheep with control sheep; and by comparing seropositive and seronegative sheep within the control group. These chapters are intended for publication in a refereed journal.

In chapter 7 the implications of the results are discussed and put in perspective with current knowledge, and some suggestions for future studies are presented.

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Chapter 2. A review of the effects of *Leptospira* spp. infection on farmed ruminants production

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2.1. Introduction

Leptospirosis is a zoonotic, ubiquitous disease. Although *Leptospira* have long been recognised as a cause of clinical disease and abortion in animals, its complex epidemiology, involving maintenance hosts and accidental hosts (Hathaway 1981), implies that subclinical and production effects can occur..

This chapter reviews the effects of *Leptospira* on animal production, namely those that can have adverse economic impact at farm and industry levels. These include reproductive inefficiency from breeding to weaning, mortality, growth retardation and decrease in milk production. This review is limited to farmed ruminants, with a focus on extensive and pasture-based systems including dairy and beef cattle, sheep, goats and farmed deer.

In this review, importance is given to the design of studies cited and the robustness of evidence provided to assess causative inferences as accurately as possible. Comparative studies (cohort, case-control, controlled challenge studies) give more robust evidence than case description and uncontrolled challenge studies. Controlled vaccination studies were reviewed to evaluate the efficacy and effectiveness of vaccination in reducing production-limiting effects, but more importance was given to studies looking at the effect of infection itself.

For this review, the search terms (leptospir*) AND (production OR growth OR reproduction OR weigh* OR fertility OR abortion) AND (cattle OR cow* OR beef OR sheep OR heifer* OR calf OR calves OR deer OR camel* OR goat OR lamb*) were used on Scopus, Web of Science and SciQuest search engines. The titles of all results until 2015 included were browsed to eliminate articles not fitting with the scope of the review. Articles not accessible from Massey University or not freely accessible as well as those in another language than English, French, Spanish or Portuguese were also eliminated. Then abstracts were reviewed and only comparative studies were retained. Exceptionally, detailed case studies and outbreak reports were selected if they provided enough details not available otherwise.

According to conventional classification of host types based on the epidemiology and ecology of *Leptospira* and leptospirosis, more severe effects should be expected in “spillover” hosts than in a *Leptospira*-adapted “reservoir” hosts (Hathaway 1981). The effects are reviewed by animal species and by serovar, with a focus on Hardjo, for which cattle, sheep and likely deer are maintenance hosts. The identification of serogroup, serovar, type and genomospecies was interpreted in the context of the sometimes limited information available in some of the cited articles. Further, some of the classifications given in older articles can vary from current convention (Levett 2001), as they were published before the introduction of molecular methods which allowed for distinction between different genomospecies and subtypes, for example the

distinction of Hardjo-bovis and Hardjo-prajitno within serovar Hardjo. The classification and terminology in those articles were retained for this review.

In comparative studies cited where no measure of statistical significance for the difference between proportions was given, a p-value was calculated using Pearson's chi-square when possible, when the study was done on one herd or flock, but not when the study was done across farms as it was impossible to account for within-farm clustering.

2.2. **Reproduction**

A summary of on-farm cattle studies of the effects of exposure to *Leptospira* spp. on production with a comparative, prospective, observational design is presented in Table 2-1. No similar study in small ruminants or deer was found.

2.2.1. **Conception and establishment of pregnancy**

Failure to conceive is difficult to distinguish from early fetal loss in extensive pastoral systems, since, if performed, pregnancy diagnosis, usually early in gestation, is the first recorded reproductive outcome. Most data on the effect of *Leptospira* on the ability of reproductive females to get pregnant arises from dairy cattle for which close monitoring and frequent use of artificial insemination is conducted, and in which pregnancy is used as a proxy for conception.

2.2.1.1. Dairy cattle

Work conducted in the 1990's showed that *L. interrogans* serovar Hardjo decreased or delayed the chances of a cow being pregnant at pregnancy diagnosis. In 207 artificially inseminated dairy cows in USA with synchronised oestrus and manually checked for pregnancy 150 days later, those with a Hardjo MAT titre ≥ 100 were twice less likely (95% CI 1.01-4.24) to be diagnosed pregnant, irrespective of the number of inseminations used (Guitian *et al.* 1999). They also required on average one more breeding than seronegative cows to conceive. Similar effects were reported in UK by Dhaliwal *et al.* (1996a) in 673 non-seasonal calving dairy cattle from four farms using artificial insemination and one farm using natural mating followed for three consecutive years. Those authors looked at "conception rate" defined as "the percentage of inseminations resulting in a calving or abortion", considering the cows that had signs of recent abortion at pregnancy diagnosis as having conceived. The method for pregnancy diagnosis was not reported and the pregnancy diagnosis was done between three months and six months of pregnancy, hence it is questionable how accurate the method was to detect animals that conceived but subsequently aborted. This may have led to misclassification bias. The conception rate of Hardjo seropositive cows was lower by 8.2 to 16 percentage points ($p < 0.01$) at MAT titres ≥ 10 and ≥ 100 , respectively, measured on the day of pregnancy diagnosis. The proportion of cows that required only one insemination to get pregnant was lower in cows with MAT titre ≥ 100 than in seronegative cows by 14.5 percentage points ($p < 0.05$).

Since the distinction between *L. interrogans* Hardjo and *L. borgpetersenii* Hardjo is recent (Marshall *et al.* 1985, Ramadass and Marshall 1990), little can be inferred about any difference of effects between the Hardjo serovar of these two serogroups. All studies cited above refer to *L. interrogans*, but it is unclear whether the genomospecies was confirmed by a molecular method or not. Little is known of the effect of *L. borgpetersenii* Hardjo strain Hardjo-bovis on conception rate but a recent vaccination study in USA in which pregnancy rates measured by manual or ultrasonic pregnancy diagnosis 32-40 days after artificial insemination were compared between 986 cows vaccinated at the end of lactation and 908 control cows in a herd with a prevalence of Hardjo MAT titres ≥ 100 of 13-15% during the study period suggests no effect (Plunkett *et al.* 2013).

The effects of Hardjo on the time from calving to return to oestrus or oestrus manifestation in dairy cattle are less clear than the effects on pregnancy rates and on number of service to conception. Guitian *et al.* (1999) found no association between *L. interrogans* serovar Hardjo MAT titre ≥ 100 and calving to first insemination interval (CFII). However, Muñoz-Zanzi *et al.* (2004) also in USA found a CFII six days longer in heifers from two dairy herds with Hardjo titre ≥ 400 than seronegative heifers. In that study, 5% of the abnormally long time interval to first insemination was attributable to Hardjo, while 13% was attributable to infection by the Bovine Viral Diarrhoea (BVD) virus.

Other serovars have also been incriminated in delaying oestrus of dairy cows. Seropositivity to *L. interrogans* serovar Pomona (MAT titre ≥ 400) was associated with a seven day delay to first insemination in 182 nulliparous heifers in Muñoz-Zanzi *et al.* (2004). In a Spanish study (Gutián *et al.* 2001), a farm-level prevalence of MAT titres ≥ 30 for Bratislava, Grippotyphosa and *L. interrogans* Hardjo of 8.6%, 6.3 % and 0.5%, respectively, was found in herds with “abnormally” (no definition given) long calving to conception and inter-calving intervals, suggesting that Bratislava and Grippotyphosa could also be associated with subfertility or reduced ability to maintain early pregnancy in cattle.

2.2.1.2. Beef cattle

The effects of *Leptospira* on beef cattle conception are less clear than for dairy. There was no difference in the proportion of 712 Brazilian animals scanned pregnant 60 days after the end of the natural breeding period (Fava *et al.* 2004) or of 358 cattle at an unreported scan time (Genovez *et al.* 2001) between cows seropositive (MAT titre ≥ 100 and ≥ 50 , respectively) or negative for *Leptospira* spp. at the beginning of the breeding period. Heuer (2007) using data from 656 New Zealand farms found no difference in pregnancy rates adjusted for the length of the breeding period, measured by scanning, between cows seropositive and seronegative for Hardjo-bovis or Pomona (MAT cut-off not given). However, in a USA study, herds where Hardjo-bovis was shown to be

present by MAT (titre ≥ 50) and immunofluorescence on urine had lower pregnancy rates 70 days after artificial insemination as measured by manual pregnancy checking than negative herds (Kasimanickam *et al.* 2007). However, these data on both dairy and beef cattle describe only association, hence caution must be used when making inference.

2.2.1.3. Small ruminants and deer

Little data are available about the effect of *Leptospira* on conception and early pregnancy in small ruminants. To our knowledge, no study has been conducted to evaluate the association between *Leptospira* and the establishment of pregnancy and pregnancy rates in sheep.

A case of reproductive problems in goats in Brazil, manifested by embryonic resorption detected by ultrasound, was attributed to serovar Icterohaemorrhagiae (Martins *et al.* 2012). It was responsive to the combination of dihydrostreptomycin treatment and vaccination but since no animals were kept as unvaccinated and/or untreated controls, it is impossible to assess whether the discontinuation of embryonic resorption was due to vaccination, to the normal course of the outbreak, or due to something other than *Leptospira*. Also in Brazil, Lilenbaum *et al.* 2008 identified *Leptospira* (MAT titres ≥ 400 for any of Hardjo, Shermani, Grippytyphosa, Icterohaemorrhagiae, Autumnalis, Castellonis and Bratislava and PCR positivity of vaginal and semen samples) from 13 goat herds and seven sheep flocks with a history of return to estrus and low conception rates, though no details were provided on how the reproduction outcomes were measured. That study also lacked a control group, hence inference about the cause of the reproductive problems is difficult.

No effect of *Leptospira* on early gestation pregnancy rate was observed in New Zealand red deer by Subharat *et al.* (2011), who compared ultrasound scan pregnancy rates 28-60 days after breeding between 125 vaccinated and 127 unvaccinated deer on four farms exposed to *L. borgpetersenii* serovar Hardjo tpe Hardjo-bovis from the beginning of the study.

2.2.1.4. Pathogenesis of early reproductive effects

The pathogenesis of failure to conceive and/or keep a conceptus until pregnancy diagnosis due to leptospirosis is still not well understood. Endometritis as a result of retention of fetal membranes is a common feature of leptospirosis abortion outbreaks and may be a cause of subsequent lowered conception rate (Diesch *et al.* 1967; Hanson *et al.* 1972; Ellis *et al.* 1985a; Kingscote and Wilson 1986). However, in an experimental *L. interrogans* serovar Hardjo challenge on 22 pregnant heifers where a wide range of reproductive symptoms including abortion and still birth were observed, fetal membrane retention was not detected (Ellis *et al.* 1986a).

L. interrogans serovar Hardjo can be found in the uterus of apparently healthy pregnant or non-pregnant cows (Ellis *et al.* 1982; Ellis *et al.* 1986b). Genital location of

L. borgpetersenii serovar Hardjo in cattle has also been reproduced experimentally but with less success than renal location (Zimmerman *et al.* 2013).

Genital localization of *Leptospira* also occurs in sheep and goats (Lilenbaum *et al.* 2008; Arent *et al.* 2013). Histological changes in the genital tract of ewes experimentally infected with Pomona were reported by Dozsa and Sahu (1970). They observed that the superficial layers of the endometrium presented temporary vacuolisation and disorganisation before returning to normal. However, the alignment of uterine epithelium is important at the time of migration and implantation of the embryo in the uterus which requires a modification of the lining of the uterus (Gaviria and Hernandez 1994). Hence modifications due to leptospiral infection could interfere with normal implantation, resulting in apparent infertility.

Uterine infection was not detected in 93 non-pregnant and 27 pregnant red hinds tested by culture and PCR in New Zealand, which suggests that the reproductive effects of *Leptospira* would be different in deer than in cattle and sheep, or that the strains present on these farms do not localize in the genital tract. However, 1/27 fetuses collected approximately at 3 months of pregnancy, was PCR positive, showing that *Leptospira* still have the potential for some reproductive effects early in pregnancy (Subharat *et al.* 2010).

2.2.2. Fetal leptospirosis and its effects: abortion and stillbirth

Abortion can be defined as “the expulsion before full term of a conceptus incapable of independent life” and stillbirth as “the expulsion of a full-term dead fetus” (Mickelsen and Evermann 1994). They are more noticeable to the farmer than failure to conceive, failure to implant or early embryonic or fetal resorption and are usually classified as clinical signs.

2.2.2.1. Fetal leptospirosis in cattle

2.2.2.1.1. *On-farm animal level studies*

Detailed on-farm case studies exist in large numbers in the published literature, but the level of evidence or inference about the causation of abortion varies greatly from the simple observation of high MAT titres to the combined use of paired serology, fetal bacteriology and exclusion of other common infectious causes. Only publications providing sufficient evidence for *Leptospira* as a causal agent of abortion are used in this review.

In New Zealand dairy cows (Carter *et al.* 1982) showed that Pomona seroprevalence (MAT titre ≥ 200) in aborted cows was higher than in non-aborting animals from the same herds (85% vs. 37%, respectively, with no measure of statistical significance given), but the seroprevalence of *L. borgpetersenii* serovar Hardjo was similar (32% vs. 25%). The annual incidence of abortion ranged 1-14%. In a prospective USA study, Guitian *et al.* (1999) found no association between

seropositivity (MAT titre ≥ 100) to *L. interrogans* serovar Hardjo within 40 days of calving and abortion in the following pregnancy.

An English study of 2994 cows of unknown production type based on serum samples collected for the brucellosis eradication scheme (Little *et al.* 1980) reported that the prevalence of Hardjo (Hebdomadis) titres ≥ 100 was higher in aborted than in non-aborting cows (28% vs. 19%, respectively). However no statistical significance measurement was provided and it was unclear whether the non-aborting cows came from the same herds as the aborting cows. The association between seropositivity and abortion did not vary with the age of cow, but a non-significant trend suggesting that abortions were more frequent in winter and spring months was observed. In a Mexican study, (Cordova-Izquierdo *et al.* 2008), a higher seroprevalence of Tarassovi, Bratislava, Canicola, Hardjo, Wolfii, Pomona, Grippotyphosa, with MAT titres ranging 100-400, was observed in aborting cows than in control cows from the same dairy herd (42% vs. 0%, $p=0.003$). However, since no dominant serovar could be identified, the actual etiology of abortion remains in question. In a Spanish report, antibody against serovar Bratislava was found significantly more frequently in dairy cows that had aborted than in control cows (9.7% vs. 3.4%, respectively, MAT titre ≥ 300), with an attributable fraction of 69%, suggesting that this serovar also can be a cause of abortion (Atxaerandio *et al.* 2005).

Elder *et al.* (1985) investigated serum samples from 1371 aborted and 7254 non-aborted cows from different herds in Queensland, Australia. They found that beef cattle with a Pomona titre of at least 3000 had a 26% (95% CI 19-34) probability of having aborted, and those with *L.interrogans* serovar Hardjo at the same cut-off had a 15% (95% CI 10-22) probability of having aborted. In dairy cattle, the probabilities were 58% (95% CI 44-71) and 46% (95% CI 32-60) for Pomona and Hardjo, respectively. This was later supported by New Zealand data (Sanhueza *et al.* 2013) which showed that beef cows seropositive for Hardjo-bovis or Pomona were more likely to have aborted than seronegative animals from the same farm (OR=1.84, 95% CI 1.01-3.33, MAT titre ≥ 384 and 14.91, 95% CI 1.73-128.84, MAT titre ≥ 768 , respectively, for each serovar).

The different observations on the association between *L.interrogans* serovar Hardjo seropositivity and abortion between studies could be partially explained by the timing of blood sampling: Guitian *et al.* (1999) used a prospective design observing the effect of being seropositive before or at the beginning of pregnancy while the Little *et al.* (1980) and Sanhueza *et al.* (2013) studies were case-control studies and blood was collected soon after abortion.

These studies support that both Hardjo and Pomona are associated with abortion in dairy and beef cattle, but that exposure during pregnancy is likely required for abortion to occur, since infection before pregnancy appears not to result in abortion.

2.2.2.1.2. *Herd level studies*

To our knowledge, few case-control studies using the herd as the study unit have been conducted although this is not the optimal design for inferences of causation due to the high risk of confounding. Furthermore, herd level studies give variable results depending on the serovar studied. In Colombian and Scottish studies, farms with a high occurrence of stillbirths and abortions (incidence of 10% or more and 1-14%, respectively) were more likely to be seropositive for *L. interrogans* serovar Hardjo or for the Hebdomadis serogroup (Ellis and Michna 1976b; Otte *et al.* 1995). A USA study showed no difference in within-herd Pomona seroprevalence (MAT titre \geq 100) between herds with and without reproductive deficiency measured as an index taking into account the proportion of cows that had 9 months of pregnancy during a year, hence looking at both failure or delay to get pregnant and abortion (Lingard and Hanson 1961). However, these studies did not allow determination of whether infection occurred before or after abortion, so prospective studies at the animal level should be regarded as more robust.

2.2.2.1.3. *Experimental abortion studies in cattle and pathogenesis of leptospiral abortion and stillbirth*

Several experimental challenge studies in pregnant cows and heifers attempted to demonstrate leptospiral abortion in cattle and provide more information on the pathogenesis of leptospiral abortion, stillbirths and weak calves. However, most did not use control animals, so if abortion was observed in those studies, it could not be concluded definitively that *Leptospira* were the cause.

In controlled experiments, Ellis *et al.* (1986a) used intra-placentome *L. interrogans* serovar Hardjo infection and successfully infected fetuses and demonstrated abortion, still birth, weak calves and mummified fetuses, while Murphy and Jensen (1969) demonstrated fetal death from 15 days following intravenous Pomona challenge.

A selected sub-set of typical examples of uncontrolled trials either succeeded (Morse and McNutts 1956; Ferguson *et al.* 1957; Fennestad and Borg-Petersen 1958; Ellis and Michna 1977; Thiermann 1982; Ellis *et al.* 1985b) or failed (Sullivan 1970; Smith *et al.* 1997) to produce abortion and fetal leptospiral infection. The route of challenge, serovar, strain pathogenicity, the pregnancy stage, a possible previous exposure to *Leptospira* or the age of the animals could influence the outcome of the challenge. Ellis *et al.* (1985b) showed that variation in pathogenicity may exist between strains within serovar since some strains of Hardjo caused abortion while others targeted mainly the urinary tract.

Infected weak calves, premature calves and stillbirth, usually concurrent with abortions, have been reported, in uncontrolled experimental challenge studies using serogroup Hebdomadis (Ellis and Michna 1977), serovar Hardjo (Sullivan 1970;

Thiermann 1982; Ellis *et al.* 1985b; Ellis *et al.* 1986a; Bolin *et al.* 1989) and serovar Saxkoebing (Fennestad and Borg-Petersen 1958).

2.2.2.1.4. *Fetal leptospirosis and associated lesions*

Observations of fetal infection by Levatidi silver staining, immunofluorescence, dark-field examination, culture and culture after inoculation of 2-day-old chicks or chinchilla from natural outbreaks of abortions and milk drop that were attributed to *L. interrogans* Hardjo or Pomona in New Zealand, USA and Australia (Te Punga and Bishop 1953; Podgwaite *et al.* 1955; Dacres and Kiesel 1958; Slee *et al.* 1983) and the experimental induction (Murphy and Jensen 1969) of vertical transmission and fetal infection supports that *Leptospira* could be a cause of abortion in cattle. In those studies leptospires were found in almost all fetal organs including kidney, lung, adrenal gland, stomach content, blood, cavity fluids, liver, spleen, brain and eye. Serogroup Icterohaemorrhagiae was also isolated from the aqueous humour, peritoneal fluid, cerebrospinal fluid, blood and kidney of an aborted fetus in UK (Ellis *et al.* 1977), suggesting that this serovar can also cause abortion in cattle.

Gross lesions in the fetus are not pathognomonic for leptospiral infection and are sometimes absent (Fennestad and Borg-Petersen 1958). Expelled fetuses sometimes showed only autolytic deterioration or mummification. Presence of red to yellow fluid in the thoracic and abdominal cavities, generalized oedema, enlarged spleen, enlarged, friable, yellow liver, “pulpy” kidneys have been frequently reported in fetuses naturally or experimentally infected with Pomona and Sejroe/Hardjo/Hebdomadis (Te Punga and Bishop 1953; Ferguson *et al.* 1957; Fennestad and Borg-Petersen 1958; Murphy and Jensen 1969; Ellis and Michna 1976a). Ellis *et al.* (1977) also reported enlarged liver, petechial haemorrhages in several organs including the brain and oedema in a naturally aborted fetus from which serovar Icterohaemorrhagiae was isolated.

Histological examinations of fetuses from naturally occurring abortions or from experimental challenge studies have been reported by Fennestad and Borg-Petersen (1958), Murphy and Jensen (1969), Ellis and Michna (1976a) and Slee *et al.* (1983). Interstitial nephritis, cellular infiltration of the kidneys and liver was seen associated with Pomona, Saxkoebing, Sejroe and Hardjo. Leptospires often had a perivascular distribution in several organs, likely causing localized haemorrhages (Ellis and Michna 1976a; Ellis *et al.* 1977). These lesions suggest that fetal leptospirosis is similar to the disease observed in adults.

2.2.2.1.5. *Localization of leptospires in placenta*

Leptospires have also been commonly reported in amniotic fluid and the placenta in cotyledons, caruncles and the materno-fetal junction, in aborting cows as well as in cows that produced healthy calves (Murphy and Jensen 1969; Ellis and Michna 1977). Degenerative lesions including vacuolization, disruption of the epithelium, separation of the fetal villi from the maternal crypts and necrosis occurred within 2 to 6 weeks of experimental infection with Pomona (Morter *et al.* 1958). These observations suggest

that leptospiral infection could lead to disturbance of the exchanges between the dam and the fetus, but whether these lesions are the cause of fetal death and abortion should be confirmed by more controlled studies.

2.2.2.1.6. *Fetal immunity*

Some calves survive infection *in utero*. Fetuses infected *in utero* were able to produce antibodies in the last trimester of gestation (Fennestad and Borg-Petersen 1958; Ellis and Michna 1977), and those authors suggested they could be protective. Antibodies were found in fetuses whose dams were experimentally challenged with serovar Saxkoebing survived to birth. They had lesions of interstitial nephritis but no leptospire were identified, suggesting that they were infected but survived the infection. However, no inference about the cause of nephritis was possible, since no control animals were used (Fennestad and Borg-Petersen 1958). In this study (Fennestad and Borg-Petersen 1958), cows challenged with Pomona or Sejroe aborted seronegative fetuses, with interstitial nephritis and leptospire identified in the fetal kidneys. Birth of previously exposed but healthy calves is thus possible, but little is known of the subsequent health, production performance or the epidemiological relevance of animals infected *in utero*.

High MAT titres were also found in aborted fetuses (Ellis *et al.* 1977). In a UK case-control study (Ellis *et al.* 1978) titres against Hebdomadis or Icterohaemorrhagiae serogroups ranging from 10 to 300 000 were found in 6.9% of naturally aborted fetuses but in no healthy fetus.

The pathogenicity of leptospiral abortion, stillbirths and weak calves is still unclear. Fetal leptospirosis and degenerative changes in the placenta are involved, but they are not always reported in abortion attributed to *Leptospira*, suggesting that other mechanisms could also be involved.

2.2.2.2. Fetal leptospirosis in small ruminants and other species

Fetal *Leptospira* infection also occurs in sheep, deer and goats. In UK and Spain, leptospire of Hebdomadis and Australis (including serovar Bratislava) serogroups have been identified by direct culture or guinea pig passage of fetal organs or fluids from naturally aborted and stillborn sheep fetuses and Pomona, Sejroe and Icterohaemorrhagiae serogroups have been similarly identified in goats, or by immunofluorescence in fetuses with Pomona isolated from ewes (Ellis *et al.* 1983; Leon-Vizcaino *et al.* 1987). However, while a controlled experimental infection with *L. interrogans* Hardjo of seronegative ewes 60 to 90 days pregnant produced abortion in 2/6 ewes it failed to result in fetal infection, as investigated by immunofluorescence, and no gross lesions could be seen in the aborted fetuses (Andreani *et al.* 1983). Ovine fetal infection with Pomona was demonstrated 21 days after inoculation via the middle uterine artery (Smith *et al.* 1966). Resulting fetal lesions were similar to those observed in cattle in both natural and experimental infection. Leptospire were also isolated from

the placentae suggesting that the pathogenesis of leptospiral abortion and perinatal losses in sheep is similar to that in cattle.

Abortion investigations in New Zealand sheep (West *et al.* 2004) revealed a Pomona seroprevalence (MAT titre ≥ 100) in aborting hoggets of 90% (48 animals sampled) compared with 73% in hoggets lambing (30 animals sampled) suggesting that this serovar was involved in abortion although the difference was not significant (Pearson's chi-square p value=0.12) and the exact MAT titre or an additional blood sample were not available.

Leptospira were detected by PCR in 1/27 healthy farmed red deer fetuses at slaughter of hinds estimated to be in the first three months of pregnancy. The hind was positive (MAT titre 196) for *L. borgpetersenii* serovar Hardjo. An investigation on four New Zealand farms naturally exposed to *L. borgpetersenii* serovar Hardjo showed no difference in abortion rates between vaccinated and control hinds (Subharat *et al.* 2010, 2011). Those observations suggest that leptospiral infection with *L. borgpetersenii* serovar Hardjo type Hardjo-bovis or with Pomona of hinds does not affect survival of the fetus. However, more research needs to be undertaken to validate this conclusion, particularly focussing on fetal infection in late pregnancy.

2.2.2.3. Relative importance of *Leptospira* as a cause of abortion

In a case-control study in south west England of a population of around 450,000 cows from herds accredited under the brucellosis eradication scheme (Little *et al.* 1980), 28% of 1471 cows experiencing abortion were positive for serovar Hardjo by complement fixation test. By comparison 19% of 1435 cows that calved normally were Hardjo positive. Hence, considering the incidence of abortion in the whole population of cattle in south west England was low (0.3%) and assuming all abortions were submitted for analysis, around 11% of abortions could be attributed to Hardjo. In an Australian study 2% of 265 abortions in 226 farms were linked to *L. interrogans* Hardjo and 2% to Pomona, determined by silver staining on the fetus, and MAT on the cows to determine the serovar (Slee *et al.* 1983). In a report from Brazil, *L. interrogans* Hardjo, Pomona or Wolfii were suspected in 60% of the 120 abortions recorded in 10 dairy herds, measured by a 4-fold increase in MAT titre between the day of abortion and 15 days later, culture on fetal kidneys and/or silver staining on fetal kidneys (Langoni *et al.* 1999). Serovar Bratislava was incriminated as the causal factor of abortion in 69% of the seropositive (MAT titre ≥ 300) cows that aborted in a Spanish case-control study involving 144 cases of abortion and 380 controls in 48 dairy herds with 5.1% of the study animals seropositive (Atxaerandio *et al.* 2005). In a New Zealand case-control study (Sanhueza *et al.* 2013), 4.7% of 379 abortions in 45 beef cattle herds could be attributed to *L. borgpetersenii* serovar Hardjo type Hardjo-bovis and 3.6% to Pomona, while 3.5% were attributable to BVDV and 3.0% to *Neospora caninum*,

During 973 outbreaks of abortion in sheep and 262 in goats in Spain (Leon-Vizcaino *et al.* 1987) investigated by dark field microscopy and immunofluorescence on

fetuses, evidence of *Leptospira* infection was detected in 1.7% of the ovine outbreaks and 2.6% of the caprine outbreaks. Serovar Pomona was identified in more than 60% of the outbreaks attributed to *Leptospira* in both species. Among 553 aborted or stillborn ovine fetuses submitted to a diagnostic laboratory in South Dakota between 1983 and 1986 (Kirkbride and Johnson 1989), none was positive for *Leptospira* by immunofluorescence, while 38.3% were positive for *Toxoplasma*, 30.0% for *Campylobacter*, 7.5% for *Chlamydomphila*, 5.9% for *Salmonella*, 5.5% for *Arcanobacterium pyogenes* and 4.7% for *Pasteurella*. Out of 326 of these fetuses that had serum tested by MAT against Pomona, *L. interrogans* Hardjo, Grippotyphosa, Canicola and Icterohaemorrhagiae) with a cut-off titre of 10, 27 (8.3%) were positive, and of these 27, 10 had another infectious agent identified as the cause of abortion. In 382 aborted ewes from 100 flocks in Slovakia that were tested serologically (Špilovská *et al.* 2009), 2.9% had MAT titres between 800 and 1600 (serovar panel not given), for 24.3% positive against *Toxoplasma gondii*, 13.6% against *Chlamydomphila abortus* and 3.7% against *Neospora caninum*.

To our knowledge, no similar study has been conducted in deer, but work by K, Patel (pers. comm.) with comparative design looking at the aetiology of abortion in New Zealand farmed deer is currently ongoing at IVABS, Massey University.

2.2.3. Clinical disease and mortality between birth and weaning

Experimental infection of calves up to eight months of age reported between 1941 and 1957 were reviewed by Fennestad (1963) but no information on the use of control animals was given. That review reported that mortality or severe clinical disease was observed in calves infected with Pomona and Grippotyphosa but not with Sejroe. Controlled experimental subcutaneous infection of seronegative calves (test and cut-off not described) at around three months of age with serogroup Australis resulted in mild symptoms and no mortality (Spradbrow 1965), but a similar experiment using serovar Pomona, subcutaneously or intravenously, on seronegative calves aged one week to seven months showed a larger variety of symptoms from mild febrile disease to death, including haemolytic disease with hemoglobinuria in calves aged less than two months while febrile disease occurred in all age groups (Spradbrow and Wright 1963). Death seems more frequent with Pomona than Hardjo and younger animals seem more susceptible. Little and Salt (1976) experimentally challenged two calves (age unknown but they were fed milk) with Pomona (challenge route not described), reporting febrile symptoms and haemoglobinuria and interstitial nephritis without being able to identify leptospire in kidneys by staining and culture. However, at post mortem 11 or 35 days after inoculation, symptoms had stopped and no gross lesion could be detected.

Field observations of New Zealand calves exposed to *L. borgpetersenii* serovar Hardjo type Hardjo-bovis showed no symptoms and no mortality (Cordes *et al.* 1982). By contrast Sutherland (1949) reported acute haemolytic disease associated with fever and mortality, sometimes called “red water” associated with high Pomona titres in Australia. This report mentioned a “high morbidity” (numbers not given) with a sudden

appearance, more seriously affecting young calves under 3 to 4 months of age. Necropsy of calves dying after natural exposure showed icterus, enlarged liver and spleen, and renal lesions similar to what was described in fetal infection.

Controlled experimental challenge of 12-week-old lambs with serovar Pomona (Hodges 1974) showed a variety of symptoms in lambs, from no apparent clinical manifestation, to death. This is consistent with on-farm observations of mortality in lambs naturally exposed to Pomona in New Zealand and USA (Hartley 1952; Smith and Armstrong 1975; Vermunt *et al.* 1994).

Subharat *et al.* (2011) observed a reduction in mean weaning rates but not in calving rates in New Zealand farmed red deer herds exposed to *L. borgpetersenii* serovar Hardjo type Hardjo-bovis.

2.3. Clinical disease and mortality in adults

Experimental infection of adult cattle between 1948 and 1957 was reviewed by Fennestad (1963) but no information on the use of control animals was given. Severe haemolytic disease followed by death was observed in one of six reported trials with serovar Pomona, but not in one reported trial with Icterohaemorrhagiae, Sejroe and Saxkoebing. These observations suggest that clinical disease and mortality are less common in adult cattle than in calves, but are still possible since little is known about the actual incidence on farms. This is supported by on-farm reports in New Zealand in which despite a sometimes high Hardjo and Pomona seroprevalence, clinical disease is rarely observed (Cordes *et al.* 1982). Marshall and Mantkelow (2002) report the involvement of Pomona in several outbreaks of “redwater” in New Zealand cattle in the 1950s.

Occasional mortality and haemolytic disease associated with Pomona, Grippotyphosa and *L. interrogans* serovar Hardjo titres ranging 200 to >2000 was reported in sheep on New Zealand and USA farms (Hartley 1952; Schmitz *et al.* 1981). However, experimental challenge of pregnant ewes with *L. interrogans* Hardjo and of recently weaned sheep with Pomona did not reveal clinical sign other than pyrexia (Webster and Reynolds 1955; Andreani *et al.* 1983). In the absence of controlled observational studies in sheep, the real occurrence of clinical disease and mortality is still unknown in this species.

Controlled experimental intraperitoneal challenge of goats with Pomona and *L. interrogans* Hardjo (Tripathy *et al.* 1985b) resulted in a febrile syndrome, but with no renal lesion in spite of isolation of Pomona from the kidney. Similar experiments (Tripathy *et al.* 1985a) revealed interstitial nephritis in 6/9 goats challenged with *L. interrogans* Hardjo and 1/5 with Swajizak but no clinical signs were observed. Furthermore, during an outbreak of reproductive problems attributed to serovar

Icterohaemorrhagiae in Brazil, no other clinical signs were reported (Martins *et al.* 2012).

A review of veterinary quarterly laboratory reports of *Leptospira* infection in farmed deer in New Zealand between 1987 and 1992 (Wilson and McGhie 1993) showed that jaundice was observed in 73% of the 70 submissions, sudden death in 40% and redwater in 33%. A review of data from the same origin covering the years between 1980 and 2005 as well as clinical and research reports (Ayanegui-Alcerreca *et al.* 2007) described a range of symptoms from depression to jaundice, haemoglobinuria and death attributed mainly to serovar Pomona, as well as one case of jaundice and redwater with death to Copenhageni. Renal and vascular lesions were also reported from animals in herds seropositive for Hardjo-bovis and Ballum.

2.4. Milk production

Early case reports of high *L. interrogans* Hardjo titres during outbreaks of milk drop and mastitis in dairy cattle in Australia and UK (Corbould 1971; Hoare and Claxton 1972; Ellis *et al.* 1976; Higgins *et al.* 1980; Pearson *et al.* 1980) described consistent symptoms. Milk drop was usually observed before the reproductive symptoms, if they occurred. Up to half of the milking herd was affected and milk production dropped by up to 50-90% in 24h. Sometimes complete agalactia was observed. All four quarters were generally affected, with no sign of inflammation. Authors described the udder as “slack and flabby” or “flaccid”. The cows recovered in 7 to 14 days. Some case reports estimate that the expected annual production of an Australian herd can drop by 30% (Sullivan and Callan 1970) or 8400-19550 litres in UK herds of around 250 cows where the expected milk production was not reported (Higgins *et al.* 1980) during an Hardjo outbreak. However, these descriptive studies, while giving details on the course of supposed leptospirosis outbreaks, did not give evidence of causal association between infection by *Leptospira* and milk production drop.

In a UK cross-sectional study (Dhaliwal *et al.* 1996b) cows with a *L. interrogans* Hardjo titre >10 or >100 did not show a lower estimated 305 day milk production (calculation method not described) compared with seronegative cows. However the 305-day milk production was estimated only from the lactation stage and the milk production on the day of sampling, and the definition of seropositive cows did not allow differentiation of cows currently infected and previous exposure, hence hiding a possible difference between cows currently infected and healthy cows.

Serovars Hardjo and Pomona have been isolated from the milk of cows with mastitis, for which all other causes were excluded, during experimental challenge and in an Australian herd naturally exposed (Gillespie and Kenzy 1958; Sullivan and Callan 1970). In these cases of supposed leptospiral mastitis, the milk was of low quality, thick, sometimes blood-tinged or with yellow spots (Sullivan and Callan 1970; Hoare

and Claxton 1972; Ellis *et al.* 1976; Higgins *et al.* 1980). A high somatic cell count measured by California Mastitis Test was also a recurrent finding. Mastitis due to Hardjo has been reproduced experimentally by intravenous inoculation of cows with serovar Hardjo-prajitno and Swajizak (Thiermann 1982). However, in a study of Hardjo and Pomona on 16 New Zealand dairy herds (Carter *et al.* 1982), the authors reported that they could not find any evidence of mastitis in any herd.

Subacute mastitis and reduction of milk production was observed in goats experimentally infected on the day of parturition with *L. interrogans* Hardjo and Pomona (Tripathy *et al.* 1985a; Tripathy *et al.* 1985b). The decrease in milk production started 4 days after inoculation and was present for 4 to 5 days. However, evidence that *Leptospira* were the cause of the mastitis was inconclusive as one control animal also presented a mild mastitis. Agalactia, reversible in 3-4 days, has also been reported on UK farms in ewes seropositive for Hardjo, leading to death of young lambs soon after birth (McKeown and Ellis 1986).

2.5. Growth and live weight

2.5.1. Weight at birth

In Brazil, Fava *et al.* (2004) recorded no significant live weight difference between calves born from cows seropositive at the beginning of the breeding period for *Leptospira* spp. (MAT titre ≥ 100 for serovars Hardjo, Wolfii, Shermani, Tarassovi, Autumnalis, Australis, Pyrogenes, Andamana, Whitcombi, Hebdomadis, Bratislava or Castellonis) and seronegative cows. Holroyd (1980) did not report a weight difference between calves born from cows vaccinated against *L. interrogans* Hardjo and control cows in an Australian herd with a history of Hardjo exposure, but no information on the exposure to *Leptospira* during the trial or on sero-status at vaccination was given. In a UK study of stillborn and weak calves Smyth *et al.* (1999) showed that calves with a placenta or any organ positive by immunofluorescence staining of tissues for *Leptospira*, were 6 to 10 kg lighter than the immunofluorescence negative calves. This suggests that intrauterine infection could lead to lighter animals and that the infection of the placenta may have an impact on the calf's weight that is equivalent to infection of the calf *per se*.

No data were found on the effect of *Leptospira* on birth weight in small ruminants and deer.

2.5.2. Growth of young and weight of adults

No difference in growth rate of Australian cattle has been described between calves born from cows vaccinated at the beginning of breeding and during pregnancy and control cows (Holroyd 1980). That study was repeated over six years but no measure was taken to account for a possible clustering between years, the vaccination protocol of heifers and cows was unclear, and no information on the exposure on the farm during the study years was given. Seropositivity at the beginning of a three-month

breeding period to Hardjo, Wolfii, Shermani, Tarassovi, Autumnalis, Australis, Pyrogenes, Andamana, Whitcombi, Hebdomadis, Bratislava or Castellonis serovars did not influence average daily weight gain during the breeding season and body condition score at the beginning and at the end of the breeding season of beef cows from one Brazilian herd (Fava *et al.* 2004).

In New Zealand farmed deer, Ayanegui-Alcerreca (2006) showed that 10-month-old deer showing culture and/or dark field microscopy evidence of leptospiral infection in urine and kidneys during the growth cycle averaged 3.71 kg ($p < 0.05$) lower weight gain in the previous 6 months than leptospiral negative deer in a herd with serological evidence of dual Hardjo-bovis and Pomona exposure. Subharat *et al.* (2012) supported this finding, showing a significant overall higher mean weight gain in vaccinated than control deer from 4 to 12 months of age, with the greatest difference of up to 26 g/day in herds with the highest Hardjo-bovis and/or Pomona seroprevalence in controls, and with the effect most evident during the period of highest exposure rate as shown by serial serological testing.

In a controlled experimental Pomona vaccine trial in sheep, with restricted feeding, Webster and Reynolds (1955) reported that the observed weight loss occurred almost two months earlier in the 20 unvaccinated animals than in the 20 vaccinated sheep, suggesting that the effect of *Leptospira* may be increased by stress. A difference of 2 pounds (0.9 kg) in average group weight was observed, although no measure of variability or statistical significance was given. In an experimental challenge of eight sheep aged 4-8 months with *L. borgpetersenii* serovar Hardjo by naso-conjunctival instillation (Fang 2014), five animals lost an average of 5.2kg over 6 weeks while two others gained 2.5 kg and one died, but no control animals were used.

Table 2-1: Summary of cattle studies with a comparative, observational, prospective design done on-farm providing quantification of the production effect of exposure to *Leptospira* showing type, sample size, serovars tested and seroprevalence, timing of measurement of exposure, method and time of measurement of the production effect and observed effect of exposure on production.

Reference	Type	Sample size	Serovar(s) tested and seroprevalence	MAT cut-off	Timing of measurement of exposure	Method and time of measurement of the production outcome	Observed effects of exposure on production outcome
Gutián <i>et al.</i> 1999	Dairy	1 herd 207 cows	<i>L. interrogans</i> serovar Hardjo 4.3%	100	Within 40 days of the first calving	Measured in the following reproduction cycle Manual pregnancy diagnosis 45 days and 150 days after artificial insemination	<ul style="list-style-type: none"> - Time from calving to first breeding: no difference, value not given, p=0.47 - Time from calving to conception: +37 days, p=0.019 - Chances to be diagnosed pregnant at 45 days half those of seronegative, p<0.05 - Number of breeding per conception: +1.3, p=0.003 - Abortion rate between 45 and 150 days of pregnancy: -12 percentage points, p=0.34
Muñoz-Zanzi <i>et al.</i> 2004	Dairy	2 herds 381 heifers	<i>L. interrogans</i> serovar Hardjo 8.4% <i>L. interrogans</i> serovar Pomona 6.8%	400	At 10-12 months of age	Manual pregnancy diagnosis around 45 days after insemination	Time from 10 months of age to first insemination <ul style="list-style-type: none"> - Hardjo: +6 days, p=0.066 - Pomona +7 days, p=0.099
Genovez <i>et al.</i> 2001	Beef	1 herd 109 cows	Icterohaemorrhagiae, Canicola, Pomona, Grippotyphosa, Hardjo and/or Wolfii 62.5% Hardjo 50.0% Wolfii 35.8%	50	October in the previous year (date relative to the production cycle unknown)	One insemination followed by bull Pregnancy diagnosis by ultrasound (at unknown pregnancy stage)	Pregnancy rate +3 percentage points, p>0.05
del Fava <i>et al.</i> 2004	Beef	1 herd 712 cows	Australis 2.5% Bratislava 1.1% Autumnalis 3.7% Castellonis 0.1% Whitcombi 1.3% Hebdomadis 3.7% Pyrogenes 2.3% Hardjo 37.7% Wolfii 35.7% Shermani 6.6% Tarassovi 3.1% Butembo, Bataviae, Canicola, Cynopteri, Grippotyphosa, Copenhageni, Icterohaemorrhagiae, Javanica, Panama, Pomona 0%	100	Beginning of the breeding season	Pregnancy diagnosis (method unknown) 60 days after the end of the breeding period	<ul style="list-style-type: none"> - Pregnancy rate: -1.3 percentage points, p>0.05 - Calving rate: -0.3 percentage points, p>0.05 - Stillbirth rate: -0.2 percentage points, p>0.05 - Daily weight gain during the breeding season: +2.64 g/day, p=0.61 - Body condition score at the end of the breeding season +0.06 (on a scale from 1 to 9), p=0.67 - Weight of calf at birth: -0.06 kg, p=0.89

2.6. Effectiveness of vaccination in preventing or reducing production losses

Most vaccination studies focus on the efficacy in reducing renal carriage or shedding in urine while few looked at the effect on production outcomes. In a randomized controlled trial with experimental challenge 12 months after vaccination (Zimmerman *et al.* 2013), vaccination prevented *L. borgpetersenii* Hardjo genital carriage in vaccinated heifers (0/18 culture positive in vaccinated group vs. 5/18 in control group), which suggests that, at least in experimental conditions, vaccination might prevent potential reproductive effects associated with genital carriage of leptospires.

2.6.1. Vaccination to prevent production losses

The results of on-farm vaccination trials in dairy cattle, beef cattle and farmed deer are presented in

Table 2-2. To our knowledge, no similar study exists in sheep or goats.

The absence of significant difference in reproductive outcomes in USA by Kasimanickam *et al.* (2007) and by Plunkett *et al.* (2013) suggests either that the Hardjo strain present may have been less pathogenic for cattle than *L. interrogans* serovar Hardjo, that the vaccine was not effective against production losses, or that the challenge on the control animals was not high enough to create a difference. Dhaliwal *et al.* 1996c observed a significant association between vaccination and some reproductive outcomes in UK dairy cattle. However, no information on the timing and serostatus at vaccination and on the method of measurement of the production outcomes was given.

In a case-control study in Chilean dairy cattle (Gädicke and Monti 2013), bivalent *L. interrogans* Hardjo-bovis-Pomona vaccination was associated with a significantly reduced risk of abortion (OR=0.61, 95% CI 0.41-0.92, corresponding to a decrease in abortion rate of 2.35 percentage points).

Studies on New Zealand deer farms (Subharat *et al.* 2011, 2012) showed no effect of vaccination on pregnancy rates in early gestation and at term but an increase in weaning rates, suggesting that calf survival from birth is affected by *Leptospira* infection, as well as an increase in average daily gain (

Table 2-2). The difference reached 26 g/day between 4 and 12 months old on the farm with the highest exposure measured by MAT titres and PCR on urine. Crude preliminary economic analysis using these reproduction data in deer showed a return on investment depending on seroprevalence of up to 1209% in a high prevalence setting (Wilson *et al.* 2011). With the production data used in this model, vaccination would become cost-effective when seroprevalence was 19% or more. However, vaccination must be undertaken before exposure so it is not possible to predict that an economic response will occur since prevalence varies between season and between farm. To our knowledge, no study quantifying the effects of leptospiral vaccination on sheep has been conducted. A complete economic analysis of leptospiral vaccination should also account for the effects on human health and this work is currently in progress (J. Sanhueza, EpiCentre, Massey University, pers comm).

Table 2-2: Summary of published studies of on-farm natural challenge vaccination trials in cattle and deer, with part of the herd vaccinated and with quantification of the effects of vaccination on production showing species and type, sample size, serovar(s) used in the vaccine, vaccination protocol, evidence of challenge in the control group, method and time of measurement of the production outcome, and observed effects, with p-value for the effect of vaccination on the production outcomes.

Reference	Species and type	Sample size	Serovar(s) in the vaccine	Vaccination protocol	Seroprevalence	Method and time of measurement of the production outcome	Observed effects of vaccination on production outcome
Dhaliwal <i>et al.</i> 1996c	Dairy cattle	3 herds 200 vaccinated, 215 non-vaccinated cows	<i>L. interrogans</i> serovar Hardjo	- 2 injections 4 weeks apart, booster a year later age - Reproduction stage and serological status NA	37-73% of MAT titre ≥ 10 at the beginning of the study similar at the end	NA* (herd records)	- Milk production: -179 kg, $p > 0.05$ - Calving to first service interval: -2.3 days, $p > 0.05$ - Calving to conception interval: -1 day, $p > 0.05$ - Inter-service interval: -1.4 days, $p > 0.05$ - Services per conception: -0.09, $p > 0.05$ - Pregnancy rate after first insemination: +17.0 percentage points, $p < 0.01$ - Overall pregnancy rate: +19.3 percentage points, $p < 0.001$ - Abortion rate: +0.5 percentage points, $p > 0.05$ - Culling rate: -19.6 percentage points, $p < 0.001$
Plunkett <i>et al.</i> 2013	Dairy cattle	1 herd 986 vaccinated, 908 placebo cows	<i>L. borgpetersenii</i> serovar Hardjo	- 2 injections 28-35 days apart - First injection with antibiotics - Second injection at least 2 weeks before insemination	13% of MAT titre ≥ 100 at the beginning of the study similar at the end	- Visual inspection and tail chalk removal for estrus - Conception defined as the last insemination before positive pregnancy diagnosis - Manual or ultrasound pregnancy diagnosis 32-40 days after insemination - Second pregnancy diagnosis (time unknown) to detect abortion	- Pregnancy rate: +0.5 percentage points, $p > 0.05$ - Abortion rate: -0.2 percentage points, $p > 0.05$ - Pregnancy rate in heifers only: +0.1 percentage points, $p > 0.05$ - Breeding to conception interval: value not given, $p > 0.05$
Holroyd 1980	Beef	1 herd	<i>L. interrogans</i>	- 2 injections 5-6	“serological evidence”,	- Manual	- Abortion rate: -2.2 percentage points, $p < 0.01$

	cattle	6 years 127 to 344 animals per year, half vaccinated	serovar Hardjo	weeks apart, mid- pregnancy - Heifers joining the herd also vaccinated at breeding - Unclear whether same animals followed all along the observation period	no details given	pregnancy diagnosis 2 months after start of breeding and then every 6 weeks - Perinatal losses defined as losses in the first 48 hours after calving, then postnatal losses until weaning - Birth weight of calves measured 3 times weekly	- Perinatal loss: -0.0 percentage points, $p>0.05$ - Postnatal loss: +0.8 percentage points, $p>0.05$ - Birth weight of calves of study animals: -0.5 kg, $p>0.05$ - Average daily weight gain of calves of study animals between birth and weaning: -0.00 kg/day, $p>0.05$
Kasimanickam <i>et al.</i> 2007	Beef cattle	8 herds 741 vaccinated, 705 placebo	<i>L.</i> <i>borgpetersenii</i> serovar Hardjo	- 2 injections 4 weeks apart - Second injection with antibiotics - Second injection at least 1 month before estrus synchronization	7-39% Hardjo MAT titre ≥ 200 on farms positive for immunofluorescence on urine, 6-20% on farms negative for immunofluorescence	- Fixed-time insemination followed by bull 2 weeks later - Manual or ultrasound pregnancy diagnosis 70 and 100-130 days after insemination	On farms positive for immunofluorescence on urine - Pregnancy rate after artificial insemination: +0.6 percentage points, $p>0.05$ - Overall pregnancy rate: -0.3 percentage points, $p>0.05$ - Calving rate after artificial insemination: -0.2 percentage points, $p>0.05$ - Overall calving rate: -1.3 percentage points, $p>0.05$
Ayanegui- Alcerreca 2006	Deer	1 herd 97 vaccinated hinds, 93 control for reproduction effects 69 vaccinated progeny, 75 control for weight effect	<i>L.</i> <i>borgpetersenii</i> serovar Hardjo <i>L. interrogans</i> serovar Pomona <i>L. interrogans</i> serovar Copenhagueni	- 2 injections 4 weeks apart - Vaccination before calving for repro - At 7 months old for growth - Evidence of shedding in vaccinated group	- Hardjo seroprevalence (MAT titre ≥ 24) >20% from 12 months old onwards, reached >40% - Pomona seroprevalence (MAT titre ≥ 48) >20% from 9 months old onwards, reached >80% - Copenhagueni seroprevalence (MAT titre ≥ 48) >20% from 11 months old onwards	- Udder palpation at weaning	- Weaning rate: +9 percentage points, $p=0.04$ - Weight gain between 4 and 10 months old: no difference, value not available
Subharat <i>et al.</i>	Deer	4 herds,	<i>L.</i>	- Antibiotics to all	- Combined	- Pregnancy	- Pregnancy rate: -5.9 percentage points, $p>0.05$

2011		yearling hinds 125 vaccinated, 127 control	<i>borgpetersenii</i> serovar Hardjo <i>L. interrogans</i> serovar Pomona	study animals - 2 injections 4-6 weeks apart, starting at 15 months old	with other untreated animals after breeding - Prevalence of Hardjo MAT titre ≥48 ranged 0-88% at the beginning of the study, 21-47% a year later - No evidence of exposure to Pomona	scanning around 1 months after breeding - Udder and abdomen palpation just before calving - Udder observation and palpation at weaning	- Pregnancy rate at term: +1 percentage point p>0.05 - Weaning rate: +6 percentage points, p=0.015
Subharat <i>et al.</i> 2012	Deer	5 herds 217 vaccinated, 218 control	<i>L.</i> <i>borgpetersenii</i> serovar Hardjo <i>L. interrogans</i> serovar Pomona	- Antibiotics to all study animals - 2 injections 4 weeks apart, starting at 3 months old	- Combined with other untreated animals at 6 months old - Prevalence of Hardjo MAT titre ≥48 0% at the beginning of the study, 4-78% at the end - No evidence of exposure to Pomona	- Weighing at 4, 9 and 12 months old	- Average daily weight gain on one farm between 4 and 12 months old: +26 g/day, p=0.049 - Overall average daily weight gain between 9 and 12 months old: +31 g/day, p=0.011

*NA Not available

2.6.2. Vaccination in response to production losses

In cattle, a reduction of symptoms was reported when using vaccination and antibiotics in the face of an abortion storm in cattle with Pomona in New Zealand and Canada (Mackintosh *et al.* 1980; Kingscote and Wilson 1986; Gilmour 2007), on USA farms with a history of abortion and subfertility with a high presence of serovar Hardjo and on farms with a history of Grippotyphosa abortions (Hanson *et al.* 1972). However, these reports do not allow differentiating between the effect of vaccination, the effect of antibiotics or the natural course of the outbreak.

The combination of antibiotics and vaccination resulted in a 90% reduction of fertility and abortion problems associated with *Icterohaemorrhagiae* in Brazilian goats (Martins *et al.* 2012). Vaccination alone was followed by the reduction of agalactia and perinatal mortality associated with *L. interrogans* Hardjo in UK sheep seven days after vaccination (McKeown and Ellis 1986), but without a control group the reduction of symptoms cannot be attributed to vaccination. However, in an investigation of an outbreak of mortality in two New Zealand flocks of lambs on a farm just after weaning attributed to Pomona (Hilson 2006), mortality stopped after the use of antibiotics and management of pasture, without the use of vaccination. This approach was chosen because the cost of vaccination of lambs could not be justified given that lambs were to be slaughtered before vaccinal immunity could be established.

Vaccination after diagnosis involves vaccinating at least some animals already infected. Vaccination was shown to reduce shedding in urine of 80-100% provided that the animals were vaccinated before exposure, while persistent shedding was reported when the animals were already shedding at vaccination (Heuer *et al.* 2012). In New Zealand deer on farms with a Hardjo-bovis seroprevalence ranging 10-58% and Pomona 0-95% at the time of vaccination (Ayanegui-Alcerreca 2006), deer vaccinated with a trivalent Hardjo-Pomona-Copenhageni vaccine were shedding for an average of 19 days (median 0 days) and for an average of 47 days (median 7 days) in control deer, measured by dark-field microscopy, urine and kidney culture, suggesting that vaccination of previously exposed animals could reduce the length of shedding but not suppress it. In Australian cattle vaccinated with two injections 6 weeks apart and an booster a year later after exposure to *L. interrogans* serovar Hardjo (Hancock *et al.* 1984), shedding in urine measured by dark field microscopy decreased from 47% (9/19) before vaccination to 27% (4/15) 22 weeks after vaccination. At the same time, shedding in unvaccinated cows went from 68% (15/22) to 44% (4/9), suggesting that the decrease of shedding was due to the natural course of the disease in this herd. Hence, an optimal vaccination program on farms exposed to *Leptospira* should be planned long-term and should focus on replacement groups before exposure to ensure maximum protection, and/or associate vaccination with treatment to clear infection.

The risk of a publication bias is also high, with reports of ineffective vaccination being less likely to be published.

Information is lacking on the cost-effectiveness of vaccination to prevent reproduction losses in beef cattle and sheep, and on its effect on growth losses.

2.7. Conclusion

The combination of evidence in the published literature of on-farm observations, on-farm analytical studies with sometimes high (>2) strength of association, prospective studies where exposure precedes the production loss as reported in Table 2-1, experimental studies and biological plausibility as shown by macroscopic and microscopic lesions (Bradford Hill 1965) provide supportive evidence for serovars Hardjo and Pomona to be a likely cause of production impairment in dairy cattle, with reduced pregnancy rates, increased abortion, perinatal and post-natal losses and reduced birth weight and milk production. These serovars are widespread globally and associated with economic losses for the farmer, and are often reported as one of the major infectious causes of reproduction impairment. Vaccination has been shown to be an effective control measure in limiting these losses in cattle. Other serovars could be implicated but more evidence is needed.

Less information on the production effects, vaccination efficacy and cost-efficiency, and the epidemiology of *Leptospira* infection is available for beef cattle, but available evidence suggests similar effects to those in dairy cattle. Robust vaccination studies in farmed deer showed that production, namely weaning rate and growth, can be improved by vaccinating against Hardjo, providing indirect evidence that *Leptospira* are indeed a production limiting infection in deer. Data are lacking in sheep and goats but *L. interrogans* serovar Hardjo and Pomona are also likely associated with abortion and milk production, and post-natal mortality for Pomona appears likely. Nevertheless, comparative studies between infected and control animals, and between vaccinated and control animals are needed in species other than dairy cattle to achieve more robust conclusions.

While fewer studies were looking at the effect of Pomona than Hardjo, this serovar seemed to be linked with more serious reproductive effects when it was present. Clinical manifestations were also more likely in calves, lambs and adults infected with Pomona than with Hardjo. Other serovars have also been reported in studies looking at impaired reproduction, but robust evidence is lacking to conclude that these serovars lead to production losses.

Variability of the effect of any given serovar was often observed between studies, possibly due to differences in study design but also possibly because of variability in the pathogenicity between local strains. Difficulty was encountered in interpreting different studies because of the terminology used, so future reports should be rigorous in accurately and fully describing the serovar, genomospecies and/or strain to allow future reviews to be more robust and conclusive. This review also highlighted the need for

studies with a robust comparative design to be able to more precisely quantify effects of *Leptospira* infection on production.

2.8. References

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Chapter 3. Serological patterns, antibody half-life and shedding in urine of *Leptospira* spp. in naturally exposed sheep

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3.1. Abstract

3.1.1. Aims

To study the epidemiology of *Leptospira* spp. in naturally infected sheep on commercial farms in New Zealand including within-farm prevalence, longitudinal pattern of exposure as measured by serology, shedding in urine, and longevity of antibody titres.

3.1.2. Methods

A longitudinal study was conducted on eight commercial sheep farms from September 2011 to January 2014. Blood samples from 115 to 217 ewe lambs on each farm were collected at intervals of 2-11 months and analysed by Microscopic Agglutination Test (MAT) to assess exposure to *Leptospira borgpetersenii* serovar Hardjo and *Leptospira interrogans* serovar Pomona. Urine from 98 animals was tested by real-time PCR to estimate shedding prevalence. The half-life of agglutinating antibodies against each serovar was estimated based on a subset of 185 sheep for Hardjo and 21 for Pomona, and the sero-status of the animals lost to follow-up was compared with the animals remaining in the study to identify a potential bias in seroprevalence estimations.

3.1.3. Results

Exposure to Hardjo occurred in animals aged 8-17 months on all farms except one where 2-3-month-old lambs were exposed. Seroprevalence reached 79-100% by 17-22 months. Pomona exposure occurred on three farms in animals aged 4-14 months, and seroprevalence reached 21-54%. Most seroconversions for both serovars occurred from late autumn to early summer at 7-15 months of age. A 3-76% seroprevalence range for Hardjo was observed on all farms, for up to three months, likely due to maternally-derived immunity. The Pomona seroprevalence in lambs aged up to three months ranged from zero to 15%.

The half-life of antibody was estimated to be 6.7 months for Hardjo and 6.3 months for Pomona. Between 11% and 88% of sheep on each farm were real-time PCR positive for leptospires in urine. All but one of the real-time PCR positive animals were seropositive for Hardjo. The animals that were lost to follow-up had a higher geometric mean titre for Pomona than those remaining in the study on the farms where Pomona exposure was observed.

3.1.4. Conclusions

This study is the first to investigate the epidemiology of leptospirosis due to Hardjo and Pomona in New Zealand sheep flocks. It demonstrated seasonal exposure from autumn to early summer in young animals, a wide range of within-flock serological and shedding prevalence, and gives an estimation of the half-life of MAT titres in sheep. More extensive data is needed to fully understand the epidemiology of leptospirosis in sheep flocks across New Zealand, and, along with economic analysis, to

justify and design cost-effective and efficient control measures to protect human and animal health.

3.2. Key words

Leptospirosis; sheep; epidemiology; seroprevalence; Hardjo, Pomona; titre; antibody; half-life; PCR; urine

3.3. Abbreviations

GMT	Geometric Mean Titre
ICC	Intra-Class Correlation
MAT	Microscopic Agglutination Test

3.4. Introduction

Leptospira borgpetersenii serovar Hardjo type Hardjo-bovis (Hardjo) and *Leptospira interrogans* serovar Pomona (Pomona) are the two most common serovars in New Zealand livestock. Hardjo is maintained by sheep (Cousins *et al.* 1989; Gerritsen *et al.* 1994) and cattle (Ellis *et al.* 1981, Faine *et al.* 1999), and while the pig is conventionally regarded as the maintenance host for Pomona, sheep (Morse *et al.* 1957; Hodges 1974) and cattle (Carter *et al.* 1982, Kingscote and Wilson 1986) are commonly infected and shed the bacteria.

A large-scale cross-sectional study conducted in 2010 over the North and South Islands in New Zealand, using a titre cutpoint of ≥ 48 , showed that 91% and 74% of sheep flocks had at least one adult animal that was seropositive to serovars Hardjo and Pomona, respectively, and 43% and 14% of the sheep had a titre ≥ 48 for serovars Hardjo and Pomona, respectively (Dreyfus 2013). This contrasts with previous cross-sectional studies, using the same titre cut point, conducted on lambs and ewes slaughtered at a North Island meat plant that reported a farm prevalence of 46 and 51%, and a sheep seroprevalence of 19 and 4% for serovars Hardjo and Pomona, respectively (Blackmore *et al.* 1982). Similarly Dorjee *et al.* (2008) reported a farm prevalence of 33 and 4% and a sheep prevalence of 5 and 1% for serovars Hardjo and Pomona, respectively, in mostly lambs from different regions of both islands. Both farm and animal seroprevalence increased with the age of the animal. While serovar Hardjo is usually reported to be more prevalent than Pomona, Fang *et al.* (2014a) reported similar sheep-level prevalence for both serovars (37% for serovar Hardjo, 35% for Pomona and 16% of dual seroprevalence).

Within infected farms, the seroprevalence for both Hardjo and Pomona is variable. Prevalence less than 50% was usually reported (Blackmore *et al.* 1982; Dorjee *et al.* 2008; Dreyfus 2013), but more recently Fang *et al.* (2014a) found a within-farm

seroprevalence ranging from 40 to 95% for Hardjo and/or Pomona. On farms known to be undergoing an outbreak, up to 84% of mixed-age ewes were found seropositive for Hardjo, and 22% for Pomona (Blackmore *et al.* 1982).

Fang *et al.* (2014a), using a real-time PCR assay, reported 31% of sheep excreting leptospire in urine, and a within-farm shedding prevalence ranging 5-69%. Those authors also showed that sheep with Hardjo titres ≥ 48 were 12.5 times more likely to be shedding leptospire than seronegative sheep. A previous study using culture failed to reveal the presence of bacteria in urine of sheep (Blackmore *et al.* 1982).

Little is known about the epidemiology of leptospirosis in sheep flocks as acknowledged by Martins and Lilenbaum (2014). Some studies report clinical disease and deaths in lambs as young as two to four months due to serovar Pomona (Hartley 1952; Vermunt *et al.* 1994; Dorjee *et al.* 2005). Another study showed high Hardjo seroprevalence in hoggets and ewes but no titres in lambs (Blackmore *et al.* 1982). Those authors demonstrated the risk period for infection to be between nine and 24 months. Little information is available on maternally-derived antibody protection or on the age at which lambs become susceptible to leptospirosis infection.

Hence, other than cross-sectional serological studies of serovars Hardjo and Pomona, along with reports of clinical occurrences, little is known about leptospirosis or its epidemiology and production effects on commercial sheep farms in New Zealand. This paper presents descriptive data from a longitudinal study of production effects of leptospirosis in sheep flocks (chapters 5 and 6) to improve understanding of the epidemiology of leptospirosis in this species. The objectives were to determine the within-farm prevalence and longitudinal exposure pattern of Hardjo and Pomona using serology, to evaluate the titre decay rate, to characterise *Leptospira* spp. shedding in urine, and to evaluate a possible bias in seroprevalence and mean titre due to animals lost to follow-up, on naturally infected sheep on New Zealand commercial farms.

3.4.1. Study design

This study was an opportunistic analysis based on data from animals from a field trial of the potential effects of leptospirosis on sheep growth and reproduction involving vaccination of a subset of sheep, conducted from 2011 to 2013 (chapters 5 and 6).

The study, designed as a cohort study, was conducted on eight commercial farms with sentinel animals sampled between five and eight occasions for up to 28 months to assess leptospirosis exposure. Data presented in this paper were from non-vaccinated control animals only. In this study, “lambs” refers to animals up to seven months old and “hoggets” refers to animals seven to 19 months old.

3.5. Material and methods

3.5.1. Farms and animals

Six farms were located in the North Island and two in the South Island (Figure 3-1). Farm descriptions, as provided by the farmers, are presented in Table 3-1. All the animals were farmed under typical New Zealand commercial farming conditions, and grazed on pasture all year round.

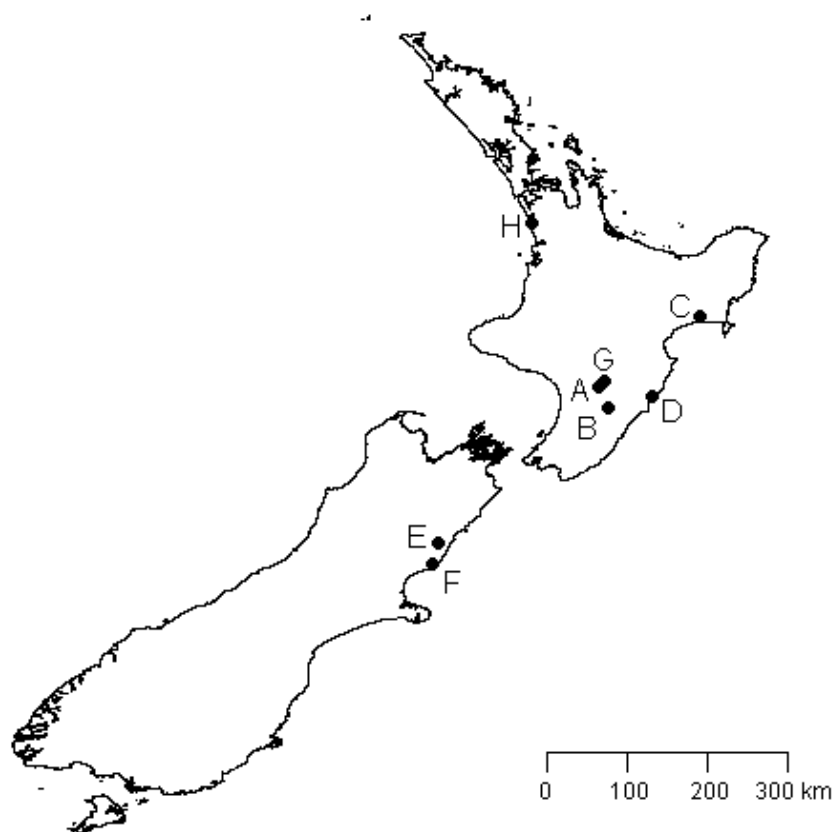


Figure 3-1: Location of the eight study farms A to H

Farms A to G were selected in June 2011 from a previous study on Johne's disease and leptospirosis (Dreyfus 2013) in which they were seropositive for Hardjo and/or Pomona, and where the farmer agreed to take part in this study, primarily investigating the effect of leptospirosis on growth and reproduction. The farms with the largest flock sizes were contacted, and since they all agree to take part, they were recruited for the study. Farm H was selected in 2012 based on its recent history of suboptimal hogget reproductive performance and the known presence of Hardjo and Pomona on the farm. Hence, no random process was used to select the farms for this study.

Between 115 and 217 ewe lambs on farms A-G in 2011 (Table 3-2) were randomly selected at tail docking in spring, at a median age of 2 to 6 weeks, and identified by ear tag with a unique number. The study period, September 2011 to

January 2014, included summer 2012-2013 which was recorded as the driest in 40 years, as measured by the soil moisture deficit, between mid-October and mid-April (Porteous and Mullan 2013). In February 2013, another 198 hoggets were enrolled on farm H at weaning, with a median age of 23 weeks.

3.5.2. Blood and urine collection

Animals were blood sampled on the dates shown in Table 3-2. Blood was collected by jugular venipuncture using a 1 inch 20 G needle and a CAT Plus Blood Collection Tube without anticoagulant (BD Vacutainer®) labelled with the animal identifier. Blood samples were transported on ice to the ^mEpiLab, Massey University where they were stored at +4°C until processing, usually within 24 hours of collection. The samples were centrifuged at 1400 g for 10 minutes. The sera were diluted at 1/6 with 0.9% saline and stored at -20°C on microplates until the Microscopic Agglutination Test (MAT) was performed, one week to 18 months after blood collection.

Urine from between four and 16 animals per farm, for a total of 98 randomly selected animals was collected in a sterile 60 ml container labelled with the animal identifier between 41 and 90 weeks after the beginning of the study. Urination was stimulated by placing a hand over the nostrils until urine was voided. Urine samples were transported on ice and stored at +4°C until processing. The samples were processed and the DNA was extracted within 24 hours of collection.

Table 3-1: Description of the eight study farms

Farm	District	Species	Number of breeding ewes	Sheep breed	Lambing period	Area (ha)	Topography	Soil
A	Manawatu	Sheep, cattle	6000	Romney/composite	01/09/2011-20/10/2011	1900	rolling to medium/steep hills	mainly sandy loam, 14 different types of soil in farm
B	Tararua	Sheep, cattle	8500	Perendale/composite	15/09/2011-03/11/2011	2016	steep hills	Taihape silt loam, Matamau silt loam
C	Wairoa	Sheep, cattle	4700	Romney Coopworth cross	01/09/2011-20/10/2011	1050	flat to steep hills	mudstone, Gisborne ash
D	Central Hawke's Bay	Sheep, cattle	4500	Romney Texel cross	20/08/2011-05/10/2011	1416	flat to rolling hills	clay based soil
E	Hurunui, Canterbury	Sheep, cattle, deer	10400	Romney	15/09/2011-20/10/2011	6130	flat to high country	clay based with some limestone
F	Hurunui Canterbury	Sheep, cattle, deer	10000	Lincoln Merino cross	15/09/2011-20/10/2011	2973	flat to rolling hills	sandy clay, lime based loams, peat loams, clay loams
G	Manawatu	Sheep, cattle, deer	1050	Romney	10/09/2011-30/10/2011	408	easy to moderate hills	Whetukura silt loam, Kiwitea loam & Mangatea hill soils
H	Waikato	Sheep, cattle,	7800	composite Romney Finn Coopworth	15/08/2012-30/09/2012	2500	easy to steep hills	sandstone, siltstone, sandy loam, silt loam, clay loam

3.5.3. Microscopic agglutination test

Serum samples were tested in the ^mEpiLab. The MAT was conducted as described by Fang *et al.* (2014b), based on the method reported in Adler *et al.* (1982). Briefly, two-fold dilutions in 0.9% saline of each serum sample were created, from 1:24 to 1:3072. The samples were then incubated for 1.5-4 hours with live *Leptospira* cultures of the tested serovar. Each dilution of each sample was then checked under a dark-field microscope for the presence of agglutination or lysis. Standard *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona antisera were obtained from the WHO/FAO/OIE leptospirosis reference laboratory, Brisbane, Australia. The *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona used for the test were hamster passaged strains that were provided by Schering- Plough, Wellington, New Zealand. The test was considered positive for the highest dilution where 50% or more of the leptospire were agglutinated or lysed.

3.5.4. Real-time PCR

Urine samples were centrifuged at 10600 *g* for 20 min, and sediment re-suspended in 200 µl of PBS, after discarding the supernatant. The DNA was then extracted using the QIAamp ® DNA Mini Kit (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand) as per manufacturer's instructions, for a final volume of 200 µl of DNA template solution. The samples were then kept at -20°C until the PCR was run.

The real-time PCR used was adapted from Subharat *et al.* (2011) and Fang *et al.* (2014b). The PCR reaction solution was made of 5 pmoles of primers targeting the *gyrB* gene (Slack *et al.* 2006), of sequence 5'-TGAGCCAAGAAGAAACAAGCTACA-3' (2For) and 5'-MATGGTTCCRCTTTCCGAAGA-3' (504Rev), 2.4 µmoles of SYTO9 (Invitrogen Corp., Carlsbad, CA, USA), 12.5 µl of a commercial mastermix (Roche LightCycler 480 Probes Master 04707494001, Roche Diagnostics GmbH, Mannheim, Germany), 2 µl of the DNA preparation and double distilled water for a total volume of 25 µl.

The PCR was run on a Rotor-Gene Q (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand). An initial denaturation of 10 min at 95 °C was followed by 40 cycles, consisting of 10 seconds of denaturation at 95°C, 20 seconds of annealing at 63°C and 10 seconds of elongation at 72°C. The melting temperature was measured by monitoring the fluorescence on the green channel, every 0.2°C from 78°C to 90°C. A positive control, consisting of either DNA extracted from a live culture of a Copenhageni strain isolated from sheep in New Zealand, or DNA extracted from sheep urine inoculated with a live culture of a New Zealand strain of *L. borgpetersenii* serovar Hardjo was used for each PCR run, as well as a negative control made of double distilled water. Confirmation of positive samples was determined by comparing the melting temperature with the positive control.

3.5.5. Statistical analysis

3.5.5.1. Log titre and geometric mean titre

If X was the MAT titre of an individual animal, the log titre Y was calculated as follows:

$$Y = \log_2(X/12) \quad (1)$$

The MAT titre was obtained from the log titre by using the relationship

$$X=2^Y * 12 \quad (2)$$

The geometric mean titre (GMT) was calculated as follows, using all animals with a MAT titre of 24 and above:

$$\text{GMT}=2^{\bar{Y}} * 12 \quad (3)$$

where \bar{Y} was the arithmetic mean log-level titre of lambs at one sampling point of a farm. Animals for which no antibodies were detected were excluded from the calculation of the GMT. These formulae ensured that a round log titre correspond to a tested dilution (1 for 1:24, 2 for 1:48 etc.). The GMT was calculated for each sampling date, stratified by farm. The 95% confidence interval for the GMT was calculated at the log level, and then the lower and upper boundaries were back-transformed.

3.5.5.2. Seroprevalence

Seroprevalence was calculated for each farm on every sampling date, for Hardjo only, Pomona only, and both serovars, as the proportion of animals sampled on that day that had a titre ≥ 48 , which is the cut-point currently recommended for Hardjo in sheep (Blackmore *et al.* 1982). The 95% confidence intervals were calculated using a simple asymptotic method without continuity correction (Newcombe 1998).

3.5.5.3. Titre pattern with age

The log titre, as defined in equation (1), was modelled separately for each serovar as a function of the median age of the sheep in months, using a zero-inflated Poisson regression (Hilbe 2007). Animals with dual Hardjo-Pomona titres were included in both models. Animals with no detectable antibodies were assigned a log-titre of 0. The binary part modelled the probability of a sheep to be exposed to leptospirosis and the count part the expected log titre of a sheep given that it was exposed.

Hence, the zero-inflated Poisson probability function can be defined as followed, with Y being the observed count (log MAT titre defined in equation (1)), Φ the probability of a sheep to be unexposed (mathematically, this corresponds to “extra-zeros” compared to a Poisson model), and λ the expected log MAT titre given the sheep was exposed:

$$P(Y_i = y | \Phi_i, \lambda_i) = \begin{cases} \Phi_i + (1 - \Phi_i)e^{-\lambda_i} & \text{if } y = 0 \\ (1 - \Phi_i) \frac{\lambda_i^y e^{-\lambda_i}}{y!} & \text{if } y > 0 \end{cases}$$

The model assumes that unexposed sheep will not have detectable MAT titres. The following set of predictors was used:

$$\begin{aligned} \text{logit}(\Phi_i) &= \alpha_1 + \alpha_2 \text{age} + \alpha_3 \text{age}^2 + \alpha_4 \text{farm} + \alpha_5 \text{farm} * \text{age} \\ \log(\lambda_i) &= \beta_1 + \beta_2 \text{age} + \beta_3 \text{age}^2 + \beta_4 \text{farm} + \beta_5 \text{farm} * \text{age} \end{aligned}$$

The final model was checked by fitting a zero-inflated negative binomial regression to detect possible presence of additional over-dispersion. The Intra-Class Correlation (ICC) of the residuals within sheep was calculated from a one-way analysis of variance as described by Shrout and Fleiss (1979) to confirm the absence of further correlation in the data.

The mean predicted log titres were then predicted as a function of age and from <1 to 28 months and farm and bootstrap 95% confidence intervals of the predictions were calculated using 1000 replications.

Loess curves were fitted with the smoothing function being the log titre predicted from the zero-inflated Poisson model as a function of age, with a span of 0.7 for individual farm plots and 0.75 for all farms.

3.5.5.4. Titre decay and antibody half-life

To model the titre decay, animals aged >3 months that had a titre of 768 to 1536 for Hardjo or for Pomona were selected, with the assumption that sampling happened close to the date of the actual peak titre. Animals from which no data were available after the assumed peak were removed from the analysis. The time was set to zero on the day of the observed maximum titre, and subsequent samplings were dated as the time in months since the maximum titre. The dependent variable 'log titre' for Hardjo or Pomona, calculated using equation (1), was regressed on time to estimate the average monthly change in log titre after the observed peak titre. The possible violation of independence was evaluated by looking at the effect of adding farm as a fixed-effect and/or sheep as a random-effect on the regression coefficient and model fit.

The half-life of the antibodies in months, assuming an exponential decrease, was then calculated as the inverse of the linear regression coefficient for time, which was the monthly rate of antibody decay. The shape of the decay curve indicated the approximate duration of MAT antibodies.

3.5.5.5. Leptospiral shedding in urine

The shedding prevalence of *Leptospira* spp. was calculated on each farm A to H with the corresponding 95% confidence intervals. The GMT was calculated within each farm for serovars Hardjo and Pomona for animals that were positive or negative as determined using the real-time PCR assay.

To examine the relationship between titre and probability of urine shedding, a logistic regression model was developed with the PCR result (positive/negative) as the binary dependent variable, and a categorical variable for serological status (negative, positive for Hardjo only or positive for Hardjo and Pomona, with no animal being positive for Pomona only) and the median flock age at the time of sampling in months as explanatory variables. A random effect was added to account for clustering within farm. Titre cut-offs for serological status from 48 to 1536 were tested to look for the best association between MAT titre and shedding, and the model with the best fit as measured by the lowest Akaike information criterion was selected. When the presence of leptospirosis on a farm could not be confirmed by the observation of seroconverting animals, the risk of false positive PCR results was increased, so the PCR results for this flock on this date were not included in the model.

3.5.5.6. Animals lost to follow-up

As the number of animals present at each sampling date varied greatly, the effect of the loss to follow-up on the prevalence and mean titre was assessed by comparing the seroprevalence at titre 48 and the GMT between animals that remained in the study, animals that were culled, and animals that were missing for an unknown reason on the following sampling date. Animals missing for unknown reasons included unreported culling and deaths. The seroprevalence was compared using Fisher's exact test, stratified by farm and date. The GMT was compared using a 2-sided t-test at the log level, stratified by farm and date.

3.5.5.7. Statistical packages

The data were stored in a Microsoft Excel spreadsheet, and all the analyses were conducted in R v 3.0.3 (R Development Core Team, 2014; R Foundation for Statistical Computing, Vienna, Austria) using packages "pscl" v1.04.4, "lme4" v1.1-5 and "ggplot2" v0.9.3.1.

3.5.6. Animal Ethics

This study was approved by the Massey University Animal Ethics Committee under protocol 11/40.

3.6. Results

3.6.1. Descriptive analysis: seroprevalence and GMT

Farms A to G all had seropositive animals at ages 1-6 weeks at the beginning of the study, when seroprevalence ranged from 2.8% to 75.6% and from 0% to 15.4% for Hardjo and Pomona (including animals with dual titres), respectively (Table 3-2).

Hardjo seroprevalence, either alone or as a co-infection with Pomona, subsequently decreased to less than 5% at the second (2-3 months of age) or third sampling (6-8 months of age) on all but farm F where the seroprevalence for Hardjo remained above 10% for at least 14 months, the end of the study. From the fourth sampling onwards, all farms had a high Hardjo seroprevalence, reaching at least 80% when dual infections were included. The GMT for Hardjo ranged from 41 to 162 at the first sampling and decreased to less than 60 at the second sampling on all but farm F where it increased to 553. The observed maximum Hardjo GMT ranged from 400 to 1935. Among the 769 animals that remained present throughout the study, between zero and 15 animals per farm, for a total of 35 sheep across all farms never presented a Hardjo titre ≥ 48 after exposure started on the farm.

The seroprevalence for Pomona, including dual seroprevalence, rose above 10% on three farms (B, C and H), observed in animals aged 8–14 months, and the maximum prevalence ranged 20.8-54.1% when including dual infections. The GMT for Pomona ranged 24-96 at the first sampling. The observed maximum Pomona GMT on those farms ranged 166-768. Among the 769 animals that were present throughout the study, between 42 and 126 animals per farm for a total of 587 sheep across all farms never presented a Pomona titre ≥ 48 after exposure started on the farm.

Table 3-2: Date of blood sampling, approximate age, number of sheep sampled on eight farms and total number and proportion seropositive (microscopic agglutination test titre ≥ 48) for *Leptospira borgpetersenii* serovar Hardjo only, for *Leptospira interrogans* serovar Pomona only and both Hardjo and Pomona, geometric mean titre (GMT) for Hardjo and Pomona and p value of a t-test comparing Hardjo and Pomona GMT.

Farm	Date/s*	Approx. age (weeks)	Number sampled*	Number seropositive (seroprevalence %)						GMT [95% CI]			
				Hardjo only		Pomona only		Hardjo and Pomona		Hardjo	Pomona		
A	1/10/2011	1	194	122	(62.9)	1	(0.5)	3	(1.5)	162	[134-197]	73	[29-183]
	15/12/2011	12	182	9	(4.9)	0	(0)	0	(0)	29	[26-33]	NA	
	02/08/2012	45	171	28	(16.4)	6	(3.5)	4	(2.3)	856	[525-1395]	41	[35-48]
	01/11/2012	58	106	83	(78.3)	0	(0)	0	(0)	308	[257-369]	NA	
	03/04/2013	79	108	82	(75.9)	0	(0)	3	(2.8)	434	[370-509]	32	[25-43]
B	22/11/2011	6	210	27	(12.9)	2	(1.0)	0	(0)	69	[51-93]	34	[23-50]
	17/01/2012	14	192	0	(0)	4	(2.1)	0	(0)	NA		38	[29-51]
	30/05/2012	33	176	6	(3.4)	20	(11.4)	3	(1.7)	946	[365-2447]	665	[384-1150]
	05/12/2012	60	141	78	(55.3)	1	(0.7)	59	(41.8)	408	[353-471]	104	[85-127]
	16/04/2013	79	136	72	(52.9)	2	(1.5)	58	(42.6)	301	[257-352]	82	[68-100]
	30/07/2013	94	139	57	(41.0)	14	(10.1)	57	(41.0)	160	[134-192]	100	[82-121]
	04/12/2013	112	122	59	(48.4)	4	(3.3)	56	(45.9)	221	[187-261]	71	[60-84]
	14/01/2014	118	98	46	(46.9)	5	(5.1)	41	(41.8)	252	[198-320]	76	[61-96]
C	17/10/2011	3	207	100	(48.3)	10	(4.8)	22	(10.6)	71	[62-81]	55	[44-69]
	01/12/2011	10	205	94	(45.9)	0	(0)	0	(0)	58	[50-67]	24	[NA]
	26/04/2012	31	188	0	(0)	1	(0.5)	0	(0)	NA		26	[22-32]
	06/11/2012 + 23/11/2012	58	63+109	137	(79.7)	0	(0)	32	(18.6)	293	[264-326]	166	[105-262]
	28/03/2013	79	77	61	(79.2)	0	(0)	16	(20.8)	436	[372-510]	127	[75-214]
D	30/09/2011 + 06/10/2011	6	79 + 102	5	(2.8)	2	(0.8)	0	(0)	41	[26-64]	24	[24-24]
	24/11/2011	14	164	2	(1.2)	1	(0.6)	0	(0)	36	[21-63]	192	[NA]
	27/03/2012	31	161	2	(1.2)	3	(1.9)	0	(0)	152	[25-933]	48	[NA]

	09/08/2012	51	158	12	(7.6)	9	(5.7)	0	(0)	1935 [1315-2847]	51 [40-66]
	18/12/2012	69	111	95	(85.6)	0	(0)	2	(1.8)	572 [459-712]	76 [15-390]
E	25/10/2011	3	217	164	(75.6)	0	(0)	1	(0.5)	109 [94-125]	96 [NA]
	19/12/2011	11	207	35	(16.9)	0	(0)	0	(0)	34 [31-38]	NA
	02/05/2012	30	197	2	(1.0)	0	(0)	0	(0)	272 [9-8107]	NA
	04/04/2013	79	134	124	(92.5)	0	(0)	6	(4.5)	344 [295-400]	30 [25-35]
	18/07/2013	94	136	127	(93.4)	0	(0)	5	(3.7)	400 [347-462]	36 [27-49]
F	28/10/2011	4	190	35	(18.4)	0	(0)	0		90 [65-125]	NA
	20/12/2011	11	169	18	(10.70)	0	(0)	0		553 [318-962]	NA
	03/05/2012	31	163	31	(19.0)	5	(3.1)	2	(1.2)	484 [338-692]	52 [38-71]
	20/11/2012 + 07/12/2012	59	111 + 45	107	(68.6)	4	(2.6)	2	(1.3)	409 [345-483]	30 [25-37]
	24/01/2013	69	52	47	(90.4)	0	(0)	5	(9.6)	239 [208-274]	54 [41-70]
G	19/10/2011 + 7/11/2011	2	70 + 45	26	(22.6)	5	(4.3)	0	(0)	57 [45-73]	63 [29-139]
	12/12/2011	10	114	17	(14.9)	0	(0)	0	(0)	52 [39-70]	NA
	10/04/2012	27	111	0	(0)	1	(0.9)	0	(0)	NA	96 [6-1453]
	01/08/2012	43	111	3	(2.7)	1	(0.9)	0	(0)	110 [23-531]	38 [19-75]
	14/11/2012 + 19/11/2012	58	78 + 14	75	(81.5)	1	(1.1)	1	(1.1)	306 [231-406]	68 [54-87]
	05/02/2013	70	68	65	(95.6)	0	(0)	0	(0)	534 [453-631]	24 [NA]
H	12/02/2013	23	198	3	(1.0)	8	(2.7)	0	(0)	48 [48-48]	35 [27-46]
	20/03/2013	28	177	0	(0)	1	(0.6)	0	(0)	24 [NA]	768 [NA]
	16/05/2013	36	169	5	(3.0)	5	(3.0)	0	(0)	59 [31-111]	43 [43-81]
	01/07/2013	42	173	19	(11.0)	27	(15.6)	5	(2.9)	620 [343-1122]	368 [271-499]
	18/12/2013	67	109	48	(44.0)	0	(0)	59	(54.1)	386 [324-461]	254 [185-348]

*Where sheep were selected on two occasions, both dates and number of lambs selected per occasion are given

NA: Not applicable

3.6.2. Titre pattern with age

The predicted mean log titres for Hardjo with increasing age within each farm are presented in Figure 3-2. The shapes of the curves are similar across farms. On all farms the mean predicted titre decreased between five and 10 months of age, but subsequently reached a maximum at around 15 months. Average titres on farm B began to decline from about 18 months and continued to decline by around one dilution over one year. No decline was observed on other farms, although the sampling period was shorter on those farms. Sampling on farm H started at about six months of age, when the average titre was close to zero.

The predicted mean log titre for Hardjo for the first two years of life for the sheep across all farms is presented in Figure 3-3. The average log titre started from up to three in animals aged 1-3 months (spring), and decreased through summer to zero at around five months-of-age (early autumn). The increase of the average log titre, indicating seroconversion, was observed between eight and 17 months of age, mainly through winter and spring. The average titre reached a maximum at around 17 to 22 months of age, and then starting to decrease until the last observation at 28 months of age. The maximum predicted average log titre was around 5 (1:384), with individual farm prediction reaching 6 (1:768) for farm G.

The predicted mean log titre for Pomona with increasing age within each farm is presented in Figure 3-4. The predicted mean log titre showed different patterns across farms, with a marked increase only on farms B, C and H. The increase in average titre started at varying ages between farms: around five months on farm B, and 10 months on farms C and H. The highest predicted maximum average log titre, 2.5 (corresponding to a titre of 68), was on farm H. The average log titre stopped rising from around 20 months on farm B, and remained elevated for the duration of sampling.

The predicted mean titre for Pomona for the first 2 years of life for the sheep across all farms is presented in Figure 3-5. The mean log titre was close to zero on most farms at docking time in spring, apart from farm C where it reached 0.5 (corresponding to a titre of 17). An increase in average log titre was observed on farms B, C and H. The beginning of this increase started in autumn and up to the following spring, in animals 4-14 months of age. No clear maximum or subsequent decline was observed in the overall smoothed prediction. The maximum predicted average log titre was around 1.5 (corresponding to a titre of 34) at the end of the study in 27-month-old ewes, with the individual farm maximum reaching 2.5 (corresponding to a titre of 68) on farm H.

The ICC of the residuals within sheep showed that no further correlation was present for either Hardjo or Pomona models.

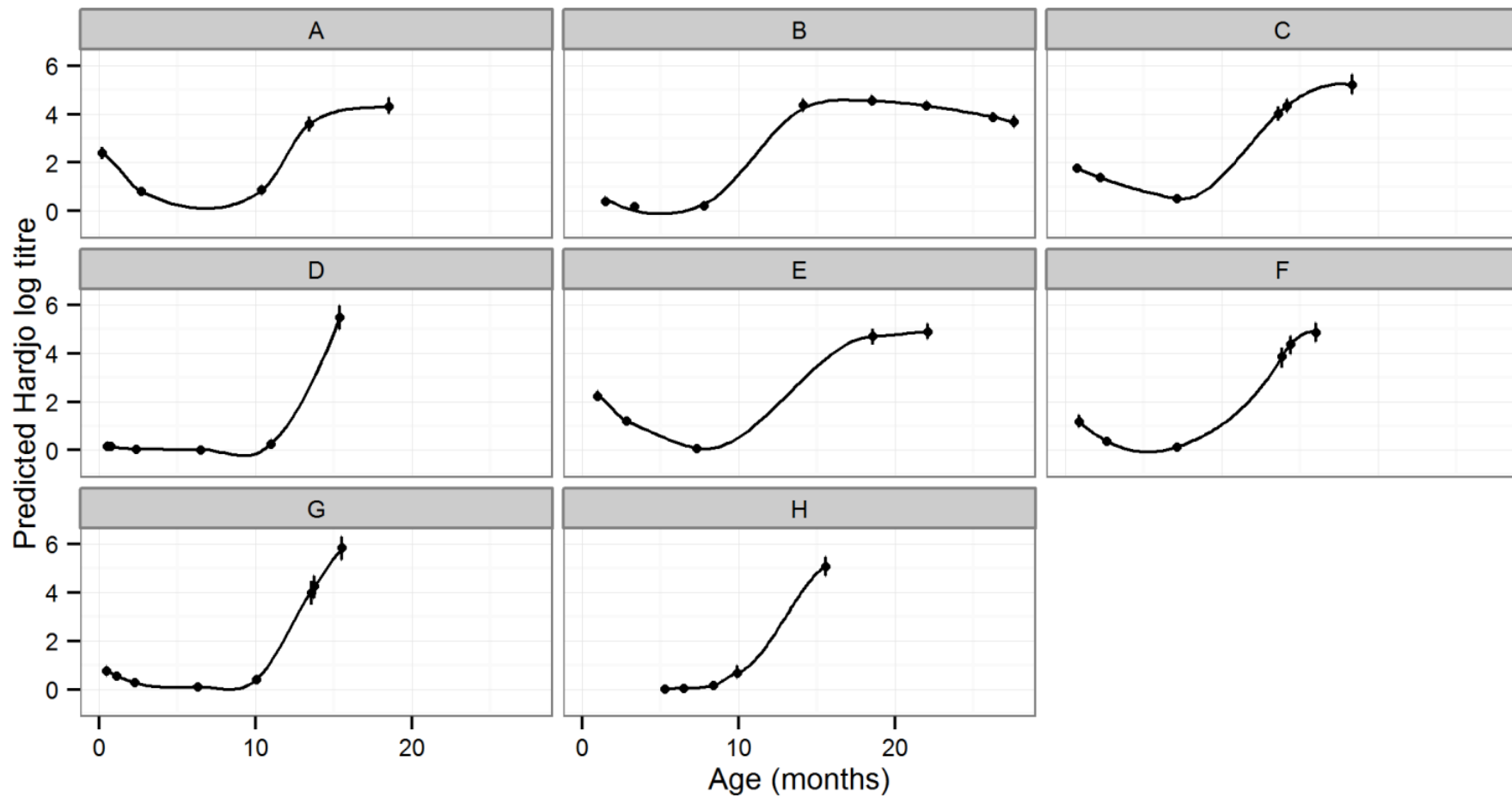


Figure 3-2: Predicted mean log titre for *Leptospira borgpetersenii* serovar Hardjo as a function of sheep age on eight different farms (A–H), with Loess smoothing and bootstrap 95% CI around predicted values.

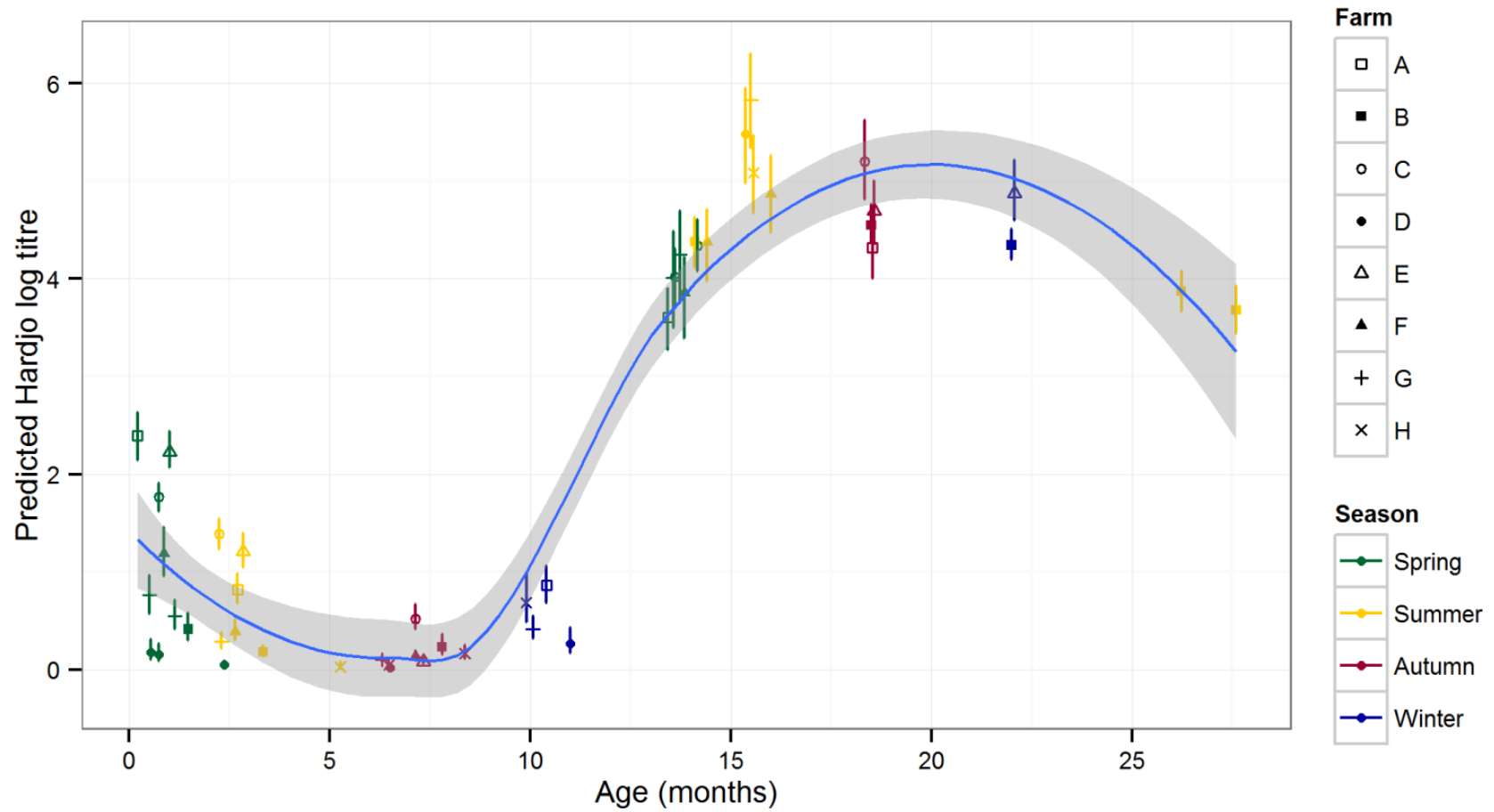


Figure 3-3: Predicted mean log titre for *Leptospira borgpetersenii* serovar Hardjo including all farms as a function of sheep age, with Loess smoothing and bootstrap 95% CI around predicted values

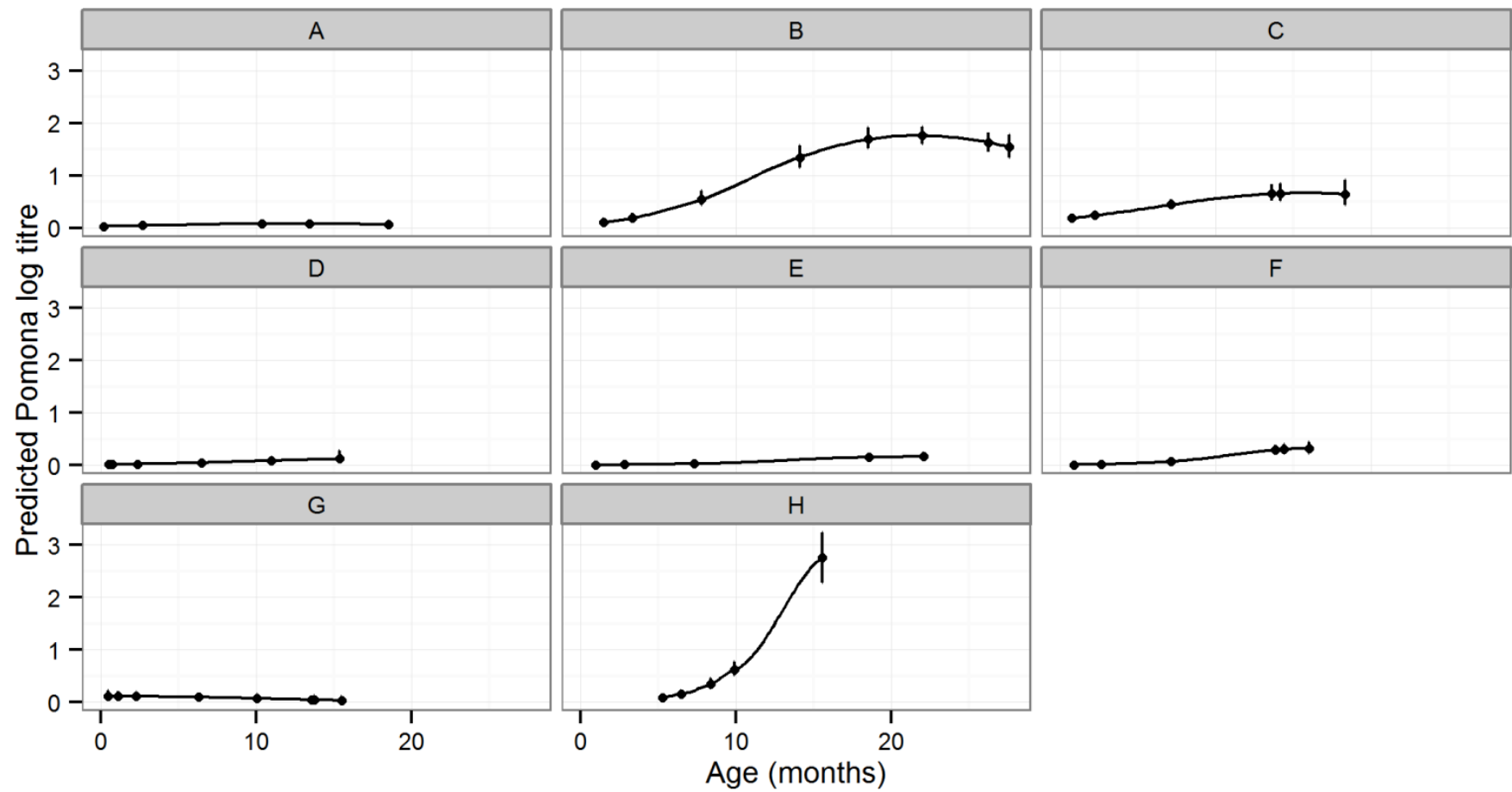


Figure 3-4: Predicted mean log titre for *Leptospira interrogans* serovar Pomona as a function of sheep age on eight different farms (A–H), with Loess smoothing and bootstrap 95% CI around predicted values

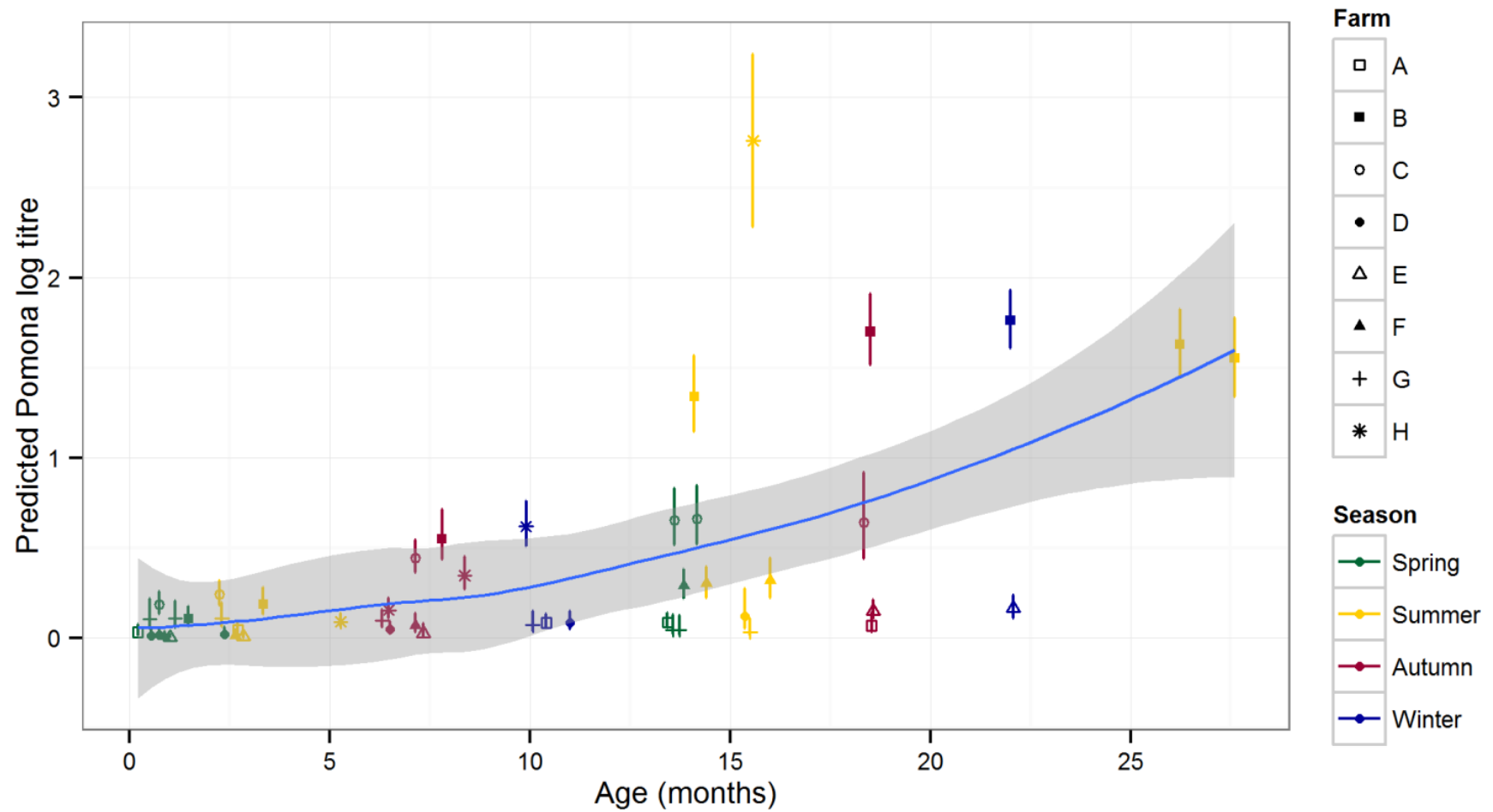


Figure 3-5: Predicted mean log titre for *Leptospira interrogans* serovar Pomona including all farms as a function of sheep age, with Loess smoothing and bootstrap 95% CI around predicted values

3.6.3. Titre decay and antibody half-life

There were 185 sheep from farms A-H with titres 768 or 1536 for Hardjo, and 21 sheep from farms B, C and H with titres 768 to 1536 for Pomona. Between one and four serology results per animal were available over periods of 1–14 months after the peak titre for Hardjo. One to five serology results were available over periods of two to 20 months after the peak titre for Pomona. Adding farm as a fixed-effect in the model did not significantly improve the fit and did not change the regression coefficient or its standard error, thus showing that adjustment for clustering within farm can be omitted. The models were thus kept as simple linear regressions with only time (months) as the variable of interest. The regression coefficients are presented in Table 3-3. The log titre for Hardjo declined by 0.15 each month for the 14 month period and for Pomona by 0.16 for the 20 month period, assuming a constant decrease. The corresponding estimated half-life was 6.7 [95% CI: 5.8-7.9] months for Hardjo antibodies and 6.3 [95% CI: 4.8-9.0] months for Pomona antibodies. Hence, the antibody duration depends on half-life and titre value at peak (Figure 3-6).

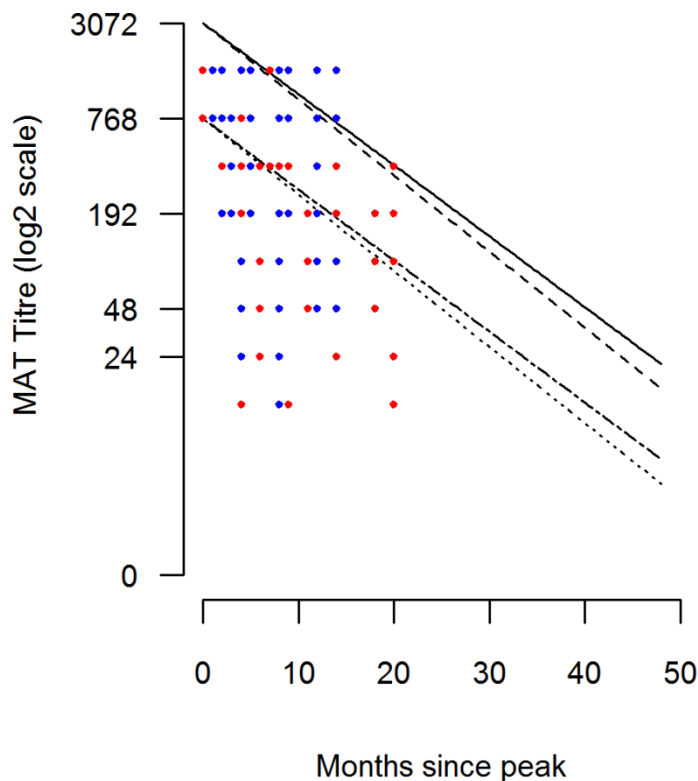


Figure 3-6: Predicted linear regressions of log titre for *Leptospira borgpetersenii* serovar Hardjo (plain line with peak at 3,072 and dot-dash line with peak at 768) and *L. interrogans* serovar Pomona (dashed line with peak at 3,072 and dotted line with peak

at 768), as a function of time in months after a peak in naturally infected sheep. The blue dots are the observed Hardjo titres and the red dots the observed Pomona titres.

Between 0 and 9 animals per farm, for a total of 18 sheep across all farms had a Hardjo titre <48 at the final sampling but presented a titre ≥ 48 that could not be attributed to maternally-derived antibodies at least once. The maximum titre of these animals ranged 48-3072. Between 1-9 animals per farm, for a total of 35 sheep across all farms had a Pomona titre <48 at the final visit but presented a titre ≥ 48 that could not be attributed to maternally-derived antibodies at least once. The maximum titre of these animals ranged 48-768.

Table 3-3: Linear regression coefficients and estimated half-life of MAT-titres for Hardjo and Pomona

Hardjo			
	Estimate	Standard error	p
Intercept	6.023	0.073	$<10^{-15}$
Time (months)	-0.149	0.012	$<10^{-15}$
Adjusted R ²	0.235		
Titre half-life (months)	6.7 [5.8-7.9]		
Pomona			
	Estimate	Standard error	p
Intercept	5.642	0.269	$<10^{-15}$
Time (months)	-0.160	0.024	$<5.81 \times 10^{-9}$
Adjusted R ²	0.353		
Titre half-life (months)	6.3 [4.8-9.0]		

3.6.4. Shedding in urine

Urine from 17 animals from farms D and G was collected before the observation of seroconversion on the farm and were thus not included in the model. All 17 were real-time PCR negative, and the GMT for Hardjo and Pomona was zero on both farms. Results for the prevalence of leptospiral shedding in urine for the remaining farms are presented in Table 3-4.

On farms where seroconversion was observed, 50 of the 81 sheep urine samples were real-time PCR positive. The within-farm shedding prevalence ranged from 11% to 88%. Among these PCR positive animals, 1(2%) was seronegative for both Hardjo and Pomona. Twenty two of 31 PCR negative sheep were seropositive for Hardjo. All animals with a Pomona titre also had Hardjo titre, and the animals seropositive for Pomona and PCR positive were all from farm H. Three animals from farm B were seropositive for Pomona and none were shedding (Table 3-4).

Sheep that were positive for shedding of leptospire in urine were observed between 0 and 8 months after exposure was observed on the farm, as defined for this

study by the date when seroprevalence rose above 10% after having decreased to less than 5%, apart from farm F where the animals were considered to have been exposed from the beginning of the study.

The results of the logistic regression model examining the relationship between titre and probability of urine shedding are presented in Table 3-5. The model using titres ≥ 48 provided the best fit of the data, showing that the real-time PCR results were best explained when 48 was used as the lower limit for the definition of seropositivity. The animals with a Hardjo titre ≥ 48 only, or both Hardjo and Pomona titres ≥ 48 , were more likely to be real-time PCR positive than seronegative animals, with OR=52.8 (95% CI=7.5-1108.6) and OR=21.0 (95% CI=2.0-538.5) respectively.

1.1.1. Animals lost to follow-up

Among the 1523 ewe lambs enrolled, 769 were still present at the end of the study, 304 had been reported as culled by the farmer, and 450 were missing for unknown reasons. The prevalence of lambs that were seropositive for serovar Hardjo, and the GMT for serovars Hardjo and Pomona, on farms where there was a statistically significant difference between animals that were retained in the study and those lost to follow-up are presented in Table 3-6.

No difference in Pomona seroprevalence was observed on any farm between lost to follow-up animals and those remaining in the study. No difference in GMT for Pomona was observed between retained and lost to follow-up sheep on farms A, D, E, F and G, which were those where there was no increase in predicted mean titre for Pomona with age. No difference in Pomona GMT was observed on farm H, where there was an increase in predicted mean titres with age (Figure 3-4).

There was no difference in seroprevalence and GMT for Hardjo and Pomona between the animals known to have been culled for impaired productivity and the animals that remained in the study.

Table 3-4: Number of sheep urine real-timePCR positive and seropositive for Hardjo and Pomona

Farm	Sampling date	No. sampled	Hardjo serology						Pomona serology			
			Number PCR positive	Months since farm exposure ^a	Number positive at ≥48	GMT [95% CI]	Number PCR negative	GMT [95% CI]	Number positive at ≥48	GMT [95% CI]	Number positive at ≥48	GMT [95% CI]
A	1/11/2012	8	7	3	7/7	384 [212-695]	1/1	768 [NA]	0/7	0	0/1	0
B	30/07/2013	9	2	14	2/2	96 [25-373]	6/7	242 [154-380]	0/2	24 [NA]	3/7	610 [246-1508]
C	6/11/2012	4	3	0	3/3	242 [154-380]	1/1	48 [NA]	0/3	0	0/1	0
D	9/08/2012	5	0	NA ^b	NA ^b	NA	0/5	0	NA ^b	NA ^b	0/5	0
E	18/07/2013	9	1	4	1/1	192 [NA]	8/8	384 [246-599]	0/1	0	0/8	24 [NA]
F	20/11/2012	12	5	6	4/5	146 [53-402]	1/7	96 [NA]	0/5	0	0/7	24 [24-24]
G	1/08/2012	12	0	NA ^b	NA ^b	NA	0/12	0	NA ^b	NA ^b	0/12	0
G	14/11/2012	16	14	0	14/14	848 [555-1296]	1/2	192 [NA]	0/14	0	0/2	0
H ^c	13/08/2013	15	13	1	9/9	1536 [926-2548]	1/2	1536 [NA]	7/9	517 [121-2199]	0/2	24 [NA]
H ^c	18/12/2013	8	5	5	3/3	768 [351-1683]	3/3	610 [388-959]	1/3	1536 [NA]	2/3	272 [9-8107]
Total		98	50	0-14	43/44	552 [394-773]	22/31	328 [230-469]	8/44	312 [84-1160]	5/31	121 [36-401]

^a Months since the prevalence rose above 10% for either Hardjo or Pomona on the farm

^b The urine was collected before the observation of seroconversion

^c Not all animals were blood sampled

Table 3-5: Coefficients of the mixed logistic regression with real-time PCR status of exposed sheep as the outcome, farm as a random effect and using 1:48 as a cut-off for seropositivity

Fixed effect	β	Standard error of β	p
Intercept	3.19	1.60	0.047
Age (months)	-0.39	0.09	<0.001
Hardjo alone	3.97	1.17	<0.001
Hardjo and Pomona	3.04	1.54	0.022
Random effect: Farm	Variance=0		

Table 3-6: Significant or marginally non-significant ($p < 0.1$) difference in Hardjo seroprevalence (titre ≥ 48) and GMT and Pomona GMT between animals subsequently retained in the study (retained) and lost to follow-up (lost), and associated p-value for Fisher's exact test or t-test

	Farm	Date when loss to follow-up recorded	Remained	Lost	p-value
Hardjo seroprevalence (titre ≥ 48)	F	Nov-Dec 2012	66/105 (63%)	45/53 (85%)	0.005
	G	Dec 2012	1/3 (33%)	65/68 (96%)	0.01
	H	July 2013	4/15 (27%)	9/106 (8%)	0.06
Hardjo GMT	A	Aug 2012	6.6	2.0	<0.001
	C	Nov 2012	4.8	4.5	0.09
	F	Nov 2012	5.4	4.9	0.07
Pomona GMT	B	May 2012	4.7	7.0	0.006
		Jul 2013	2.9	3.9	0.04
		Dec 2013	2.5	3.0	0.007
	C	Dec 2011	1.0	2.2	<0.001
		Nov 2012	3.8	4.8	0.08

3.7. Discussion

Our results provide what is, to our knowledge, the first detailed study of the within-farm prevalence and longitudinal exposure pattern to *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona on a sample of sheep farms in New Zealand.

While flocks contained sheep vaccinated for leptospirosis, the impact of vaccination on the epidemiology of infection would have been small if any. This is because the number of lambs vaccinated represented between 0.9 – 5.2% of the adult sheep population, and therefore less than half those proportions of total lamb and ewe number. It was not possible to accurately estimate the proportion of vaccinated animals within each management group because group size and allocation of trial animals

changed according to management requirements. Nevertheless, the proportion would invariably have been small.

Monitoring of MAT titres in sheep for up to 27 months revealed a pattern of Hardjo seroconversion that was similar on all farms. The animals were first exposed when 8-17 months old which seasonally coincided with a period from the end of winter to early summer. This timing concurs with that reported by Dorjee *et al.* (2008) showing an increase in seroprevalence between July and November. It confirms that hoggets are generally more likely to be Hardjo seropositive than lambs, as previously shown by Dorjee *et al.* (2005). However, while that seems to be the usual pattern, variation does exist, since early Hardjo infection of 3-month-old lambs was observed on farm F. Exposure to Pomona started at a similar age (4-14 months), but titres increased at a lower rate and steadily over the entire observation period of about two years.

Seasonality of infection could be related to the ecology of *Leptospira* and a wetter climate in winter and spring. Animal and human outbreaks have been associated with climatic conditions that are favourable for *Leptospira* survival in the environment, such as heavy rains and floods (Hartley 1952; Miller *et al.* 1991; Vermunt *et al.* 1994; Dorjee *et al.* 2008; Hartskeerl *et al.* 2011). It has been demonstrated that winter provides good conditions for survival of Pomona in soil and environmental transmission (Hellstrom and Marshall 1978). However, the different exposure patterns for Hardjo vs. Pomona suggest that ecological determinants (e.g. survival, shedding, host preference) may be serovar specific. Maternal antibodies for serovar Pomona were observed on only one farm and on six farms for Hardjo. The within-farm seroprevalence rose above 10% on only three farms for Pomona but reached 80% on all farms for Hardjo, contributing to evidence of a different ecology between these two serovars.

Outside of New Zealand, reports of leptospirosis in sheep are often associated with clinical manifestations and link infection and clinical disease with rainfall. Hardjo has been reported in USA to infect and be excreted by six- to nine-month-old lambs in winter in association with reports of flooded pastures, and possibly causing clinical disease (Schmitz *et al.* 1981). In Canada, Pomona was reported in lambs as young as two months at the end of winter, with observation of “exceptional amounts of rainfall in the preceding 3 months”, associated with a seroprevalence of 16% in an adult flock (Smith and Armstrong 1975). However, prevalence studies in animals less than one year old are rarely available. In England and Wales, 24% of flocks were reported to be exposed to Hardjo but the regional seroprevalence varied greatly between 11% and 55%. The within-flock prevalence ranged from 5% to 95% (Hathaway *et al.* 1984). However, the lack of information, especially in older studies, about the infecting genomospecies makes the comparison of effects between countries difficult.

In Brazil, the reported sheep-level seroprevalence ranged from 5% in semi-arid regions (Alves *et al.* 2012) to 32-47% in tropical regions (Martins *et al.* 2012, de Carvalho *et al.* 2014) and the flock level seroprevalence from 28% in semi-arid regions

to 81% in tropical regions. Hardjo was reported as one of the most frequent serovars in tropical regions, along with Grippotyphosa and Icterohaemorrhagiae. Pomona was also reported in these areas, but with prevalence less than 5%. Some authors suggest that Hardjo infection in sheep is independent of season and rainfall (Melo *et al.* 2010, Martins and Lilenbaum 2014). In semi-arid regions, the most prevalent serovars in sheep are Autumnalis, Andamana and Sentot, which are non-adapted serovars and likely cause sporadic infection. In Trinidad (Suepaul *et al.* 2011), with a tropical climate, the seroprevalence was estimated at 5% with Icterohaemorrhagiae and Autumnalis being the only serovars reported, and the seroprevalence was not associated with the age of the animals, suggesting sporadic infection. However these studies were based on serological evidence only and isolation would be needed to confirm the serovar repartition. Pomona is rarely reported and isolated in sheep worldwide (Martins and Lilenbaum 2014).

Co-grazing of hoggets in their first gestation with other reservoir species can also be suggested as a risk factor for exposure, as has been observed for deer (Subharat *et al.* 2012b). Conflicting evidence exists on the effect of co-grazing sheep with other species on Hardjo seropositivity. In Brazil, Genovez *et al.* (2011) showed that grazing sheep with cattle increased the risk of being positive for Hardjo and in the United Kingdom Hathaway *et al.* (1984) suggested that sheep flocks were more likely to be Hardjo positive when the cattle density increased, although that relationship was not confirmed statistically. On the other hand, evidence for transmission across species was not observed in a multi-species prevalence study in New Zealand by Dreyfus (2013). However, the seroprevalence observed in this study was high (97% of seropositive sheep flocks), thus limiting the power of the study to find significant risk factors. A change of wildlife reservoir behaviour, bringing them closer to pasture (Eymann *et al.* 2007) is also plausible although the present *Leptospira* status and serovars distribution in wild animals in proximity to livestock in New Zealand is unknown.

The within-farm Hardjo prevalence rose from less than 5% to 80-100% over a ten-month period, suggesting a high infection pressure, probably exposure to a common source of infection such as contaminated water source, and not only animal to animal transmission (Thrusfield 2005). The within-farm prevalence was higher in our study than observed by others in New Zealand, mostly less than 40% (Blackmore *et al.* 1982; Dorjee *et al.* 2008; Dreyfus 2013), higher than that observed in UK by Hathaway *et al.* (1984) with *L. interrogans* serovar Hardjo type Hardjoprajitno, but consistent with the upper values of within-line prevalence found in New Zealand by Fang *et al.* (2014a). However, those studies were conducted on fewer animals of a different age, including mixed-age sheep which could have seroconverted and reverted to non-detectable titre levels over time.

A recent New Zealand abattoir survey (Fang *et al.* 2014) found a higher Hardjo seroprevalence in lambs aged four months or less (49%) than 13-16 month-old hoggets (37%) and mixed age ewes (19%) (F. Fang, personal communication¹). These findings are consistent with what was observed in the present study in lambs under three months of age. Farms had 13% to 76% of the 2-6-week-old lambs with a MAT titre ≥ 48 at the first sampling at tail-docking. Apart from farm F, seroprevalence on all farms subsequently dropped to under 5%, suggesting that these early titres were due to maternal antibodies. These likely maternal antibody titres were observed for up to three months in some lambs, hence longer than suggested by previous studies (Andreani *et al.* 1983), but less than the six months observed in cattle (Hellstrom 1978). Is it still unclear whether these apparently maternally-derived antibodies are protective against infection and whether the presence of newly acquired agglutinating antibodies is directly correlated with protection against infection, as has been suggested in cattle (Zuerner *et al.* 2011). The observed trend on farms also suggests that from spring to autumn, lambs were either protected or not exposed. The rapid seroconversion in late autumn to early summer suggests that either flock immunity was poor or exposure suddenly increased during this period.

The pattern of seroconversion on farm F was different to that on other farms, with the first Hardjo seroconversions observed between tail docking in spring and weaning of 3-month-old lambs in summer, as shown by a higher Hardjo GMT at weaning than on the other farms. However, the seroprevalence at that time was low (10%) and was less than 20% in the following autumn. Hence, most seroconversion occurred at the same period as on the other farms, between late autumn and early summer.

A similar seasonal trend for the first exposure was also present for Pomona. Seroconversion was observed only on three farms, and exposure started from autumn to spring. The seroprevalence in lambs aged four months or less was less than 5% apart from one farm where it reached 15% before declining to under 5%. This differs from that observed by Fang *et al.* (2014) in an abattoir study, where lambs up to four months old had a seroprevalence of 35%. The spring seroprevalence observed in 13-16-month-old hoggets and mixed-age ewes concurred with the present study (37% and 36%, F. Fang, personal communication). To our knowledge, little has been published on the seroprevalence of serovar Pomona in different age groups of sheep or on maternally derived immunity for this serovar.

The within-farm seroprevalence for Pomona was between 20% and 50% where present, and mostly as a dual Hardjo-Pomona infection. Seroprevalence was less than for Hardjo, and varied between farms as observed in earlier studies (Blackmore *et al.* 1982; Dorjee *et al.* 2008; Dreyfus 2013). Maternal antibodies for Pomona were

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observed on only one farm and on six farms for Hardjo. The within-farm seroprevalence rose above 10% on only three farms for Pomona and but reached 80% on all farms for Hardjo, contributing to evidence of a different ecology between these two serovars.

Leptospira serovars Hardjo and Pomona, which belong to different serogroups, are not known to cross-react in the MAT, which was shown to have an overall serogroup specificity of 75% in the convalescent phase, with high variability between serogroups (Levett 2003). Among the six serovars known to be endemic in New Zealand (Marshall and Manktelow 2002), only Hardjo and *Leptospira interrogans* serovar Balcanica can potentially cross-react (Hathaway and Marshall 1980), but the latter is adapted to brushtail possums (*Trichosurus vulpecula*) and is expected to cause only sporadic infection in sheep (Mackintosh et al. 1981). Cross-reaction between serogroups and paradoxical reactions are also possible during the acute phase (Levett 2001), but the low number of animals with a titre ≥ 48 that became negative by the end of the study suggests that this situation was rare or absent. Hence several observations of both Hardjo and Pomona positive MATs in the same animal confirm dual infection.

Since farms in this study were not selected randomly, they may not be fully representative of New Zealand commercial sheep farms, and more work would be necessary to know how frequently early exposure similar to that observed in farm F happens. Further, this study was largely conducted in 2011-2012, hence most farms experienced only one complete yearly weather pattern. As environmental transmission is probable and influenced by wet weather, the exposure pattern is susceptible to variation between years. A longitudinal study conducted by Subharat (2010) showed that 62% of replacement flocks maintained the same Hardjo status (seropositive or seronegative) and 77% the same Pomona status across the three study years. The within-flock seroprevalence was relatively constant between years for Hardjo but differed between years for Pomona, supporting the proposition that the epidemiology of leptospirosis in sheep flocks varies from year to year and between serovars. The exceptionally dry summer in early 2013 could also have affected re-exposure of the animals and influenced the seroprevalence observed at the last samplings.

No clear cut-off currently exists to differentiate acute infection from past infection, or maternal antibodies from infection in lambs. A cut-off of 48 has been suggested to detect past exposure (Blackmore, 1982). In this study, using this cut-off in lambs would have led to misclassification as infected in all the farms due to maternal antibodies. However, the GMT for Hardjo was less than 96 on all the farms except farm A at the first sampling. On the second sampling the GMT was still less than 96 on all the farms except farm F, where seroconversion was happening. On some farms some lambs still had maternal antibodies for Hardjo at three months of age. This shows that no clear cut-off can be defined at an individual level, but using flock level data can give evidence of maternal immunity rather than exposure of lambs.

The mean predicted Hardjo titre peaked at around 20 months-of-age, and the observed GMT peak varied between 400 and 1935. This peak occurred later than the maximum observed by Blackmore (1982) who found a higher GMT in lambs less than 12 months of age than in hoggets (above 12 months old in this publication). Observed titres to serovar Pomona showed no consistent pattern with increasing age after the initial exposure due to lower seroconversion rates and not all the farms being infected with Pomona. The average predicted log titre by farm did not show any age related peak either. The observed GMT peak for Pomona varied between 166 and 665. The average titre was lower for Pomona than for Hardjo, as observed earlier (Blackmore *et al.* 1982; Dreyfus 2013). The observed peak average Pomona titre was coincidental with the observation of seroconversion, suggesting that the peak titre occurs rapidly after exposure. Fang (2014) reported that the peak titre occurred within 2 weeks after experimental infection with Hardjo or Pomona.

The age at exposure was consistent with the model of Hathaway (1981) for leptospirosis circulation in maintenance hosts, with young animals being infected before joining the breeding flock and thus potentially achieving protective immunity, before the risk period for reproductive loss, but still during the risk period for suspected reduced growth in lambs exposed to Pomona (Webster and Reynolds 1955). However, hogget breeding is becoming more popular in New Zealand, putting hoggets at risk of reproductive sub-performance due to Hardjo and Pomona exposure during their first pregnancy (Ellis *et al.* 1983, Leon-Vizcaino *et al.* 1987, West *et al.* 2004, Bruere 2013).

Significant differences in Hardjo GMT and seroprevalence between animals retained in the study and animals lost to follow-up occurred occasionally, but the size of the difference was not consistent and this was observed when sample sizes were low. Hence, these differences were likely due to a statistical type I error. It is thus unlikely that missing animals affected the Hardjo seroprevalence or GMT estimates. However, animals lost to follow-up likely caused an underestimate of the seroprevalence, GMT and transmission for Pomona, as they had a significantly higher GMT for this serovar on several occasions on two farms. This result suggests that Pomona may be associated with increased culling or mortality rates in lambs and hoggets, though this needs to be substantiated.

The within-farm urinary shedding prevalence ranged from 11% to 88%. Two of the nine animals sampled on one farm eight months after Hardjo and Pomona titres ≥ 48 were first observed were still shedding, confirming that leptospiral shedding in sheep can be persistent. All but one PCR positive animals were seropositive for Hardjo and none was seropositive for Pomona only. This strongly supports the presence of Hardjo shedding but no inference can be made about Pomona shedding based on PCR and MAT only, without confirmation of serovar by culture. The shedding prevalence was higher than the zero prevalence in urine and 20% (3/15) in kidneys from one of 54 lines observed 30 years earlier (Blackmore *et al.* 1982), or than the 21% of kidney culture rate in seropositive sheep reported more recently by Dorjee *et al.* (2008). Those two

studies used culture and dark field microscopy, so the difference could be, at least partially, due to the fact that PCR detects DNA material while culture requires live organisms, and *Leptospira* spp. are known for being difficult to grow and sensitive to transport and contamination (Smith et al. 1994). Urine shedding prevalence was also higher than the 33% observed by Fang *et al.* (2014a) who used the same real-time PCR assay as the present study but animals in that study were from different age groups including mixed-age ewes. Those authors observed a seroprevalence of 57%.

The shedding prevalence was lower on the two farms (B and E, 2/9 and 1/9, respectively) sampled during the second winter of the study, suggesting that shedding may be more frequent in ewe lambs or young hoggets than in older ewes. However, the sample size in this study was too small to provide robustness for statistical analysis. This also suggests that lambs born to hoggets would be more at risk of an early exposure. The age of the dams of the animals in this study was not available to confirm this hypothesis. More data are needed on lamb exposure, on the role of the dam's age, serological and shedding status and on the role of maternally derived immunity.

In this study one animal with a positive real-time PCR result did not have detectable antibodies for Hardjo or Pomona. Dorjee *et al.* (2008) and Fang *et al.* (2014) found that 1% (5/499) and 3% (2/71) of seronegative sheep were shedding, respectively. Shedding in seronegative goats was observed in Brazil by Hamond *et al.* (2014), where 77% of the animals were shedding yet 34% were seropositive, although those authors used a cut-off of 1:200. This higher cut-off for seropositivity could explain a weaker association between shedding and serostatus, as observed in this study where the strongest association was found at a cut-off of 48. This confirms that sheep can transmit leptospirosis without having a “diagnostic” (48) MAT titre. Shedding without Hardjo MAT titre has also been observed in cattle and deer (Subharat *et al.* 2012) which are recognised maintenance hosts for this serovar.

Sheep with a titre ≥ 48 for serovar Hardjo were more likely to have a positive real-time PCR result. This supports the findings of Fang *et al.* (2014a) who showed that the risk of shedding in Hardjo seropositive animals was 12.5 times the risk of seronegative animals. The present shedding results were obtained only on ewes 1-2 years old, with very few seronegative animals, while Fang *et al.* (2014a) also included older ewes and a lower seroprevalence. This shows that MAT can be used to predict the presence of shedding in sheep at flock level, but the test is less predictive of shedding at the individual animal level than the flock level, especially in the first year following exposure. The utility of MAT for the prediction of shedding is primarily at flock level, hence our findings support those of Fang (2014) in that MAT is a robust tool at flock level for the evaluation of the risk of infection posed to humans. Moreover, the MAT provides information on the infecting serogroup, which is not possible with the PCR tests currently available (Bezerra da Silva *et al.* 2011), without further sequencing (Perez and Goarant 2010; Subharat *et al.* 2011). The identification of shedders by PCR

is also limited by the possibility of intermittent shedding (Adler and de la Pena Moctezuma 2010).

Leptospirosis is one of the principal zoonotic public health concerns in New Zealand, with an incidence higher than in most other temperate countries (Thornley *et al.* 2002). In recent years human infection has been associated with sheep handling, especially at abattoirs (Heuer and Davies 2004, Dorjee *et al.* 2011, Dreyfus *et al.* 2014). The high seroprevalence and urinary shedding observed in the present study confirms bacterial circulation among sheep and that these animals can be a source of infection for humans.

The lack of specificity of the PCR in identifying the infecting serogroup means that the shedding sheep could actually be shedding a serovar other than Hardjo or Pomona. However, considering the high MAT seroprevalence of Hardjo, revealing a high exposure, it is very likely that the majority of urine real-time PCR positive sheep are shedding Hardjo. Our estimate of shedding prevalence is also likely to be conservative, as no specific precaution was taken to account for the possible presence of PCR inhibitors in urine causing false negatives. Nevertheless further MAT screening using a panel of other serovars is required to fully validate the range of serovars in the New Zealand sheep population.

This study provides, to our knowledge, the first estimation of half-life of agglutinating antibodies against Hardjo and Pomona in naturally exposed sheep. A half-life of 6.7 months for Hardjo and 6.3 months for Pomona would mean that for a sheep with a peak titre of 3072, it would take 47 months to become seronegative (<48) for Hardjo or 44 months for Pomona, and for a sheep with a peak titre of 384, it would take 27 months for Hardjo or 25 months for Pomona to be negative. However, on farms where both serovars were detected, the GMT was significantly higher for Hardjo than for Pomona, suggesting that the peak titre would be lower for Pomona and thus the antibodies would be detected for a shorter time than for Hardjo. Comparing these results with Fang (2014) who showed that some sheep experimentally challenged with Pomona became seronegative 42 days after infection reveals that the likely continuous re-exposure of sheep on farm, as supported by the shedding results, may play an important role in the maintenance of MAT titres. Other published work reports positive MATs for at least three months in sheep after experimental infection with Hardjo or Pomona and at least four months in sheep naturally exposed on farm to Hardjo (Hodges 1974; Andreani *et al.* 1983; Gerritsen *et al.* 1994).

This half-life estimation was calculated on animals that had an observed MAT peak of 768 to 1536 in order to capture the decrease as close as possible to the peak titre, but this could have introduced a bias due to the selection of animals with a specific immunity, possibly producing more agglutinating antibodies.

The MAT is based on the detection of agglutinating IgG and IgM (Morris and Hussaini 1974). IgM are detectable within one week after experimental infection of sheep with Hardjo (Hathaway and Marshall 1979), while IgG production peaks somewhat later. IgG are known to decrease slowly after infection and can be detected for 11 months after Hardjo infection using other serology techniques (Cousins *et al.* 1989).

Clinical disease in sheep has rarely been reported in recent studies (Dreyfus 2013) but loss to follow-up for unknown reasons was associated with a higher GMT for Pomona on two farms in this study. The frequency of exposure of young lambs to Pomona before weaning on New Zealand commercial sheep farms is unknown, but this serovar has been associated with clinical disease and mortality (Vermunt *et al.* 1994). *L. interrogans* serovar Hardjo type Hardjoprajitno has been suspected in abortions and perinatal loss in sheep (Ellis *et al.* 1983), but the precise effects of *L. borgpetersenii* serovar Hardjo type Hardjo-bovis and *L. interrogans* serovar Pomona on sheep production in New Zealand and the associated economic losses still remain to be quantified.

This study provides insight into some aspects of the epidemiology of *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona on commercial sheep flocks in New Zealand, especially on age of exposure, within-farm prevalence over time, duration of MAT titres, duration of shedding and the association between MAT antibodies and shedding. However, more studies across more farms and several years are needed to assess the effect of weather patterns and how consistent exposure is over time. Shorter sampling intervals around the periods of high risk of exposure between birth and one year of age are needed to fully understand the seroconversion pattern, assess the frequency of early exposure of lambs 4-month-old or younger and the antibody dynamics. Shedding data with short sampling interval are also needed to better estimate the risk of infection for both animals and humans.

A more detailed understanding of the epidemiology of leptospirosis due to Hardjo and Pomona in sheep along with economic analysis would allow robust and cost-effective recommendations to protect human and animal health.

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Chapter 4. Effectiveness of a commercial leptospiral vaccine on urinary shedding in naturally exposed sheep in New Zealand

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4.1. Abstract

Leptospirosis due to *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona is endemic in the New Zealand sheep flock, and is a significant human health concern in this country. An effective vaccine and vaccination strategy would protect both humans and livestock.

A total of eight farms were enrolled in research to evaluate the production effects of leptospirosis. Between four and 12 lambs were selected from each farm (total - 84) to form the vaccinated group, while between four and 16 lambs (total – 98) served as the unvaccinated control group. A commercial bivalent Hardjo/Pomona vaccine was first given to the vaccinated groups at 2-6 weeks of age, and again at 5-11 weeks and 10-18 months of age. Vaccinates and controls were grazed together. Blood was regularly collected from the control group to assess flock exposure to Hardjo and Pomona. Urine was collected from both groups six to 19 months after the second vaccination and tested by real-time PCR.

Seroprevalence at the time of first urine sampling ranged from 7.6 to 98.3% for Hardjo and from 0 to 54.1% for Pomona, showing that a strong natural challenge occurred between vaccination and urine sampling. The adjusted shedding prevalence was 45.1% (50/98) [95% CI 17.6-72.7] in the control animals and 1.8% (5/84) [95% CI 0.0-10.1] in the vaccinated animals. Data confirm that the vaccine was 100% effective in sheep on five farms where animals were vaccinated before three months of age and before natural exposure occurred, but the effectiveness was 80% [0-97] on one farm where the lambs were exposed before vaccination and 76% [29-92] on one farm where the animals were vaccinated at around six months of age. The overall vaccine effectiveness when data from all situations were pooled was 86.3% [63.6%-94.8%] despite the presence of maternal antibodies in some flocks at first vaccination and exposure prior to vaccination in some flocks. Although the presence of a serovar other than Pomona or Hardjo could not be ruled out, vaccination timing seemed to be crucial in achieving optimum reduction in shedding in urine in vaccinated sheep.

4.2. Keywords

Vaccine effectiveness, sheep, leptospirosis, shedding, Pomona, Hardjo

4.3. Abbreviations

GEE Generalized estimating equation

MAT Microscopic agglutination test

RR Risk ratio

VE Vaccine effectiveness

4.4. Introduction

Leptospirosis is widespread in New Zealand, with 97% of sheep flocks having one or more animals seropositive for serovars Hardjo or Pomona [1]. Sheep, like cattle, are reservoir hosts for serovar Hardjo and the bacteria are maintained in the kidneys [2]. They are able to shed live leptospire persistently or intermittently for at least 11 months in urine after natural Hardjo infection [3] or for Pomona, at least 102 days after artificial challenge and up to 9 months after natural challenge [4].

Leptospiral infection in sheep can have adverse economic effects by causing clinical disease, manifested by fever, jaundice, hepatic and renal dysfunction, haemoglobinuria, anaemia and lamb mortality [5-8]. Subclinical disease is also reported, with suspected impaired reproductive efficiency, abortion and agalactia [9, 10]. However, seropositive sheep can be shedding without clinical signs, and are thus exposing humans to the risk of infection.

In 2012, 113 human cases of leptospirosis were notified in New Zealand [11], a figure likely to be underestimated by 40 (95% CI 16 to 56) times [12]. Meat workers are particularly at risk when processing sheep [13-16]. Workers in one sheep abattoir were exposed to up to 54 kidney culture positive carcasses per day during high risk periods [15], and in four other abattoirs had an average annual infection risk of 11.1% [12].

Some reports suggest that human acute severe leptospirosis can be successfully treated with a combination of corticosteroids and antibiotics if diagnosed early [17, 18]. However, the disease often remains undiagnosed or is diagnosed too late due to its non-specific clinical signs. Therefore prevention of infection is the most efficient control measure. Personal protective gear has not been shown to reduce the risk of infection in meat workers [16]. No human vaccine is available in New Zealand, and protection therefore relies on avoiding exposure to infected urine. The most effective way to protect humans in New Zealand would thus be reducing or eliminating *Leptospira* shedding in farm animals by vaccination [19].

Leptospiral vaccines currently registered for livestock in New Zealand are inactivated whole-cell vaccines with an adjuvant. No cross-protection exists between serogroups, so vaccination schemes must include the serovars that are endemic in the area [20] for the specific host species involved. Only one of 161 New Zealand sheep farmers surveyed reported vaccinating their sheep against leptospirosis [12]. Vaccines have shown variable efficacy in reducing shedding or renal carriage in cattle after artificial challenge with serovars Hardjo or Pomona using various challenge routes, strains and protocols: some reports showed vaccines were efficacious [21, 22], while others showed poor efficacy [21, 23, 24], for an overall range of 0-100%. The reported efficacy in the face of natural challenge was 71 to 100% reduction in urine shedding up to one year after vaccination [25-27]. A vaccination campaign started in 1983 in New

Zealand dairy cattle was associated with a >80% reduction in the incidence of disease among dairy farmers and dairy workers [28].

Marshall et al. [29] artificially challenged sheep with serovar Hardjo 6 weeks after vaccination and found 2/9 vaccinated and 10/10 control sheep positive at kidney culture. Early studies with serovar Pomona showed a vaccine efficacy of 100% when challenge occurred 20 days, 40 days or 11 months after vaccination [4], but only dark field microscopy was used, a method which lacks sensitivity and specificity especially for low shedding rates. An experimental challenge trial on 9-11-week-old lambs [30] challenged four months after vaccination with Hardjo or Pomona showed an efficacy of 100% as measured by urine and kidney culture. Hence there is a dearth of data on either the efficacy of vaccines per se or vaccination programmes in a natural challenge, commercial sheep farming context. Studies on vaccine effectiveness for preventing shedding after natural challenge in a commercial farming environment are therefore needed as an essential step to validate the use of vaccine by sheep farmers.

This study evaluated the effectiveness of *Leptospira* vaccination in sheep in reducing shedding in urine in naturally infected commercial sheep flocks.

4.5. Materials and methods

4.5.1. Animals

From September to November 2010 (farms A-G) and in January 2013 (farm H), between 170 and 327 ewe lambs per farm from eight commercial sheep and beef farms in the North and South Islands of New Zealand (Table 4-1) were enrolled for a study of production effects of leptospirosis (Chapter 5 and 6). One third of the enrolled lambs were randomly selected to receive a bivalent commercial vaccine against *L. borgpetersenii* serovar Hardjo type Hardjo-bovis and *L. interrogans* serovar Pomona. This constituted no more than 5.2% of sheep on any farm, and no more than 1/3 of any management group at enrolment, with the intention to minimise the disturbance of natural disease dynamics. On some farms the group composition changed over time by management decision. This was independent from the vaccination status of the animals which was unknown to the farmers. Animals on Farms A-G were enrolled and vaccinated first at tail docking (2 – 6 weeks of age). On Farm H, lambs were first vaccinated at weaning (18 weeks of age).

4.5.2. Vaccine and vaccination protocol

Animals were vaccinated with a commercial bivalent (serovars Hardjo and Pomona) vaccine (“Leptavoid 2”, MSD Animal Health, batch numbers B258D2A, B403A1A and B677C1A). For each injection 2mL were injected subcutaneously by one of the authors using a vaccination gun (Vaximate™ 2 mL, ISL) and a reusable 18 gauge, 3/8 inch needle in the anterior third of the neck. The animals were individually

held by an operator on Farms A to G and were restrained in a race on Farm H. The needle was changed after approximately 15 animals or when visibly contaminated. Open vaccine packs were kept at +4°C and not used after one month after opening, according to manufacturer's instruction. They were transported to the farms in a cooler box or bag with ice packs.

The second injection was given 5-11 weeks after the first (Table 4-1), hence the protocol deviated from the manufacturer's recommendation (injections four to six weeks apart) on all but Farm H, to meet the requirement of participating farmers to coincide research procedures with usual farm operations on the animals. Vaccination hence mimicked the likely practice on commercial sheep farms. On four farms, (A, B, E and G), a third injection, approximating an annual booster, was given to the animals still in the study 41 to 75 weeks after the first injection (Table 4-1).

Table 4-1: Farm location, breed, median age and Hardjo and Pomona seroprevalence (MAT titre ≥ 48) of sheep at the first vaccination (“Leptavoid-2”, MSD Animal Health), and vaccination schedule

Farm	District	Sheep breed	Median age at first vaccination (weeks)	Seroprevalence (%) in vaccinated lambs at first vaccination		Date of first vaccination	Weeks between vaccination 1 and 2	Weeks between vaccination 2 and 3
				Hardjo	Pomona			
A	Manawatu	Romney composite	1	57.7	4.2	1/10/2011	11	44
B	Tararua	Perendale composite	6	15.9	1.9	22/11/2011	8	54
C	Wairoa	Romney Coopworth cross	3	67.0	10.1	17/10/2011	6	NA*
D	Central Hawke’s Bay	Romney texel cross	2	6.3	2.5	30/09/2011	8	NA*
E	Hurunui	Romney	4	76.9	0	25/10/2011	8	75
F	Hurunui	Lincoln merino cross	4	25.8	0	28/10/2011	8	NA*
G	Manawatu	Romney	2	18.5	1.9	19/10/2011	8	41
H	Waikato	Romney Finn coopworth composite	18	1.1	1.1	8/01/2013	5	NA*

*Not applicable: the third injection was given on the day of urine sampling, after or not given at all, so it did not contribute in the evaluation of vaccine effectiveness

4.5.3. Blood and urine sampling

A subset of both vaccinated and control sheep was randomly selected for urine collection, as described in Table 4-2. Urine was collected from 84 vaccinated and 98 control animals (Table 4-2) on the day of the third vaccination (“annual booster”) or shortly after (Table 4-1). This sample size allows for the detection of a difference of 20 percentage points in urine shedding prevalence between vaccinated and control sheep with 80% power and a significance level of 5%. Farms G and H were visited twice and different animals were sampled on each occasion. Urination was stimulated by placing a hand over the nostril until urine was voided [4] and urine was collected in a sterile 60 ml individual container, labelled with the animal unique identifier. The samples were transported in a cooler bin or bag with ice packs to the ^mEpiLab, Massey University where they were stored at +4°C until processing, within 48 hours of collection.

A blood sample was also taken from both vaccinated and control animals on the day of the first vaccination to estimate the presence of current infection or maternal antibodies. Blood was also collected at intervals of eight to 48 weeks, for up to five times before urine sampling, from control animals only, to confirm exposure to Hardjo and/or Pomona in the flock, including on the days of urine collection. Blood was collected by jugular venipuncture, using a 1 inch 20 gauge needle and a red top CAT Plus Blood Collection Tube (BD Vacutainer®).

Blood samples were transported in a cooler bin or bag with ice packs to the mEpiLab where they were stored at +4°C until processing, within 48 hours of collection. After clotting at ambient temperature, the samples were centrifuged at 1400 g for 10 minutes. The serum was diluted at 1/6 with 0.9% saline and stored at -20°C until the Microscopic Agglutination Test (MAT) was performed.

4.5.4. Laboratory analysis

4.5.4.1. Microscopic Agglutination Test (MAT)

The MAT was conducted as described by Fang [31], based on the method reported by Adler *et al.* [32]. Briefly, two-fold dilutions in 0.9% saline of each serum sample were prepared, from 1:12 to 1:1536. An equal volume of live *Leptospira* cultures of the tested serovar was added to make a final dilution series ranging from 1:24 to 1:3072. The samples were then incubated for 1.5 to 4 hours. Standard Hardjo and Pomona antisera obtained from the WHO/FAO/OIE leptospirosis reference laboratory, Brisbane, Australia. The *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona used for the test were hamster passaged strains that were provided by Schering-Plough, Wellington, New Zealand. Dilutions of each sample were examined under a dark-field microscope for the presence of agglutination or lysis. The test was considered positive for the highest dilution where 50% or more of the leptospires were agglutinated or lysed.

4.5.4.2. DNA extraction and PCR

Urine samples were centrifuged at 10600 g for 20 min, and the sediments were resuspended in 200 µl of PBS, after discarding the supernatant. The DNA was then extracted using the QIAamp ® DNA Mini Kit (Qiagen) as per manufacturer's instructions, for a final volume of 200 µl of DNA template solution. The samples were then kept at -20°C until the PCR was run.

The real-time PCR used was adapted from Subharat et al. [33] and Fang [31]. The PCR reaction solution was made up of 5 pmoles of primers (Invitrogen) targeting the *gyrB* gene (Slack et al. 2006), of sequence 5'-TGAGCCAAGAAGAAACAAGCTACA-3' (2For) and 5'-MATGGTTCRCRTTCCGAAGA-3' (504Rev), 2.4 µmoles of SYTO9, 12.5 µl of a commercial mastermix (Roche LightCycler 480 Probes Master 04707494001), 2 µl of the DNA preparation and double distilled water for a total volume of 25 µl.

The real-time PCR was run on a Rotor-Gene Q (Qiagen). An initial denaturation of 10 min at 95 °C was followed by 40 cycles, consisting of 10 seconds of denaturation at 95°C, 20 seconds of annealing at 63°C and 10 seconds of elongation at 72°C. The melting temperature was measured by monitoring the fluorescence on the green channel, every 0.2°C from 78°C to 90°C. A positive control, consisting of either DNA extracted from a live culture of a Copenhageni strain isolated from sheep in New Zealand, or DNA extracted from sheep urine inoculated with a live Hardjo culture (hamster passaged strain provided by Schering-Plough, Wellington, New Zealand) was used for each PCR run, as well as a negative control made of double distilled water.

4.5.5. Statistical analysis

4.5.5.1. Definition of flock exposure

The exposure and serological patterns are presented in detail by Vallée *et al.* [34]. To relate vaccine effectiveness to time of seroconversion, the infection status of each flock was determined using a cut point of $\geq 1:48$ [35]. Where there was evidence of non-specificity such as low prevalence with a low geometric mean titre, and non-repeatability of positivity of individuals in subsequent samples, i.e. when the serological pattern was sporadic, the flock was considered non-exposed. Flock F was already exposed to Hardjo at the time of first vaccination, but all others became exposed over time and Hardjo seroconversion was observed 13 to 67 weeks after the second vaccination. No flock was exposed to Pomona at the first vaccination and subsequently 3/8 flocks seroconverted to Pomona (Table 4-2). When seropositivity was observed before seroconversion, it was assumed to have been due to maternally-derived antibodies [34].

4.5.5.2. Shedding and vaccine effectiveness calculations

A lamb with a positive PCR result was regarded as ‘shedding’. The shedding prevalence was computed for vaccinated and control lambs within each farm. Logistic regression with farm as a random effect to account for within-farm clustering was used to calculate the shedding prevalence in each treatment group. The association of PCR positivity with MAT titres are described in details in [34].

Vaccine effectiveness (VE) was calculated for each farm where exposure was identified as described above, as $1-RR$ [36], with RR being the risk ratio of being PCR positive between vaccinated and control animals. Due to the small sample size on each farm, the confidence intervals for the RR and VE were calculated only when the point estimate of the VE differed from 100%.

To obtain RR for the calculation of vaccine effectiveness, a GEE Poisson model with robust standard errors [37] was developed with the shedding status as the outcome and vaccination status as the explanatory variable of interest. Farm was added as the clustering variable and the median flock age as a covariate. The point estimate and the confidence interval for the Risk Ratio (RR) of shedding was obtained from the model, and the Vaccine Efficacy (VE) was obtained by the formula $VE=1-RR$, applied to the confidence interval limits. Urine results collected on sampling days without prior seroconversion, hence when no challenge could be detected, were excluded from the VE model. Interactions between vaccination and farm were included to evaluate VE differences between farms.

The analysis was conducted in R v3.0.2. using packages “geepack” v1.1-6, “doBy” v4.5-10, “lme4” v1.1-5 and “epiR” v0.9-57.

4.5.6. Animal ethics

This study was approved by Massey University’s Animal Ethics Committee, under protocol 11/40.

4.6. Results

Urine sampling was undertaken 31 to 90 weeks after the first vaccination, 26 – 49 weeks after the booster (second vaccination) from flocks receiving only the initial sensitiser and booster, and 13 – 34 weeks after the third vaccination (“annual booster”) for those receiving three vaccinations before urine sampling (Table 4-2). The presence of maternal antibodies at vaccination was observed on farms A-E and G, and possibly on farm F concurrently with Hardjo exposure.

On farms D and G, the animals were urine sampled before seroconversion was observed hence data could not be used to calculate the vaccine effectiveness. Farm G

was visited again later when seroconversion was confirmed, and only the data obtained on that visit was used to calculate the farm level and overall vaccine effectiveness. Farm D was excluded from the analysis.

Vaccine effectiveness on reducing urinary shedding was 100% on all farms where animals were vaccinated before weaning and before exposure occurred (Table 4-2). Hardjo seroprevalence in the sampled control animals ranged from 89% on farm B to 100% on farms A, C and E. Three animals, all on Farm B, were also seropositive for Pomona.

On Farm F, where Hardjo exposure occurred before the second injection of vaccine and possibly before the first injection, one of 12 vaccinated animals and 5/12 control animals were PCR positive, resulting in a vaccine effectiveness of 80%. The seroprevalence for Hardjo was lower on this farm than on other seropositive farms: 5/12 of control sheep had a MAT titre ≥ 48 . Four of 12 control sheep were both seropositive for Hardjo and PCR positive, one was Hardjo seropositive and PCR negative and one was Hardjo seronegative and PCR positive.

Sheep on farm H, where the animals were vaccinated after weaning, were urine sampled on two occasions. In August 2013, three of 10 vaccinated and 13/15 control sheep were PCR positive, for a vaccine efficacy of 65% [95% confidence interval 9%-87%]. Ten of the 11 control animals from which a blood sample was taken were seropositive for Hardjo, and 6/11 were also seropositive for Pomona. Using samples collected four months later, the vaccine efficacy was 80% on that farm. All six animals from which blood was taken had a Hardjo MAT titre ≥ 48 and three were also seropositive for Pomona.

After discarding the data from Farms D and G as above, the overall cluster-adjusted shedding prevalence on farms where Hardjo and/or Pomona exposure was detected was 45.1% [95% CI 17.6-72.7] in the control group and 1.8% [95% CI 0.0-10.1] in the vaccinated group. Shedding prevalence (Table 4-2) in controls on the farms where MAT positive for Hardjo was observed ranged from 11% (1/9) to 88% (7/8).

After adjusting for clustering within-farm, the overall vaccine effectiveness in reducing shedding in urine, as measured by PCR, was 86.3% [95% confidence interval 63.6%-94.8%].

Table 4-2: Farm exposure, vaccination and sampling sequence, Hardjo and Pomona seroprevalence (titre ≥ 48) in the control group, number of sheep sampled, number of urine PCR positive for each treatment group, number MAT positive (titre ≥ 48) for Hardjo and Pomona in the vaccinated sampled animals at time of vaccination, number MAT positive (titre ≥ 48) for Hardjo and Pomona in the control sampled animals at time of urine sampling and vaccine efficacy (VE) stratified by farm

Farm	Vaccinated before weaning	Exposure on the farm	First observation of seroconversion (weeks since V1)	Number of vaccinations before urine sampling	Weeks since last vaccination	Seroprevalence in all control animals at urine sampling		No. sampled	Vaccinated			Control		VE % [95% CI]		
						Hardjo	Pomona		No. Hardjo MAT positive at V1	No. Pomona MAT positive at V1	No. PCR positive	No. sampled	No. PCR positive		No. Hardjo MAT positive	No. Pomona MAT positive
A	Yes	Hardjo	11-44	3	13	78.3	0	7	1	0	0	8	7	8	0	100
D	Yes	Hardjo	26-45	2	37	7.6	5.7	4	0	0	0	5	0	0	0	NA
E	Yes	Hardjo	27-75	3	15	97.1	3.7	9	7	0	0	9	1	9	0	100
G ^a	Yes	Hardjo	25-56	2	33	2.7	0.9	9	0	0	0	12	0	0	0	NA
G ^a	Yes	Hardjo	25-56	3	15	82.6	1.1	10	2	0	0	16	14	15	0	100
B	Yes	Hardjo Pomona	8-27	3	34	82.0	51.1	11	4	0	0	9	2	8	3	100
C	Yes	Hardjo Pomona	27-55	2	49	95.2	9.5	4	2	0	0	4	3	4	0	100
F	Yes	Hardjo (early)	0-8	2	48	69.9	3.9	12	4 ^e	0	1	12	5	5	0	80 [0-97]
H ^a	No	Hardjo Pomona	13-20	2	26	13.9 ^d	18.5 ^d	10	NA ^f	NA ^f	3	15	13	10 ^b	6 ^b	65 [9-87]
H ^a	No	Hardjo Pomona	13-20	2	44	98.2	54.1	8	NA ^f	NA ^f	1	8	5	6 ^c	3 ^c	80 [0-97]
Total								84			5	98	50	65	12	89 [66-96]

^a Different animals were sampled during the two visits on the same farm

^b 4 animals did not have a blood sample taken

^c 2 animals did not have a blood sample taken

^d seroprevalence obtained 6 weeks before urine sampling

^e on this farm, antibody observed was potentially due to infection since titres in controls persisted whereas on all other farms, titres in controls were all zero shortly after first vaccination, suggesting positivity at that time was due to maternal antibody

^f the vaccinated animals from which urine was collected were not blood sampled at V1 (the seroprevalence among the sampled animals at V1 on this farm was 1% for Hardjo and 1% for Pomona)

4.7. Discussion

To our knowledge, this study is the first to evaluate a commercial leptospiral vaccine's effectiveness against natural infection in New Zealand sheep.

The Hardjo seroprevalence ranged 76%-100% at the time when urine samples were used to calculate vaccine effectiveness. This indicates that natural challenge had occurred in non-vaccinated lambs. Since lambs were randomly allocated to vaccination or control and kept on the same pasture, it was inferred that natural challenge was similar in both groups, hence providing relevant data for determination of vaccine effectiveness.

The commercial bivalent vaccine resulted in a reduction of shedding in urine by 86.3%, and by 100% on farms where vaccination started prior to weaning and where there was no evidence of leptospiral infection at the time of first vaccination. This was despite most of the vaccination protocols implemented by the study farmers not complying accurately with the manufacturer's recommendation which prescribes two injections 4-6 weeks apart for the primary vaccination plus an annual booster. This non-compliance is often seen in the field. Several immunogenicity studies and experimental challenge studies support the use of two injections to effectively stimulate immunity using an inactivated whole cell vaccine [4, 29, 38]. In this study, the primary injections were administered up to 11 weeks apart and the annual booster up to 16 months after the first course of vaccination without reducing vaccine effectiveness. The reason for the variation from manufacturer's recommendation was that participating farmers required that vaccination timing fit in with other farm management practices. While this resulted in a sub-optimum experimental design, the data provide robust estimates of effectiveness in a practical context since the protocols adopted by farmers here were likely typical of the variation that would be expected in a commercial farming environment.

Previous studies in sheep looked at vaccine efficacy using experimental, controlled infection. Scheidy [39] and Webster and Reynolds [4] both reported an efficacy of 100% using vaccines made of inactivated *Pomona* bacterins. The first focused on the rise of body temperature, and used only one vaccine injection, while the second observed a shorter fever period in vaccinated sheep than in control sheep in two different trials and no evidence of shedding in vaccinated sheep for up to 11 months. However, urinary shedding was assessed only by oil immersion microscopy using Fontana staining, which may have a lower sensitivity and a higher detection limit than the commonly used culture and PCR. Chapman and Clough [30] also reported an efficacy of 100% as measured by urine and kidney culture, for the *Pomona* component of a commercial multivalent vaccine, using conjunctival and nasal challenge with a local *Pomona* strain given by artificial challenge.

To our knowledge, only two trials have tested the efficacy of the Hardjo component of multivalent vaccines on kidney colonization in sheep. Marshall et al. [29] used two vaccine injections one month apart, challenged the sheep intramuscularly six weeks later and isolated leptospire from the 10 control and two of the nine vaccinated sheep. However, the authors acknowledge that they possibly used a high infecting dose, thus potentially influencing the vaccine efficacy estimate. Chapman and Clough [30] used a commercial bivalent vaccine on sheep and found an efficacy of 100% measured by kidney and urine culture after conjunctival and intranasal challenge with a local strain, thus making the experimental trial closer to natural farm conditions. Those efficacy figures are in line with the field effectiveness of a bivalent Hardjo/Pomona vaccine observed in our study.

In this study, vaccine effectiveness was 100% on five out of eight farms, even when a high proportion of non-vaccinated stock were shedding and when dual Hardjo and Pomona infections were observed. The vaccinated sheep that were shedding were clustered on two farms, which suggests some farm-specific reasons other than an efficacy deficiency due to the vaccine itself. However, the number of sheep sampled on each farm was too small to allow robust statistical analysis of vaccine effectiveness between farms, or to identify any risk factor with certainty.

On Farm F, one vaccinated animal was PCR positive, as well as one animal that was seronegative for Hardjo and Pomona [34]. While Hardjo circulation was confirmed by serology on this farm, the presence of a serovar other than Hardjo or Pomona could not be ruled out. The MAT was conducted only for Hardjo and Pomona and no culture was done to isolate the shed bacteria, which would be necessary for definitive serovar identification [20]. Hence there is a possibility that the shed *Leptospira* were not a serovar used in the vaccine. Another explanation for the shedding in vaccinated sheep may be related to time of infection. On Farm F, early seroconversion was observed, showing exposure of some lambs before weaning. It is thus possible that animals were already infected when vaccinated, a circumstance known to decrease vaccine efficacy [26]. When deer received the same vaccine as used in this study before natural challenge, shedding was reduced by 100% [40]. However, when deer were vaccinated with a trivalent Hardjo-Pomona-Copenhageni vaccine (“Leptavoid 3”, MSD Animal Health) after infection, the efficacy was only 44.0% based on urine dark field microscopy and culture [41]. Hence, timing of vaccination relative to expected challenge appears an important determinant of vaccine efficacy.

On Farm H, no direct hypothesis can explain the presence of shedding in vaccinated lambs. The operator on this farm was different from the other farms, but no difference in vaccine handling and conservation or in vaccination technique was reported. The animals were older than on other farms when vaccinated, no maternal antibodies were detected and no seroconversion in control animals was observed before the second vaccine injection. The high seroprevalence for both Hardjo and Pomona on that farm on the day of urine sampling makes these two serovars the most likely cause

for seroconversion and shedding in urine. However, again the presence of a third serovar could not be ruled out. Among the other serovars reported in New Zealand livestock and wildlife, Tarassovi is maintained in pigs, Balcanica in possums and Ballum and Copenhageni in rats [42]. As sheep would be accidental hosts to those serovars, the serological and shedding prevalences are expected to be low. Few studies have looked at these serovars in sheep in New Zealand. A flock exposed to wet pastures and rodents revealed absence or seroprevalence <20% of Copenhageni, Ballum and Tarassovi [43], and a cross-sectional study conducted in 1979 reported a seroprevalence of 2.6% for Tarassovi, 2.3% for Copenhageni and 2.7% to Ballum, compared to 18.5% for Hardjo, using a MAT titre cut-off of 1:48 [35]. However, since no culture confirmation was undertaken, those serological data could include false positive MAT results, hence the low prevalence might in fact be zero. A more complete study using a broader panel for MAT and a joint use of PCR and culture [44] would be required to fully understand the limitations of the vaccination strategy used in this study to reduce leptospiral shedding.

Maternally derived immunity has been shown to potentially interfere with vaccination [45], and the vaccine's manufacturer warns against the potential interference of maternal antibodies if the vaccine is used for animals under 6-months. In the present study, depending on the farm, up to 76.9% of the vaccinated lambs were MAT positive for Hardjo and 10.1% for Pomona at the first vaccination, which was shown to be due to maternal antibodies except on farm F where challenge had likely started prior to docking, hence before the first vaccination [34]. No conclusive information is currently available about an interference of maternal antibodies with vaccine response. However, on all but Farm F and H, vaccine effectiveness was 100% when the full course of vaccination was given by weaning, suggesting no interference and indicating that lambs can be vaccinated effectively at a very young age. Moreover, the early seroconversion observed in very young lambs on Farm F may suggest that maternal antibodies were not sufficiently available or that they may not be protective against infection. Previously published leptospiral vaccine trials used seronegative animals [4, 29, 39]. Using the same vaccine as in the present study, Subharat et al. [40] carried out a vaccine efficacy trial in New Zealand farmed deer that already had maternal antibodies at the time of vaccination. The deer were naturally exposed to Hardjo and Pomona and no shedding in urine was found in vaccinated animals, suggesting that vaccine was effective in the presence of maternal antibodies in this species.

The vaccine effectiveness in reducing urinary shedding shown here supports that vaccination of domestic animals could be an efficient way of protecting people with regular contact with sheep, such as farmers, veterinarians, sheep shearers and meat workers [16, 19, 46]. This would not however, eliminate human leptospirosis in New Zealand, since a proportion of cases are due to other serovars such as Ballum and Tarassovi [11, 46]. If it was shown that these serovars could be transmitted to people via livestock, inclusion of those serovars in a vaccine could be justified.

L. interrogans serovar Hardjo, which has not been found in New Zealand, has been associated with reproductive loss in sheep [9] and with lamb mortality [8], but not *L. borgpetersenii* serovar Hardjo. However, vaccination has proved to be cost effective in farmed deer, another reservoir species, in which growth and reproductive loss have been mitigated by vaccination against Hardjo and Pomona [40, 47]. Serovar Pomona has been associated with clinical disease and mortality in lambs in New Zealand [7, 48] and elsewhere [6], as well as loss to follow-up of lambs and hoggets in a longitudinal study conducted on these farms [34].

More conclusive research is required to fully understand leptospirosis vaccination effectiveness and optimum timing for vaccination in sheep at the flock level. In our study, the lowest vaccine effectiveness was 76%. This may be sufficient for the development of herd immunity by limiting the circulation of the pathogen in the flock for a period of time. Disease transmission modelling could help to establish the minimum proportion of vaccinated sheep in a flock required to stop disease circulation. Additional work on vaccine effectiveness in the field, involving longitudinal studies with more intensive monitoring of exposure and shedding, using serology with an extended panel of serovars and a combination of urine PCR and culture, could help to understand why the effectiveness differs from farm to farm and to assess the role of other serovars in leptospirosis in sheep.

4.8. Conclusion

This study revealed an overall vaccine effectiveness of 86.3% [63.6%-94.8%] on shedding in urine of Hardjo and Pomona on commercial sheep farms, in spite of the presence of maternal antibodies at the time of vaccination and variation from the manufacturer's recommendations regarding the vaccination protocol. Vaccination of animals already infected seems to be a limiting factor for an effective vaccination program. The use of vaccination of sheep against leptospirosis is currently limited in New Zealand but the results of this study suggest that it could be used for the purpose of reducing both animal and human disease.

4.9. Contributions of the authors

E. Vallée contributed to the design of the study, recruited the farms, vaccinated the animals, collected the field samples and contributed to the laboratory analysis, statistical analysis and manuscript writing as part of a PhD programme. A.L. Ridler contributed to the design, vaccination, sample collection and manuscript preparation. Other authors were project supervisors contributing to design, analysis, interpretation, manuscript preparation and guidance with laboratory analyses.

4.10. Conflicts of interest

The company which produces the vaccine partially funded the study but was not involved in the study design, analysis, conclusions or decision to publish.

4.11. Acknowledgements

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Chapter 5. Effects of natural infection by *L. borgpetersenii* serovar Hardjo type Hardjo-bovis and *L. interrogans* serovar Pomona and leptospiral vaccination on sheep growth

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5.1. Abstract

In New Zealand, up to 97% of NZ sheep flocks are seropositive to *Leptospira borgpetersenii* serovar Hardjo and/or *Leptospira interrogans* Pomona, yet vaccination is rare. This study evaluated the impact of exposure to these serovars and of vaccination on sheep growth.

One third of 2260 ewe lambs on eight farms were randomly selected and vaccinated with a primary and booster bivalent Hardjo and Pomona vaccine starting at one month of age. Repeated blood samples were taken over one (n=6 farms, mated as hoggets) or two (n=2 farms, mated as 2-tooths) years and tested by microscopic agglutination test to assess exposure to Hardjo and Pomona. Individual weights were recorded at the same time and modelled using a multilevel linear model accounting for within-farm clustering and repeated measures. Predicted average weights were computed and compared based on the vaccination status and within the control group based on exposure status (positive for Hardjo only, Pomona only, Hardjo and Pomona and negative) for each combination of farm and weighing episode. Statistical significance of the comparison was evaluated after adjustment for multiple comparisons.

There was no difference in average weight between vaccinated and control sheep before or after vaccination in any of the flocks. The comparison between sheep seropositive for either or both serovars and seronegative sheep was inconclusive, with variations of direction and magnitude of the difference between farms and weighing episodes.

In the absence of an overall growth response to vaccination, widespread adoption of vaccination would unlikely yield an economic response at the industry level. However, the inconsistency observed when comparing animals based on their exposure status suggests that the actual effect of leptospirosis on growth is difficult to predict. A study of the effect on sheep reproduction is needed to fully assess the effect of vaccination on sheep production.

5.2. Keywords

Leptospirosis, *Leptospira*, sheep, Hardjo, Pomona, vaccine, weight, growth

5.3. Introduction

Exposure to *Leptospira* is endemic in New Zealand sheep. The most recent on-farm seroprevalence study found 91% of 162 flocks seropositive to *L. borgpetersenii* serovar Hardjo type Hardjo-bovis (Hardjo) and 74% seropositive to *L. interrogans* serovar Pomona (Pomona). At the animal level, 43% of 3,361 sheep were seropositive to Hardjo and 14% to Pomona (Dreyfus, 2013). Earlier studies of lambs at slaughter (Blackmore et al., 1982; Dorjee et al., 2008) both using a seropositivity cut-point of 48

showed a range of flock-level seroprevalence of 33-46% and 4-51% and sheep-level seroprevalence of 5-19% and 1-4% for Hardjo and Pomona respectively.

Sheep are believed to be a maintenance species for Hardjo (Cousins et al., 1989; Gerritsen et al., 1994) maintaining the bacteria in their kidneys. Being a true maintenance host would imply presenting few or no clinical signs (Hathaway, 1981; Ellis, 2015). While data are limited, no evidence of clinical effects associated with Hardjo is available, supporting the fact that New Zealand sheep appear to be a reservoir for Hardjo (Dorjee et al., 2005). Hardjo infection in sheep produces largely subclinical losses (Hathaway, 1981; Faine et al., 1999), including impaired reproduction efficiency and agalactia (Ellis et al., 1983; McKeown and Ellis, 1986). Sheep are considered accidental hosts for Pomona, despite being able to shed leptospire in urine for up to nine months after infection (Webster and Reynolds, 1955). Clinical disease is reported to be more severe in accidental hosts with manifestations including fever, jaundice, hepatic and renal dysfunction, haemoglobinuria, anaemia and mortality, all of which have been reported in lambs due to Pomona (Hodges, 1974; Smith and Armstrong, 1975; Schmitz et al., 1981; Vermunt et al., 1994).

Infection by Hardjo was shown to reduce farmed deer growth in New Zealand. Subharat et al. (2012) demonstrated that deer vaccinated at approximately three months of age had up to 31g/day higher average growth rate than unvaccinated deer exposed to Hardjo, for a final mean live weight difference of up to 6.4 kg at approximately 12 months of age. No growth effect of *Leptospira* infection has been observed in cattle (Holroyd, 1980; Fava et al., 2004). Little has been published on the effect of leptospirosis on sheep growth. In a controlled vaccine trial with experimental Pomona challenge in sheep with restricted feeding, Webster and Reynolds (1955) observed that a weight loss occurred in both groups but one month earlier in unvaccinated animals than in vaccinated lambs. The mean weight was around three pounds (1.4 kg) higher in the vaccinated than in the control group 15 weeks after challenge, although no measure of variability, precision or statistical significance was given. In this study leptospire were not detected in stained urine smears from 20 vaccinated lambs, but were detected in smears from 19/22 control lambs. Such small trials lack statistical power for showing subtle, yet possibly economically relevant sub-clinical effects.

Evaluating economic consequences of weight loss or reduced weight gain due to natural exposure would be essential in New Zealand where *Leptospira* infection in sheep is wide spread. During a study on eight commercial farms with a known endemic presence of *Leptospira*, animals were usually first exposed between eight and 15 months of age (Vallée et al., 2015) while still growing, although exposure before three months of age, earlier in the animal's growth cycle, was also shown.

Vaccination of sheep against *Leptospira* was shown to be effective in preventing urinary shedding when exposed to Hardjo and Pomona during natural (Chapter 4) or experimental (Chapman and Clough, 2014) challenge when vaccination was done

before exposure. However an economic analysis of vaccination as a control measure requires understanding the effects of infection and vaccination on production, including growth.

This paper reports on an investigation of the effects of natural Hardjo and Pomona exposure and leptospiral vaccination on sheep growth in a large-scale field trial. The objectives of this study were to compare live weights of sheep on farms naturally exposed to *Leptospira* during the growth phase with and without serological evidence of Hardjo and/or Pomona infection and to compare the mean weight of vaccinated and control sheep during the same growth phase.

5.4. Materials and methods

5.4.1. Study design, farms and animals

This combined randomized field trial and cohort study was conducted between November 2011 and December 2013 on eight commercial sheep and beef farms in the North (n=6) and South Islands (n=2) of New Zealand. A brief description of the farms is presented in Table 5-1.

Farms A to G were selected in June 2011 from a previous study on Johne's disease and leptospirosis (Dreyfus 2013) in which they were seropositive for leptospirosis, and where the farmer agreed to take part. Farm H was selected in 2012 based on its recent history of suboptimal reproductive performance in ewe lambs and the known presence of Hardjo and Pomona.

For evaluation of the effect of vaccination, an initial sample size of 786 vaccinated and 1572 control sheep was calculated assuming a difference in average daily gain of 20 g/day and a standard deviation of 100 g/day, for a power of 80% and an error of 5%. The required number of animals was doubled to account for within-farm clustering. The actual sample size was limited by mob size, duration of sampling on each farm and loss to follow-up.

Each enrolled sheep was identified with an individually numbered ear tag and randomly allocated to vaccinated or control group.

On each farm, between 55 and 106 female lambs born in spring 2011 or 2012 (Table 5-1), for a total of 730, were selected using a random number generating package. They were vaccinated with a commercial bivalent vaccine against *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona ("Leptavoid 2", MSD Animal Health, batch numbers B258D2A, B403A1A and B677C1A). For each injection, 2 mL were injected subcutaneously in the anterior third of the neck, after pushing the wool apart and making a skin fold. The first injection was given at tail-

docking on farm A-G, when the animals were aged 1-6 weeks, and at weaning (around 18 weeks of age) on farm H. The second injection was given 5-11 weeks later according to farm management practice. A third injection, approximating an annual booster, was given 10 to 18 months after the first injection to those animals remaining on the farm (Table 5-1). Data on effectiveness of the vaccination in preventing shedding in urine in these sheep are presented in Chapter 4. The proportion vaccinated constituted no more than 5.2% of total sheep on any farm, and no more than 1/3 of any management group at enrolment, to minimise the potential influence of vaccination on natural disease dynamics and therefore exposure.

Between 115 and 217 non-vaccinated control female lambs were randomly selected from the same mobs as the vaccinated lambs on each farm, for a total of 1523 control lambs.

Blood samples were collected from every control and vaccinated sheep on the day of enrolment, and then from every control sheep only, on the dates presented in Table 5-1. Blood was collected by jugular venepuncture using a one inch 20 gauge needle and a red top CAT Plus Blood Collection Tube (BD Vacutainer®). The exposure and seroconversion patterns for these two serovars are described in Vallée et al. (2015) and summarized in Table 5-1.

Live weight was measured at the dates indicated in Table 5-1 individually for each animal. Up to eight weighing episodes, determined by farmer participation, were timed to coincide with routine management events on each farm, namely docking and weaning as lambs, and subsequently when those animals were bred (joined with rams) and scanned as hoggets (around 10 months old), when their progeny were docked and weaned, and at breeding and scanning as 2-tooths (around 22 months-old). The farmer's weigh scales were used for all measurements on farm H and for all measurements other than the first occasion on farms A to G, when a domestic scale was used with the operator standing on the scale holding the lamb.

To ensure blinding, the farmers and the persons in charge of weighing were not aware of which animals were vaccinated. The farmers were informed of the flock seroprevalence but not of the individual sero-status of each sheep.

Table 5-1: Farm location, number and breed of ewes, date of weighing episodes, number of vaccinated and control sheep, Hardjo and Pomona seroprevalence (titre ≥ 48) and geometric mean titre (GMT, for animals with a titre ≥ 24) in unvaccinated controls at each sampling.

Farm	District (Island)	Number of breeding ewes	Breed	Date	Weighing episode**	Vaccination	Number vaccinated	Number of controls	Seroprevalence (%) in controls		GMT in control sheep	
									Hardjo	Pomona	Hardjo	Pomona
A	Manawatu (N)	6000	Romney/ composite	1/10/2011	LD	Sensitiser	97	197	64.6	2.0	162	73
				15/12/2011	LW	Booster	95	189	5.0	0.0	29	NA
				15/03/2012	HB		83	181	NA*	NA*	NA*	NA*
				2/08/2012	HS	Booster	84	172	17.9	5.5	856	41
B	Tararua (N)	8500	Perendale/ composite	22/11/2011	LD	Sensitiser	106	210	12.9	1.0	69	34
				17/01/2012	LW	Booster	102	193	0.0	2.1	NA	38
				30/05/2012	HB		92	175	5.1	13.1	946	665
				5/12/2012	HD	Booster	85	144	97.1	41.7	408	104
				16/04/2013	TB		73	132	95.3	43.8	301	82
				30/07/2013	TS		74	139	82.8	51.1	160	100
C	Wairoa (N)	4700	Romney/ Coopworth cross	17/10/2011	LD	Sensitiser	101	210	59.5	15.2	71	55
				1/12/2011	LW	Booster	98	207	45.6	0.0	58	NA
				26/04/2012	HB		88	190	0.0	0.5	NA	26
				6- 23/11/2012	HD	Booster	76	175	98.3	18.7	293	166
				28/03/2013	TB		39	80	100.0	21.3	436	127
D	Central Hawke's	4500	Romney Texel	30/09- 6/10/2011	LD	Sensitiser	78	180	2.8	0.0	41	24

	Bay (N)		cross	24/11/2011	LW	Booster	71	157	1.3	0.6	36	192
				27/03/2012	HB		68	158	1.2	1.9	152	48
				9/08/2012	HS	Booster	62	153	7.6	5.7	1935	51
				25/10/2011	LD	Sensitiser	109	215	76.3	0.5	109	96
E	Hurunui, Canterbury (S)	10400	Romney	19/12/2011	LW	Booster	100	203	16.3	0.0	34	NA
				2/05/2012	HB		95	200	1.0	0.0	272	NA
				4/04/2013	TB	Booster	49	114	97.2	5.5	344	30
				18/07/2013	TS		67	138	97.1	3.7	400	36
				28/10/2011	LD	Sensitiser	90	190	18.4	0.0	90	NA
F	Hurunui Canterbury (S)	10000	Lincoln Merino cross	20/12/2011	LW	Booster	84	166	10.2	0.0	553	NA
				3/05/2012	HB		77	161	19.9	4.3	484	52
				18/09/2012	HS		23	45	NA*	NA*	NA*	NA*
				20/11- 7/12/2012	HD	Booster	68	148	70.8	1.7	409	30
				19/10- 7/11/2011	LD	Sensitiser	55	114	22.8	4.4	57	63
G	Manawatu (N)	1050	Romney	12/12/2011	LW	Booster	54	112	14.3	0.0	52	NA
				10/04/2012	HB		54	112	0.0	0.1	NA	96
				1/08/2012	HS	Booster	53	111	1.8	0.9	110	38
				14/11/2012	HD		41	77	93.3	1.3	306	68
				11/01/2013	HW		48	68	95.6	0.0	534	24
H	Waikato (N)	7800	composite Romney Finn	8/01/2013	NA	Sensitiser	94	NA*	NA*	NA*	NA*	NA*
				12/02/2013	LW	Booster	91	190	0.5	3.2	48	35
				20/03/2013	HB		87	177	0.0	0.6	24	768

Coopworth	1/07/2013	HS	83	171	13.9	18.2	620	368
	18/12/2013	HW	52	105	99.0	54.1	386	254

NA: not applicable

* No blood sample was collected on that day

** LD: lamb docking, LW: lamb weaning, HB: hogget breeding, HS: hogget scanning, HD: hogget docking, HW: hogget weaning, TB: 2-tooth breeding, 8: TS-tooth scanning

5.4.2. Microscopic agglutination test

The Microscopic Agglutination Test (MAT) was conducted as described in Vallée et al. (2015), based on the method reported in Adler et al. (1982) and Fang et al. (2014). Briefly, two-fold dilutions in 0.9% saline of each serum sample were realised, from 1:24 to 1:3072. The samples were then incubated for 1.5 to four hours with live *Leptospira* cultures of either Hardjo or Pomona. Each dilution of each sample was then checked under a dark-field microscope for the presence of agglutination or lysis. The test was considered positive for the highest dilution where 50% or more of the leptospire were agglutinated or lysed.

5.4.3. Statistical analysis

Individual weights collected from the second sampling onwards were used as the outcome variable of hierarchical linear regressions. A three-way interaction of farm by age and by an exposure variable was used as a fixed effect, as well as the weight at enrolment (docking weight for farms A-G, weaning weight for farm H). Age was used as a categorical variable with seven levels: weaning, hogget breeding (when they were joined with the ram), hogget scanning, hogget docking (when their lambs were docked), hogget weaning (when their lambs were weaned), 2-tooth breeding (when they were joined with the ram), 2-tooth scanning. The exposure variable was either vaccination status (binary) or serological status for control sheep only (negative, positive for Hardjo only, Pomona only, Hardjo and Pomona). A random effect for sheep and a first order autoregressive correlation structure were used to account for the hierarchical nature of the data and for repeated weighing and sampling of the same animal (Diggle, 1988).

Differences of covariate-adjusted predicted marginal mean weights between exposure levels (control – vaccinated, Hardjo only – negative, Pomona only – negative, Hardjo and Pomona – negative) were computed from each model for the combinations of farm and age where weight was actually measured. The significance of the difference was tested by a Z-ratio, from which p-values were calculated. To test for rejection of the null hypothesis of no difference of weight, adjusted p-values (also called “q-values”) were computed using Benjamini and Hochberg’s false discovery rate method for multiple comparisons (Benjamini and Hochberg, 1995), for each set of p-values obtained from a model. A probability value of ≤ 0.1 denotes marginal non-significance, while $p \leq 0.05$ or less indicates significance.

A titre cut-point of 48 was chosen for the definition of seropositivity. A sensitivity analysis was undertaken to assess the effect of the choice of the cut point titre. All titres from 48 to 768 inclusive were tested as cut-points and the predicted weight differences between sero-positive and sero-negative sheep was recorded.

The analysis was conducted in R v3.0.3. using packages “nlme” v3.1-113 .

5.4.4. Animal ethics

This study was approved by Massey University's Animal Ethics Committee, under protocol 11/40.

5.5. Results

Seroprevalence in control sheep in each flock at each weighing episode is presented in Table 5-1. Seropositivity (MAT titre ≥ 48) for Hardjo and Pomona was detected on all farms (Table 5-1). Pomona seroprevalence was above 10% only on farms B, C and H. Hardjo seroprevalence was 5% or less at hogget breeding on all but farm A, where it was not recorded at that time, and on farm F where it was 19.9%. Hardjo seroprevalence was above 10% at hogget scanning or docking except on farm D, where the final recorded seroprevalence was 7.6% and on farm E where Hardjo seroprevalence was not recorded for these management episodes but was 97.2% at two-tooth breeding.

Adjusted predicted weights of non-vaccinated seronegative sheep, those seropositive for Hardjo only, Pomona only, or both, and vaccinated sheep are presented in Figure 5-1. Data for predicted differences between vaccinated and control sheep and between seropositive and seronegative non-vaccinated control sheep are presented in Table 5-2.

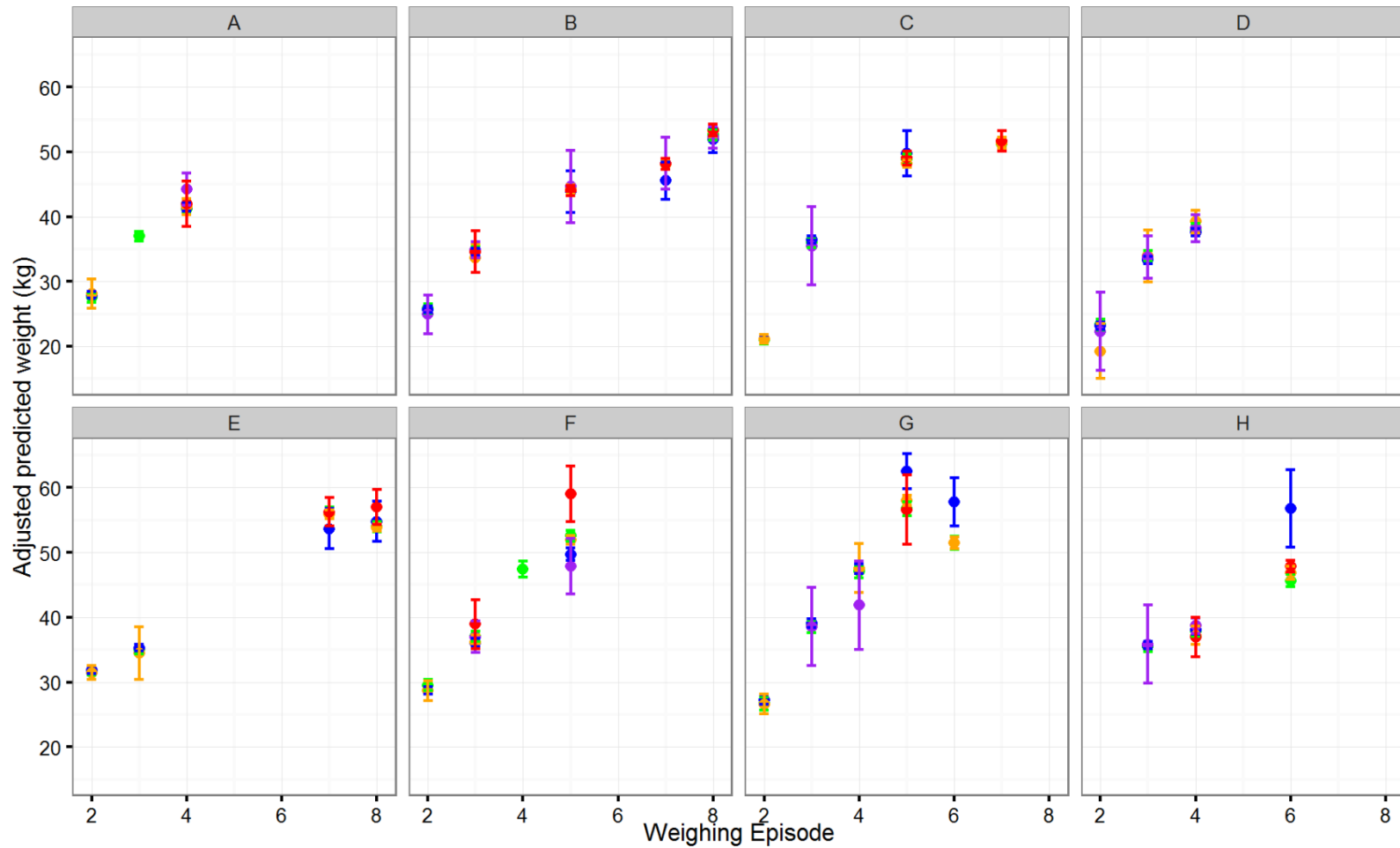


Figure 5-1: Predicted weights (kg) of ewes (Farms A-H), adjusted for farm and enrolment weight (lamb docking on all farms but H, lamb weaning on farm H), according to weighing episode (2: lamb weaning, 3: hogget breeding, 4: hogget scanning, 5: hogget docking, 6: hogget weaning, 7: 2-tooth breeding, 8: 2-tooth scanning), and stratified by status (green vaccinated, orange non-vaccinated with Hardjo titre only ≥ 48 , purple non-vaccinated with Pomona titre only ≥ 48 , red non-vaccinated with both Hardjo and Pomona titres ≥ 48 , blue non-vaccinated with Hardjo and Pomona titres < 48)

5.5.1. Effect of vaccination status on live weight

Differences of predicted adjusted mean weights between vaccinated and control sheep are presented in Table 5-2. No significant difference between vaccinated and control sheep was observed for any combination of farm and weighing episode. Non-vaccinated sheep were marginally non-significantly ($p \leq 0.1$) heavier than vaccinated sheep at hogget weaning on farm H.

5.5.2. Effect of serological status on live weight

At hogget docking on farm F, sheep seropositive (titre ≥ 48) for Hardjo alone and for Hardjo and Pomona combined were significantly heavier than seronegative sheep (Table 5-2). At hogget docking and weaning on farm G, sheep seropositive for Hardjo were significantly lighter than seronegative sheep. At hogget weaning on farm H, sheep seropositive for Hardjo alone or Hardjo and Pomona combined were significantly higher than seronegative sheep.

On farms G and H at hogget weaning, seronegative control sheep were significantly heavier than control sheep seropositive for any serovar, and heavier than vaccinated sheep. On farm F at hogget docking, sheep seropositive for Hardjo were significantly heavier than seronegative and vaccinated sheep (Figure 5-1).

5.5.3. Sensitivity analysis for titre cut-point

Changing the cut-point did not result in any change of the effect of seropositivity on live weight on farms A, B, C, D or E.

The weight difference observed on farm F at hogget docking was significant for all tested cut-points. The change in the magnitude between the smallest and the largest difference was 64%. By contrast the magnitude of the differences on Farm G, which were all negative, were reduced as the cut-point was increased, and remained significantly different from zero for three consecutive cut-points. The significant differences observed on farm H at hogget weaning at cut points 40 and 96 were not observed at higher cut-points.

Table 5-2: Predicted mean weight difference (kg) between non-vaccinated and vaccinated sheep (Vacc), and in the non-vaccinated group, between seropositive (H: Hardjo only, P: Pomona only, HP: Hardjo and Pomona) and seronegative sheep for different titre cut-points, with number of sheep seropositive at this cut-point (in brackets), by farm and by weighing episode (LW: lamb weaning, HB: hogget breeding, HS: hogget scanning, HD: hogget docking, HW: hogget weaning, TB: 2-tooth breeding, TS: 2-tooth scanning); significance level: ° <0.1, *<0.05, **<0.01, ***<0.001. A negative weight difference indicates a higher mean weight in vaccinated than in control sheep, or a higher mean weight in seronegative than seropositive sheep.

Farm	Vacc	≥48			≥96			≥192			≥384			≥768			
		H	P	HP	H	P	HP	H	P	HP	H	P	HP	H	P	HP	
A	LW	0.336			0.170 (8)			-1.039 (3)									
	HB	0.312															
	HS	0.332	0.042 (26)	2.829 (6)	0.519 (3)	0.044 (28)			0.249 (25)			0.249 (24)			0.397 (23)		
B	LW	-0.223		-0.725 (4)													
	HB	0.016	-0.964 (6)	0.329 (20)	-0.032 (3)	-1.004 (7)	0.241 (19)	0.556 (2)	-0.920 (7)	0.293 (19)	0.588 (2)	-1.047 (7)	0.212 (17)	0.628 (2)	-0.727 (6)	-0.010 (14)	-0.082 (1)
	HD	-0.038	0.341 (78)	0.784 (1)	0.185 (57)	-0.718 (88)	0.402 (1)	-0.917 (46)	-0.983 (98)	0.010 (4)	-1.019 (22)	0.185 (83)	0.357 (4)	-0.322 (7)	0.606 (57)		
	TB	-0.002	2.523 (68)	2.653 (2)	2.610 (54)	1.368 (83)	3.032 (4)	1.325 (32)	0.620 (89)	1.130 (4)	0.493 (16)	-0.893 (64)	-2.095 (3)	1.163 (1)	0.077 (34)	-0.802 (2)	
	TS	0.462	1.371 (56)	0.375 (13)	1.504 (55)	1.703 (51)	0.939 (17)	1.844 (42)	1.282 (52)	2.081 (17)	2.321 (17)	0.393 (37)	1.087 (9)	0.688 (3)	0.015 (14)	-1.587 (1)	
C	LW	0.046	0.034 (93)			0.008 (50)			0.279 (18)			0.543 (7)			3.205 (2)		
	HB	0.483		-0.983 (1)													
	HD	-0.359	-1.608 (136)		-0.695 (32)	-1.044 (140)		-1.581 (24)	-0.662 (130)		-0.350 (20)	0.200 (87)	-0.138 (6)	1.054 (9)	0.296 (26)	0.000 (9)	6.238° (2)
	TB	0.194							2.817 (60)		1.448 (11)	1.069 (51)	-5.897 (1)	1.444 (7)	0.879 (25)		

D	LW	-0.318	-3.866 (2)	-0.823 (1)		-5.029 (1)	-0.798 (1)		-0.771 (1)			
	HB	-0.623	0.701 (2)	0.484 (3)		0.691 (2)		0.679 (2)		0.678 (2)		
	HS	-0.301	1.658 (12)	0.653 (9)		1.625 (12)	-0.674 (2)	1.634 (12)		1.634 (24)		1.171 (11)
E	LW	-0.019	-0.362 (31)			-0.665 (5)						
	HB	0.200	-0.776 (2)			3.552 (1)		3.530 (1)		3.530 (1)		3.543 (1)
	TB	-0.443	2.221 (98)		2.612 (6)	0.825 (102)	0.293 (1)	0.693 (91)		0.062 (66)		0.148 (32)
	TS	0.037	-0.944 (126)		2.188 (5)	-0.177 (128)	-3.301 (1)	0.539 (122)		1.505 (91)		-0.115 (44)
F	LW	-0.809	-0.016 (17)			0.034 (16)		0.020 (16)		-0.002 (15)		-0.336 (10)
	HB	-0.684	0.094 (30)	0.897 (5)	2.882 (2)	0.138 (30)	2.952 (2)	0.440 (30)		0.175 (22)		-0.24 (13)
	HS	-1.325										
	HD	-1.237	2.157** (102)	-1.894 (2)	9.303** (2)	2.535*** (102)	-2.655 (1)		2.802*** (93)		2.801*** (74)	
G	LW	0.520	-0.686 (16)			-0.990 (6)		-0.690 (4)				
	HB	0.491		-0.528 (1)			-0.562 (1)		-0.588 (1)	1.739 (1)		1.749 (1)
	HS	0.280	0.092 (2)	-5.591 (1)		-2.370 (1)	-5.631 (1)	-2.405 (1)	-5.625 (1)	-1.277 (1)		-2.056 (1)
	HD	1.587	-4.516* (68)		-5.931 (1)	-4.540* (68)	-5.938 (1)	-4.579** (66)	-5.870 (1)	-2.704° (62)		-0.667 (45)

	HW	0.916	-6.299*			-6.316*			-3.694°			-1.322			-0.688		
			(64)			(64)			(61)			(56)			(33)		
H	HB	0.165		0.112		-0.180			-0.025			0.065			-0.252		
				(1)		(1)			(1)			(1)			(1)		
	HS	0.210	-0.833	0.679	-1.126	-0.895	0.055	-0.213	-0.880	0.356	0.497	-1.017	0.463	0.512	-0.824	0.813	
			(19)	(26)	(4)	(19)	(26)	(3)	(20)	(24)	(1)	(16)	(22)	(1)	(15)	(8)	
	HW	1.847°	-9.903*		-8.895*	-3.925	-12.813*	-3.160	-2.213	-2.990	-1.120	1.406	0.759	2.565	-0.863	-0.243	2.209
			(41)		(50)	(42)	(1)	(47)	(45)	(8)	(35)	(44)	(11)	(17)	(26)	(11)	(7)

5.6. Discussion

This is the first investigation of the potential impact of *Leptospira* exposure on sheep growth in commercial flocks in New Zealand. The study was prompted by recent reports of a high seroprevalence for *Leptospira* in the national sheep flock (Dreyfus 2013) and of a significant reduction in growth rate due to *Leptospira* demonstrated in farmed deer in this country (Subharat et al 2012). The study employed two methods for testing for growth effects, namely comparison of mean weight between vaccinated and non-vaccinated animals, and comparison between sheep experiencing or not experiencing exposure as determined serologically.

Hardjo exposure occurred on all eight farms, reaching at least 70% on six of them by the last weighing episode. On the two other farms, the seroprevalence remained lower during the study but the high GMT observed at the end of the study suggested recent exposure of the flock. The Pomona seroprevalence rose above 20% on three farms, allowing comparisons between vaccinated and control sheep to be meaningful. The vaccinated animals constituted no more than 5.2% of the sheep on each farm, and no more than one third of any management mob at the time of first vaccination or subsequently. Mobs were often rearranged according to the farmer's decision, resulting in a lower proportion of vaccinated animals, thus it is unlikely that vaccination would have provided a flock-level immunity high enough to bias the results. The chosen design also looked for a difference in live weights between seropositive and seronegative sheep, which was limited on some farms for some age groups by a very high or very low seroprevalence.

Some significant or marginally non-significant live weight differences were observed on three farms, after exposure to Hardjo and/or Pomona was observed in the flock. However, while this study showed significantly lower weight for seropositive sheep on some occasions on some farms, there were instances of higher weight in seropositive animals on other farms. Further, the study did not show any significant weight response to vaccination. Hence, this study has been unable to provide unequivocal data on the effect of *Leptospira* exposure on live weight of growing sheep and it suggests no detectable flock-level effect of vaccination. This is despite high seroprevalence in many and a number of animals high enough to allow for significant differences in live weight of 2.2 kg or more to be detected.

On farm G, sheep with a Hardjo titre ≥ 48 were significantly lighter than sheep with Hardjo and Pomona titres < 48 at hogget docking and weaning. On this farm Hardjo seroprevalence (≥ 48) rose from 1.8% at hogget scanning to 93.3% at hogget docking. A significant weight difference was still observed after increasing the cut-point, which is coherent with the fact that lower titres may represent older exposure, at which time the weight effect occurred and was not compensated for during the titre decline phase. However, no weight difference was observed between vaccinated and control sheep on this farm at the same periods, in spite of a vaccine effectiveness on shedding in urine of

100% (95% CI 20-100%) (Chapter 4). Furthermore, Figure 5-1 shows that there was no difference between vaccinated and control sheep with Hardjo titre ≥ 48 . It is unclear why the seronegative sheep on these farms were heavier than seropositive sheep as well as vaccinated sheep. However, a similar case of vaccination resulting in a reduced weight gain has been reported by (Goodwin-Ray et al., 2008) where vaccination of three-months-old lambs against *Pasteurella* using an inactivated vaccine containing cells expressing *Mannheimia haemolytica* and *Pasteurella trehalosi* antigens significantly reduced the average daily gain in the first 11 weeks post initial vaccination. Using an adjuvant placebo on the control group may have attenuated this weight difference. However, since there were only nine and four seronegative sheep at hogget scanning and hogget docking respectively, it cannot be excluded that this result occurred by chance, leading to type I error.

On farm H at hogget docking, seronegative sheep were significantly heavier than sheep with Hardjo titre ≥ 48 and both Hardjo and Pomona titre ≥ 48 by 9.9 kg and 8.9 kg, while sheep with Pomona titre only ≥ 96 were significantly lighter by 12.8 kg. However, these significant differences were not observed for higher cut-points and the vaccinated sheep were marginally non-significantly lighter than control sheep. Furthermore, there was only one sheep with Pomona titre only ≥ 96 and 14 sheep with both Hardjo and Pomona titres < 48 . No difference was observed on the two other farms with both Hardjo and Pomona exposure, suggesting that these observed differences were also a type I error. However, apart from one animal on farm F where early exposure, possibly before vaccination, was detected, all the vaccinated animals that were PCR positive for *Leptospira* in urine were detected on Farm H (Chapter 4). The presence of a serovar other than Hardjo and Pomona on this farm could explain the absence of significant difference in weight between vaccinated and control sheep, but the PCR did not allow for the identification of the serovar to confirm this hypothesis.

On farm F at hogget docking, sheep seropositive for Hardjo only were significantly heavier by 2.2 to 3.5 kg than sheep seronegative for both Hardjo and Pomona at all the tested cut-points. The number of seronegative and seropositive sheep was balanced enough to provide a meaningful comparison. However, at the same time, vaccinated sheep were heavier by 1.2 kg, although non-significantly. It is unclear why on this farm at this weighing episode unvaccinated sheep with Hardjo titres ≥ 48 were heavier than sheep seropositive for Pomona or both Hardjo and Pomona, seronegative sheep and vaccinated sheep. The Hardjo seroprevalence on that farm at hogget docking was 70.8%. Hardjo exposure started earlier on that farm than on the other farms, as indicated by Hardjo seroprevalence never being less than 10%. Further, the Hardjo GMT increased to 553 by lamb weaning while it decreased on all the other farms after the first sampling. However, in spite of the early start of exposure, the seroprevalence at or before hogget docking remained lower than on the three other farms where prevalence at hogget docking was available. This early exposure and then relatively low seroprevalence compared with the other farms suggest that the epidemiology of Hardjo infection on this farm was different, but it remains unclear why sheep seropositive for

Hardjo were heavier than all the other exposure status groups. Natural immunity against *Leptospira* relies partially on the production of antibodies (Moffat, 2007; Fraga et al., 2011). One hypothesis would be that there was a critical growth period during which Hardjo infection had an impact, and infection at an earlier stage would ensure protection during this critical period. However, more studies would be needed to explore this hypothesis. Another possibility would be some unmeasured confounder where for example some sheep would by chance ingest more wet pasture than others and therefore would be more exposed to *Leptospira* but also gain more weight.

Hence, this study, which is, to our knowledge, the first one to look at the effect of natural Hardjo and Pomona exposure on sheep growth, did not provide conclusive evidence of an effect of these serovars or of vaccination on sheep live weight, in spite of a high exposure on most study farms.

Few studies have addressed the effect of leptospirosis on cattle growth and none have directly investigated the effect of exposure on calf growth. However, studies evaluating the effect of dam's vaccination and weight gain between reproduction cycles in adult cows showed no effect of seropositivity or vaccination. Holroyd (1980) showed no difference in growth rates between calves born from cows vaccinated against Hardjo and control cows for six consecutive years, on a farm known to be exposed to Hardjo and Pomona. Fava et al. (2004) showed that seropositivity to Hardjo, Wolfii and other serogroups did not influence average daily gain and body condition score in Brazilian beef cows.

On the other hand, in New Zealand farmed deer, Ayanegui-Alcerreca (2006) found a 3.7 kg higher live weight in Hardjo seronegative one-year-old deer than in one-year-old deer seropositive with evidence of urine shedding or renal carriage. Subharat et al. (2012) showed that deer vaccinated at approximately three months of age, prior to exposure, had up to a mean of 31g/day higher growth rate, largely depending on seroprevalence, than unvaccinated deer exposed to Hardjo, for a final mean live weight difference of up to 6.4 kg at approximately 12 months of age.

Cattle (Ellis et al., 1981), sheep (Cousins et al., 1989; Gerritsen et al., 1994) and deer (Subharat et al., 2012) are known to be able to shed leptospires in urine and have a high Hardjo seroprevalence in New Zealand (Dreyfus, 2013), making the three species potential Hardjo reservoirs in this country. However, no effect on growth was detected in sheep and cattle while deer did present a growth response to vaccination. These findings suggest a different Hardjo pathogenesis in cattle, sheep and deer.

It is also possible that the chosen design did not allow for the detection of an effect if it was present. This study allowed for the detection of the effect by both an intervention (vaccination) and an observational research approach, but since it was conducted on volunteer commercial farms, it was not always possible to collect data at the desired time. Furthermore, the status of the animals was not known at the time of

vaccination, so a compromise had to be accepted between having animals old enough to be able to produce an immune response to vaccination but that were not yet exposed to Hardjo or Pomona. Subharat et al. (2012) mimicked an infection free status by treating the study animals with antibiotic and vaccinating before joining them with known naturally infected cohorts, thus having control on the pre-vaccination status and the timing of exposure in relation to vaccination. However, this was not logistically possible in our study, and it was decided to evaluate the same vaccination strategy that farmers would normally use if they decided to vaccinate their flock. The vaccine effectiveness observed on these farms (Chapter 4) suggests that maternally-derived immunity did not have an influence on the vaccine effectiveness in preventing shedding in urine.

The false discovery rate method (Benjamini and Hochberg, 1995) was used to adjust for multiple comparisons. Doing so resulted in some results being declared non-significant while they had an unadjusted p-value <0.05 . However, not adjusting for multiple comparisons would have increased the risk of detecting a significant difference by chance only. For example, for a significance limit of 0.05, for 100 tests done where the null hypothesis is true, five will be wrongly rejected and declared significant. In other words, for the vaccination model, 31 comparisons were done, as presented in table 5-2; if there was truly no effect of vaccination, without adjustment the probability of wrongly detecting at least one significant difference would be 80% ($1-0.95^{31}$). This probability would be even higher with the serostatus model where 53 pairwise comparisons were done. While traditional p-values refer to the probability of rejecting the null hypothesis given it is true, the false discovery rate refers to the proportion of rejection of the null hypothesis where the null hypothesis was actually true. Hence, setting the significance threshold at 0.05 implies that no more than 5% of the weight differences that are declared significant were actually not significant. Benjamini and Hochberg's false discovery rate method was shown to be more powerful and less conservative than commonly used adjustment methods such as Bonferroni adjustment, while still controlling the type I error (Pike, 2011; Glickman et al., 2014). Hence, it is unlikely that adjustment would have led to missing some significant differences, but type I errors are still more likely than with Bonferroni-type adjustments.

Hence, in spite of a high exposure to Hardjo on 6/8 farms and to Pomona on 3/8 farms, no evidence of weight loss or growth reduction, or evidence of an effect of vaccination on sheep growth could be shown. However, these farms likely had an endemic status for *Leptospira*. More studies are needed to assess the growth effect of *Leptospira* in a context of outbreak in a naïve flock, or the effect of the timing of exposure on growth.

5.7. Conclusion

This study provides the first estimates of the effects of natural exposure to Hardjo and Pomona on sheep growth in a large-scale field trial. However, it did not permit definitive conclusions: the observed significant differences were either not repeatable from farm to farm, or from one age group to the other, or they were not consistent in direction and magnitude between comparisons between serostatus or vaccination status.

However, that no significant difference in live weight between vaccinated and control sheep was detected, suggests that a widespread recommendation for vaccinating sheep would not result in improved growth or return on investment for the sheep industry when looking at weight gain alone. However, a complete study of production and an economic analysis of the effect of leptospiral vaccination in sheep would require inclusion of any potential effect of exposure and vaccination on reproductive outcomes.

5.8. Conflict of interest

The authors report no conflict of interest.

5.9. Acknowledgements

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Chapter 6. Effects of natural infection by *L. borgpetersenii* serovar Hardjo type Hardjo-bovis, *L. interrogans* serovar Pomona and leptospiral vaccination on sheep reproduction

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6.1. Abstract

Most NZ sheep flocks are seropositive to *Leptospira* serovars Hardjo and/or Pomona, yet vaccination is rare. This study evaluated the impact of exposure to these serovars and of vaccination, on primiparous one- (hogget) and two-year-old (2-tooth) sheep reproduction outcomes.

One third of 2236 randomly selected ewe lambs on eight farms were randomly allocated to the vaccinated group and received a primary and booster bivalent Hardjo and Pomona vaccine starting at one month of age. The other animals were left unvaccinated (controls). Repeated blood samples were taken over one year (n=6 farms, bred as hoggets) or two years (n=2 farms, bred as 2-tooths) for microscopic agglutination test to assess exposure to Hardjo and Pomona. Reproduction outcomes were the proportion of bred ewes scanned pregnant with one or more lambs, the proportion of pregnant ewes rearing a lamb to tail docking and the proportion of docked lambs that were weaned. Reproductive outcomes were adjusted for breeding weights after missing weights were imputed using multiple imputation by chained equations. Logistic models accounting for within-farm clustering were developed separately for animals bred as hoggets (7 months old) and as 2-tooths (19 months old). Odds ratios and their 95% confidence intervals were extracted to compare reproductive performance between vaccinated and control sheep, and within the control group, between seropositive (Hardjo only, Pomona only, both Hardjo and Pomona) and seronegative sheep. Odds ratios and their 95% confidence intervals were also calculated to assess the relationship between vaccination and loss to follow-up from hoggets breeding to hoggets weaning and to 2-tooth scanning.

There was no difference in pregnancy and docking rates between vaccinated and control sheep, or between seropositive and seronegative sheep. Hoggets with a Hardjo titre ≥ 1536 were significantly less likely (OR=0.41, 95%CI=0.19-0.93) to keep a lamb between docking and weaning than hoggets with both Hardjo and Pomona titres < 1536 , for an observed difference in weaning rate of up to 22.6 percentage points on one farm. A reduction of weaning rates in 2-tooths seropositive for Pomona alone and both Hardjo and Pomona was observed but this was non-significant, possibly because of a lack of power. No difference in weaning rate was observed between vaccinated and control hoggets or 2-tooths. On one farm vaccinated hoggets were less likely to be lost to follow-up (OR=0.27, 95% CI 0.08 to 0.95) between breeding and weaning.

Comparing reproduction performance of vaccinated and control sheep revealed no significant difference. However, comparing exposed and non-exposed ewes revealed a possible adverse effect of *Leptospira* on weaning rates. This suggests that a full vaccination program may result in an improvement of reproductive outcomes, possibly by providing herd immunity.

6.2. Keywords

Leptospirosis, sheep, Hardjo, Pomona, vaccine, reproduction, pregnancy, weaning rate

6.3. Introduction

Leptospira are a recognized cause of reproductive loss in sheep (Ellis, 1994; Levett, 2001; Martins et al., 2012). Evidence based on case-series, cross-sectional studies or clinical reports (Ellis et al., 1983; Leon-Vizcaino et al., 1987; West et al., 2004; Cortizo et al., 2014) have associated the presence of antibodies against *Icterohaemorrhagiae*, Hardjo, Australis and Pomona with abortion and perinatal deaths. On the other hand, little is known about the effect of *Leptospira* on the ability to conceive in this species. Genital carriage of *L. borgpetersenii* serovar Hardjo has been observed in asymptomatic ewes (Arent et al., 2013) and *Leptospira* have been identified in fetuses (Ellis et al., 1983; Leon-Vizcaino et al., 1987; Saglam et al., 2008), similar to that in cattle (Ellis et al., 1982; Ellis et al., 1986). However, longitudinal and prospective studies in sheep and experimental evidence are lacking. Andreani et al. (1983) could not reproduce fetal infection and abortion with Hardjo, and Smith et al. (1966) observed abortion after Pomona challenge but only by using uterine inoculation.

Other studies reported clinical disease expressed by fever, jaundice, hepatic and renal dysfunction, haemoglobinuria and anemia and mortality in lambs aged 2-9 months, during outbreaks due to serovar Pomona or *L. interrogans* serovar Hardjo, and in experimental infection of 3-month-old lambs (Hodges, 1974; Smith and Armstrong, 1975; Schmitz et al., 1981; Vermunt et al., 1994). Dorjee et al. (2005) reported 5-15% death rates of 1-4 week old lambs in a case study of 5 farms that experienced Pomona outbreaks.

Exposure to *Leptospira* is endemic in New Zealand sheep. The most recent on-farm seroprevalence study found 91% of flocks seropositive to *L. borgpetersenii* serovar Hardjo type Hardjo-bovis (Hardjo) and 74% seropositive to *L. interrogans* serovar Pomona (Pomona) (Dreyfus, 2013). At the animal level, 43% of sheep were seropositive to Hardjo and 14% to Pomona. Vallée et al. (2015) showed the majority of sheep were exposed between 8 - 15 months of age, which coincides with the hogget pregnancy on many farms. Evaluating potential reproduction effects due to natural exposure would be desirable in the New Zealand context where *Leptospira* exposure is so widespread.

Vaccination of sheep against *Leptospira* was shown to be efficient in reducing urinary shedding when exposed to Hardjo and Pomona during natural (Chapter 4) or experimental (Chapman and Clough, 2014) challenge. However, an economic analysis of vaccination as a control measure requires understanding the effects of infection and vaccination on all aspects of production and health, including reproduction.

This paper reports an investigation of the effects of natural Hardjo and Pomona exposure and leptospiral vaccination on sheep reproduction. The objective was to investigate whether *Leptospira* Hardjo and/or Pomona serological status or vaccination of ewes affected pregnancy and lamb tail-docking and weaning rates.

6.4. Material and methods

6.4.1. Study design, farms and animals

This study was designed as both a randomized field trial and a cohort study, conducted between November 2011 and December 2013 on eight commercial sheep and beef farms in the North and South Islands of New Zealand. A detailed description of the farms is presented in Vallée et al. (2015).

Farms A to G were selected in June 2011 from a previous study on Johne's disease and leptospirosis (Dreyfus, 2013) in which they were seropositive for leptospirosis, and where the farmer agreed to take part. This study used some of the sheep used for the concurrent growth study (Chapter 5). Farm H was selected in 2012 based on its recent history of suboptimal hogget reproductive performance and the known presence of Hardjo and Pomona on the farm. Other investigations on fetal losses in hoggets on this farm have been published elsewhere (Ridler et al., 2015).

An initial sample size of 305 vaccinated and 610 control sheep was calculated assuming a difference in pregnancy rate of 10 percentage points, for a power of 80% and a type I error of 5%. The sample size was doubled to account for within-farm clustering. The actual sample size was limited by mob size, duration of sampling on each farm, culling of non-pregnant ewes and loss to follow-up.

Each enrolled sheep was identified with an individually numbered ear tag and randomly allocated to vaccinated or control group.

On farms A to G between 55 and 106 female lambs for a total of 730 were selected among those born in spring 2011 and on Farm H 94 female lambs were selected from those born in spring 2012. Lambs were randomly selected using a random number generating package. They were vaccinated with a commercial bivalent vaccine against *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona ("Leptavoid 2", MSD Animal Health, batch numbers B258D2A, B403A1A and B677C1A). For each vaccination, 2 mL were injected subcutaneously in the anterior third of the neck, after pushing the wool apart and making a skin fold. The first injection was given at tail-docking on farms A-G, when the animals were 1-6 weeks old, and at weaning (around 18 weeks of age) on farm H. The second injection was given 5-11 weeks later. A third injection, approximating an annual booster, was given 10 to 18 months after the first injection to the animals still in the study (**Error! Reference**

source not found.) Between 24 and 84 vaccinated lambs per farm for a total of 536 were present at pregnancy diagnosis (scanning).

Control female lambs from the same mobs were randomly selected and enrolled on the day of the first vaccination. Between 115 and 217 were enrolled on each farm, for a total of 1506 and a ratio vaccinated:control of approximately 1:2. No intervention was done on the control group. Between 45 and 178 per farm for a total of 1135 control lambs were present at pregnancy scanning.

Blood samples were collected from both control and vaccinated sheep on the day of enrolment, and thereafter from control sheep only on the dates presented in **Error! Reference source not found.** Blood was collected by jugular venepuncture using a 1 inch 20 gauge needle and a red top CAT Plus Blood Collection Tube (BD Vacutainer®). The exposure and seroconversion patterns for Hardjo and Pomona are described in Vallée et al. (2015) and summarized in **Error! Reference source not found.**

6.4.2. Reproduction data collection and outcome definitions

On farms A, C, D, F, G in 2012 and on farm H in 2013 (**Error! Reference source not found.**), the ewe lambs were bred naturally at 7-8 months of age (“hogget breeding”). These animals were followed until the weaning of their first lambs around 7-10 months later (“hogget weaning”). On farms B and E the animals were bred naturally for the first time at 19-20 months of age (“2-tooth breeding”) and followed until weaning of their first lamb (“2-tooth weaning”). Hence, on each farm, reproduction performance was monitored during the first reproduction cycle. Dates of reproductive events are presented in **Error! Reference source not found.**

Pregnancy scanning was conducted using trans-abdominal ultrasonography undertaken by experienced lay scanners or veterinarians at days 45-100 of gestation. A binary status (pregnant or non-pregnant) was chosen as the outcome and pregnancy rate was calculated as the proportion of animals bred that were scanned pregnant. Non-pregnant animals were removed from the study immediately after scanning. At tail docking, when off-spring of ewes were 3-8 weeks of age, the udder of each ewe was palpated by farm staff. Based on the palpation a binary assessment was made of the lactation status of the ewe; if deemed to be lactating it was assumed she had reared at least one lamb to tail docking and if not lactating it was assumed that she did not have any live lambs at that time. Tail-docking rate was calculated as the proportion of pregnant animals that were lactating at tail-docking. This was repeated at weaning, at which time the offspring were 12-18 weeks of age. Weaning rate was calculated as the proportion of animals lactating at tail-docking that were lactating at weaning.

To ensure blinding, the farmers and the persons in charge of scanning and udder palpation were not aware of which animals were vaccinated or the serological status of non-vaccinates, but farmers were informed of the flock seroprevalence.

Table 6-1: Dates of vaccination and management events and number of animals positive (≥ 48) for Hardjo (H) only, Pomona (P) only and both H/P in controls by farm; lambing occurred as hoggets on farms A, C, D, F, G and H and as 2-tooth on farms B and E

Farm		A	B	C	D	E	F	G	H
Vaccination dates		1/10/2011	22/11/2011	17/10/2011	30/09-06/10/2011	25/10/2011	28/10/2011	19/10+7/11/2011	8/01/2013
		15/12/2011	17/01/2012	1/12/2011	24/11/2011	19/12/2011	20/12/2011	1	12/02/2013
		2/08/2012	5/12/2012	6-23/11/2012	9/08/2012	4/04/2013	20/11-7/12/2012	1/08/2012	
No. sheep (vaccinated + control) seropositive at first vaccination	Number sampled	194	210	207	181	217	190	115	198
	H only	122	27	100	5	164	35	26	2
	P only	1	2	10	0	0	0	5	7
	H and P	3	0	22	0	1	0	0	0
Management event									
Breeding	Age first breeding	Hoggets	2-tooth	Hoggets	Hoggets	2-tooth	Hoggets	Hoggets	Hoggets
	Start of breeding	1/04/2012	16/04/2013	1/05/2012	15/03/2012	1/04/2013	7/04/2012	10/04/2012	12/03/2013
	Duration of breeding	5 weeks	5 weeks	2.5 weeks	5 weeks	5 weeks	5 weeks	5 weeks	5 weeks
	Date blood sampling	NA	16/04/2013	26/04/2012	27/03/2012	4/04/2013	3/05/2012	10/04/2012	20/03/2013
	Number sampled	NA	136	188	161	134	163	111	169
	H only	NA	72	0	2	124	31	0	5
	P only	NA	2	1	3	0	5	1	5
	H and P	NA	58	0	0	6	2	0	0
Pregnancy scanning	date	2/08/2012	30/07/2013	NA*	9/08/2012	18/07/2013	18/09/2012	1/08/2012	1/07/2013
	Number sampled	171	139	NA	158	136	NA	111	172
	H only	28	57	NA	12	127	NA	3	19
	P only	6	14	NA	9	0	NA	1	27
	H and P	4	57	NA	0	5	NA	0	5

Tail-docking	date	1/11/2012	4/12/2013	6/11/2012	NA	NA	NA	14-19/11/2012	NA
	Number sampled	106	122	172	NA	NA	NA	192	NA
	H only	83	59	137	NA	NA	NA	75	NA
	P only	0	4	0	NA	NA	NA	1	NA
	H and P	0	56	32	NA	NA	NA	1	NA
Weaning	date	3/04/2013	14/01/2014	28/03/2013	18/12/2012	NA	NA	11/01/2013	18/12/2013
	Number sampled	108	98	77	111	NA	NA	68	109
	H only	82	46	61	95	NA	NA	65	48
	P only	0	5	0	0	NA	NA	0	0
	H and P	3	41	16	2	NA	NA	0	59

* Date of pregnancy scanning not available but results were recorded

NA: Not available

6.4.3. Microscopic agglutination test

The Microscopic Agglutination Test (MAT) was conducted as described in Vallée et al. (2015), based on the method reported in Adler et al. (1982) and Fang et al. (2014). Briefly, two-fold dilutions in 0.9% saline of each serum sample were realised, from 1:24 to 1:3072. The samples were then incubated for 1.5 to 4 hours with live *Leptospira* cultures of either Hardjo or Pomona. Each dilution of each sample was then checked under a dark-field microscope for the presence of agglutination or lysis. The test was considered positive for the highest dilution where 50% or more of the leptospire were agglutinated or lysed.

6.4.4. Statistical analysis

6.4.4.1. Inference of missing breeding weights

Breeding weights were considered important covariates in all reproduction models. Complete case analysis would lead to discarding less than 5% of the observations on farms A, C, D and G, 6.2% on farm H, 13.9% on farm B and 27.4% on farm E, leading to potential bias and lack of power. Separate analysis was conducted for farms breeding as hoggets (Farms A, C, D, F, G and H) and farms breeding as 2-tooths (Farms B and E).

Since the data were missing due to the sheep not being mustered on that date or, the animals were present but the field team did not have enough time to finish collecting data, missing weights were assumed to be missing at random, hence the probability of a weight not being recorded was assumed to be independent from the weight itself, the vaccination, serology or reproductive status, but dependent on the farm.

Missing breeding weights were inferred for each 2-tooth analysis model using a multiple iterative regression imputation or multiple imputation by chained equations, also called fully conditional specification (Su et al., 2011; van Buuren, 2012). No imputation was done for the hogget model, since the proportion of missing breeding weights was less than 10%. Five chains used the previous measured weight, the considered reproduction outcome (pregnancy, docking, weaning) and the exposure status (vaccination or serology status) as dependent variables to impute the missing weights using conditional probability densities. The previous weight was collected four months before breeding on farm B and 11 months before breeding on farm E. Weights variables were log-transformed to avoid imputation of aberrant weights. One thousand iterations were run to draw imputations from the specified conditional probability densities using a Gibbs sampler and the imputation process was checked visually by plotting the mean and the standard deviation of the imputed variables by chain for each iteration. The imputation resulted in five complete imputed datasets, that differed only by the value of imputed weights.

6.4.4.2. Effects on reproduction

A regression model was run on each imputed data set for the 2-tooths or the observed dataset for the hoggets, with the reproduction outcome as the dependent variable, and farm, breeding weight and exposure as independent variables. An interaction term farm by exposure status was tested by the likelihood ratio test, and removed from the model if not significantly improving goodness of fit. The reproduction outcome was a binary variable indicating whether the ewe had at least one lamb at pregnancy scanning, tail-docking or weaning. The exposure variable was a binary variable indicating whether the ewe was vaccinated, or for unvaccinated sheep only whether it was seropositive for Hardjo, Pomona, or both, or seronegative at the time where the reproduction outcome was recorded. Different titre cut-offs from 1:48 up to 1:3072 were tested for the threshold for seropositivity. The regression coefficients and their 95% confidence intervals were computed and pooled across the five different imputed datasets for the 2-tooth models (van Buuren, 2012). Finally, adjusted odds ratios for the effect of exposures on the considered outcomes and their 95% confidence intervals were calculated. These results were log-transformed for easy graphic representation.

Animals that experienced a negative reproductive outcome at pregnancy scanning or docking were removed from the other models with reproductive outcomes.

The effect of seropositivity to Hardjo or Pomona was assessed only for cut-offs and farms where the prevalence for any serovar was more than 10%, less than 95% for the 2-tooth models and 2% or more for a specific serostatus at the sampling time when the reproductive outcome was measured. The prevalence for each flock at each time point can be calculated from the numbers shown in **Error! Reference source not found..**

6.4.4.3. Effects of vaccination on cumulated loss to follow-up

An animal was defined as lost to follow-up if it disappeared from the study for any reason other than culling after a negative reproductive outcome.

The effects of vaccination on cumulated loss to follow-up were calculated between hogget breeding and hogget weaning on farms A, B, C, D, G and H and between hogget breeding and 2-tooth scanning on farms B and E. Logistic regression was used for each period with binary data indicating if the animal was lost to follow-up during the interval under study as the dependent variable, and vaccination status, weight at hogget breeding (around seven months of age) and farm as fixed effects. Two

interactions, “farm by vaccination status” and “farm by breeding weight”, were added. Odds-ratios and the corresponding 95% intervals were calculated.

For all analyses a p-value less than 0.05 was considered significant. The analyses were conducted in R v3.0.3 using package “mice” v2.22 and “ggplot2” v1.0.1. This study was approved by Massey University’s Animal Ethics Committee, under protocol 11/40.

6.5. Results

6.5.1. Reproductive rates

The observed pregnancy, tail-docking and weaning rates by farm, by vaccination status, and by serostatus for the control animals, are presented in Table 6-2. Pregnancy rates ranged from 64% to 100%, tail-docking rates from 79% to 93% and weaning rates from 81% to 96%.

The interaction term “farm by exposure” was non-significant in all of the tested models.

6.5.2. Relationship between vaccination and reproduction

No association was observed between vaccination and reproductive performance for either age group (Figure 6-1).

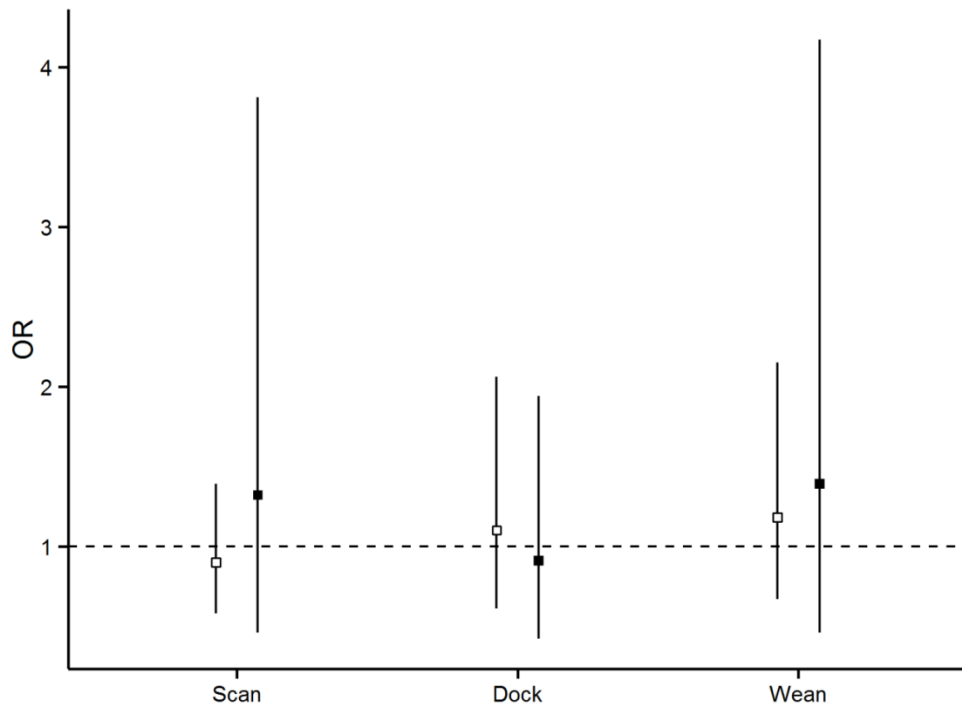


Figure 6-1: Odds ratios (with 95% confidence intervals) of the effect of vaccination on the presence of a live lamb at different reproduction events (scan: at pregnancy scanning, dock: from scanning to tail-docking, wean: from tail-docking to weaning) for both age groups (□: hoggets, ■: 2-tooth). Odds ratios are adjusted for breeding weight.

6.5.3. Relationship between Hardjo serology and reproduction

The associations between Hardjo serostatus for different titre cut-offs and reproduction outcomes are presented in Figure 6-2. Hoggets with a Hardjo titre ≥ 1536 (67 animals) at weaning were significantly less likely (OR=0.41, 95%CI=0.19-0.93) to keep a lamb between docking and weaning than hoggets with both Hardjo and Pomona titres < 1536 . The corresponding observed difference between seronegative (both Hardjo and Pomona titres < 1536) and seropositive (Hardjo titre ≥ 1536) sheep in weaning rate ranged from -10.3 percentage points on farm G to +22.6 percentage points on farm H.

Table 6-2: Reproduction outcomes (number with positive outcome/number tested) for vaccinated sheep, and control sheep stratified by Hardjo (H) and Pomona (P) serostatus at the time of measurement of the outcome within each farm. Note: The number by serostatus does not always sum to total control due to some missing sheep, or sample mis-labelling. Lambing occurred as hoggets on farms A, C, D, F, G and H and as 2-tooth on farms B and E.

Farm	Outcome	Vaccinated		Control			
		Total	H<48 and P<48	H only ≥48	P only ≥48	H and P ≥48	
A	Pregnancy	61/84	129/177	92/130	22/28	4/6	3/3
	Tail-docking	55/58	105/114	21/23	77/83	NA	NA
	Weaning	53/55	100/105	20/21	72/76	NA	2/2
B	Pregnancy	72/75	141/148	10/11	55/57	13/14	54/57
	Tail-docking	54/67	101/123	3/3	49/59	4/4	44/56
	Weaning	45/50	85/98	6/6	41/45	3/5	34/41
C	Pregnancy	52/83	114/178	NA	NA	NA	NA
	Tail-docking	41/50	87/113	NA	66/83	NA	19/26
D	Pregnancy	51/65	128/161	108/136	9/12	7/9	NA
	Weaning	36/44	93/108	12/14	78/91	NA	2/2
E	Pregnancy	68/70	132/138	4/4	121/127	NA	5/5
F	Pregnancy	24/24	45/45	NA	NA	NA	NA
G	Pregnancy	42/52	85/112	81/107	3/3	0/1	NA
	Tail-docking	34/41	73/81	5/5	64/72	NA	1/1
	Weaning	31/33	66/72	1/3	60/64	NA	NA
H	Pregnancy	62/83	130/176	88/119	10/18	21/26	4/5
	Weaning	45/52	90/114	2/2	37/47	NA	46/58

NA: Not applicable

6.5.4. Relationship between Pomona serology and reproduction

The associations between Pomona serostatus for different titre cut-offs and the reproduction outcomes are presented in Figure 6-3. No significant association was observed between Pomona serology and any reproduction outcome.

6.5.5. Relationship between dual Hardjo-Pomona serology and reproduction

The associations between dual Hardjo-Pomona seropositivity for different titre cut-offs and reproduction outcomes are presented in Figure 6-4. No significant association was observed between dual Hardjo-Pomona serology and any reproduction outcome.

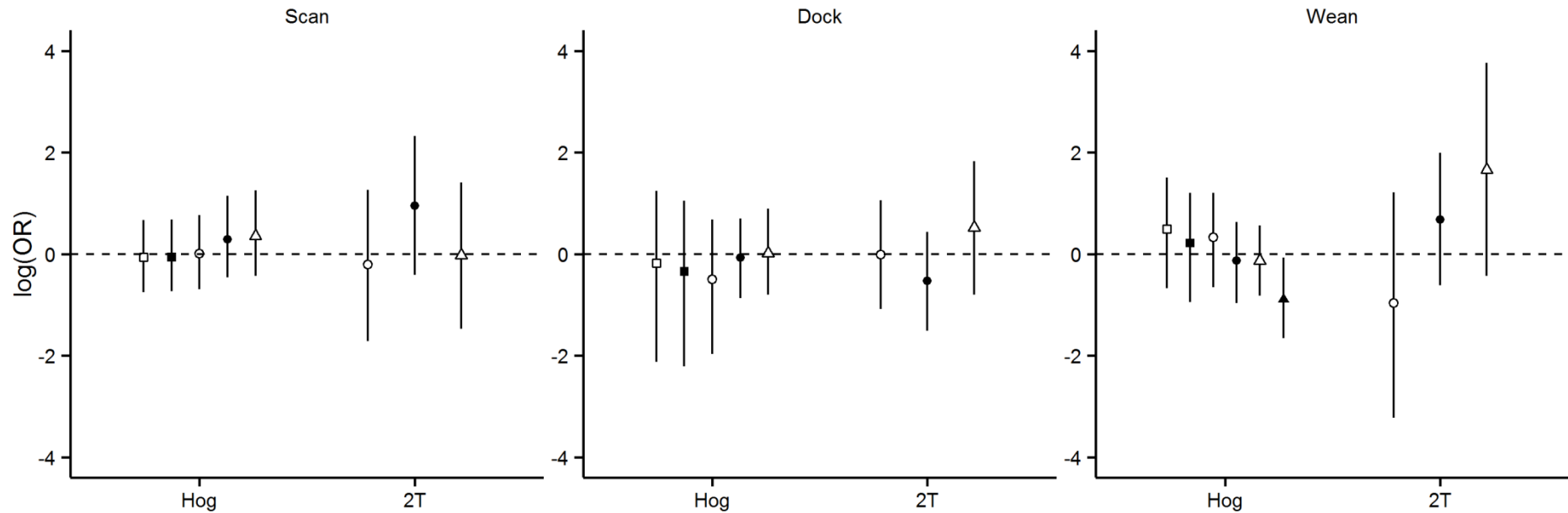


Figure 6-2: Log-transformed odds-ratios adjusted for breeding weight of the effect of Hardjo serostatus of control ewes compared with seronegative ewes using different cut-offs (\square : 48, \blacksquare : 96, \circ : 192, \bullet : 384, \triangle : 768, \blacktriangle : 1536) on having a live lamb for each reproduction outcome (scan: from breeding to scanning, dock: from scanning to tail-docking, wean: from tail-docking to weaning) for both age groups (Hog: hoggets, 2T: 2-tooth), with 95% confidence intervals.

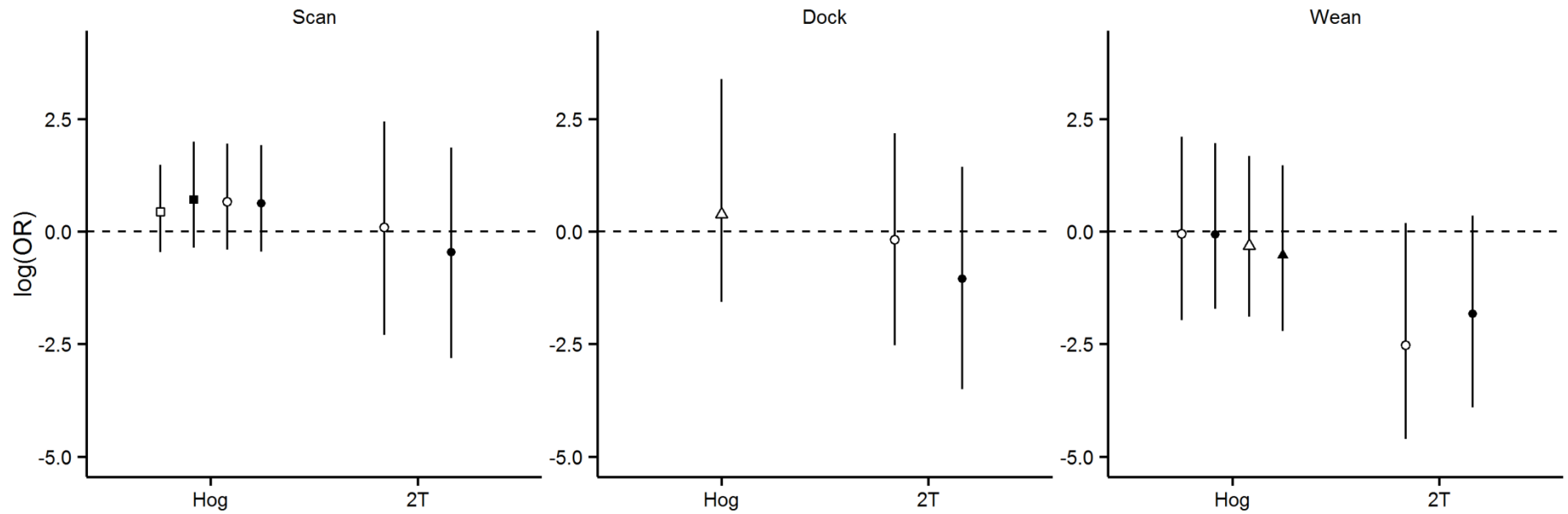


Figure 6-3: Log-transformed odds-ratios adjusted for breeding weight of the effect of Pomona serostatus of control ewes compared with seronegative ewes using different cut-offs (□: 48, ■: 96, ○: 192, ●: 384, △: 768, ▲: 1536) on having a live lamb for each reproduction outcome (scan: from breeding to scanning, dock: from scanning to tail-docking, wean: from tail-docking to weaning) for both age groups (Hog: hoggets, 2T: 2-tooth), with 95% confidence intervals

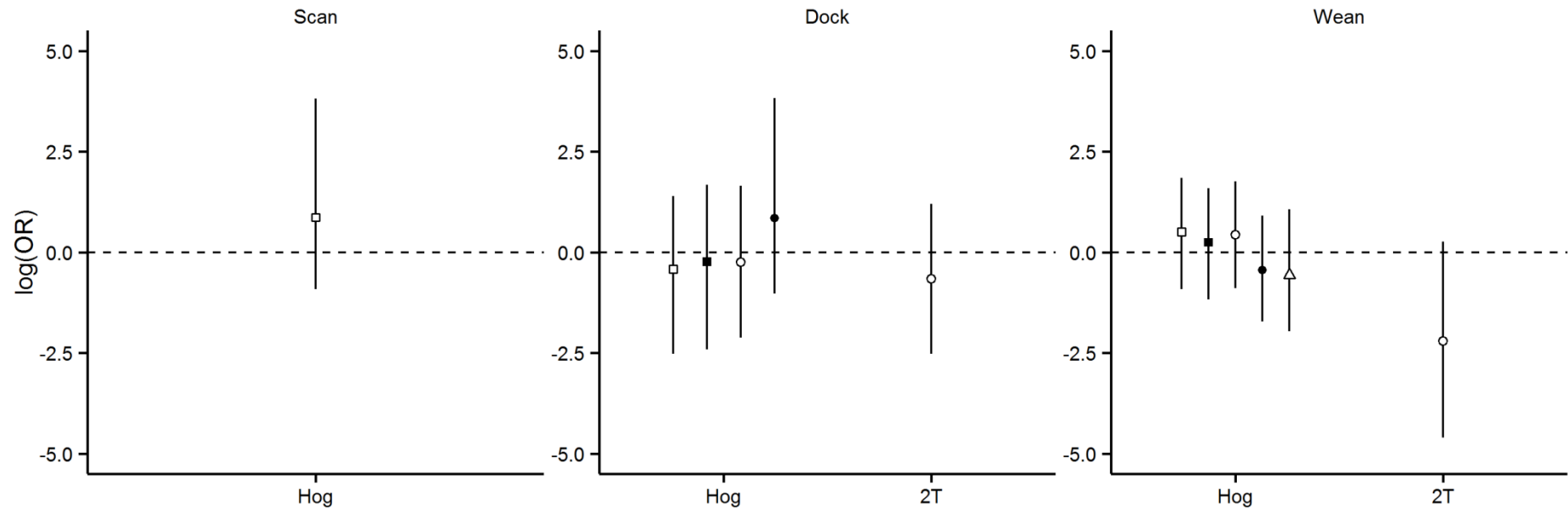


Figure 6-4: Log-transformed odds-ratios adjusted for breeding weight for the effect of dual seropositivity Hardjo-Pomona of control ewes compared to seronegative ewes using different cut-offs (\square : 48, \blacksquare : 96, \circ : 192, \bullet : 384, \triangle : 768) on having a live lamb for each reproduction outcome (scan: from breeding to scanning, dock: from scanning to tail-docking, wean: from tail-docking to weaning) on study farms, for both age groups (Hog: hoggets, 2T: 2-tooth), with 95% confidence intervals.

6.5.6. Relationship between vaccination and loss to follow-up

Between 9% (10/114, farm G) and 25% (39/157, farm C) of the ewes were lost to follow-up between hogget breeding and hogget weaning. On farms B and E, 18% (47/268) and 32% (95/295), respectively, were lost to follow-up between hogget breeding and 2-tooth scanning.

Vaccinated sheep were less likely to be lost to follow-up between hogget breeding and hogget weaning on farm A only (OR=0.27, 95% CI 0.08 to 0.95). On this farm, 7% (4/59) of vaccinated and 17% (21/126) of control sheep were lost to follow-up during this period. Vaccination was not associated with loss to follow-up between hogget breeding and 2-tooth scanning on farms B and E (Figure 6-5).

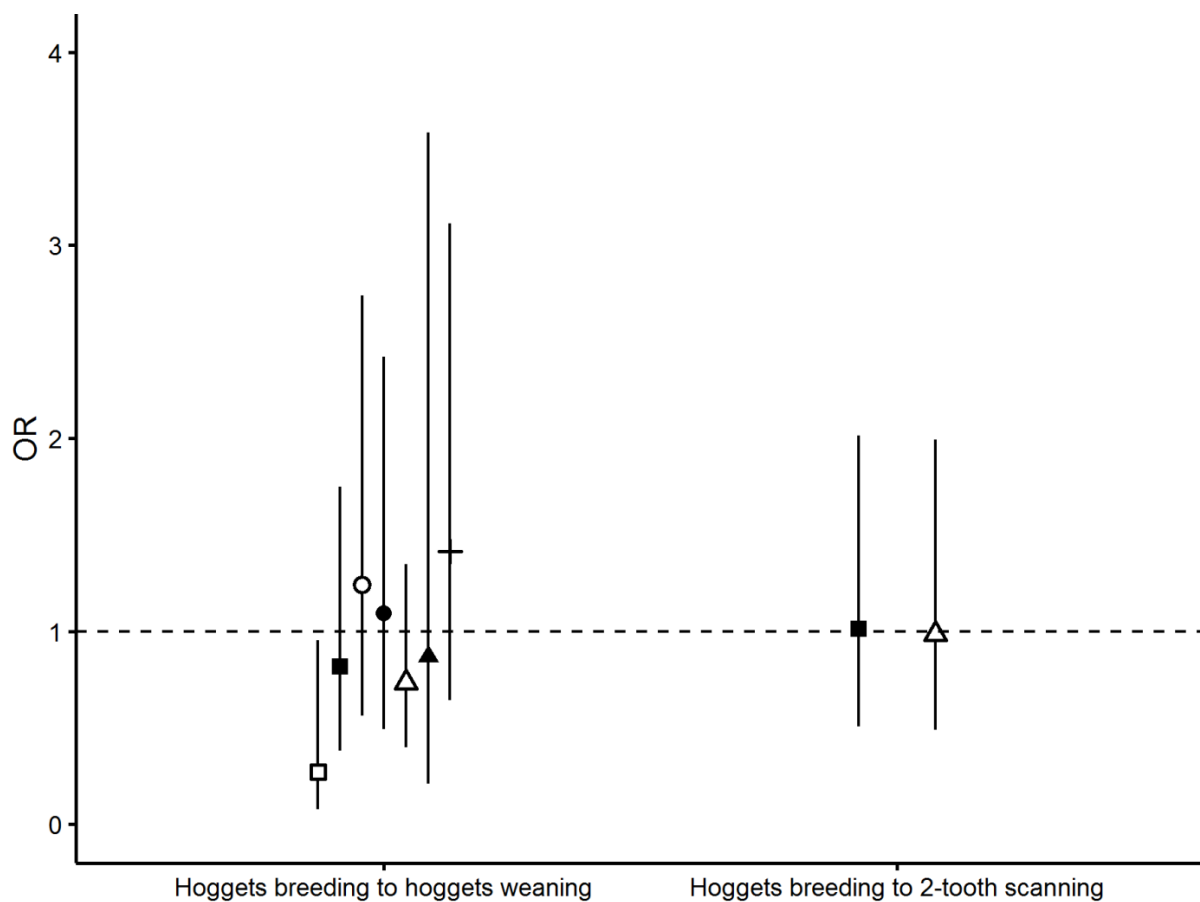


Figure 6-5: Odds-ratio adjusted for breeding weight of the effect of vaccination on loss to follow-up from hoggets breeding to hoggets weaning and to 2-tooth scanning on the different study farms (□: A, ■: B, ○: C, ●: D, △: E, ▲: G, +: H) with 95% confidence intervals

6.6. Discussion

The observed exposure rates observed allowed some insight into the potential effects of natural exposure to Hardjo and Pomona on sheep reproduction. No association between seropositivity or vaccination and scanning rates or docking rates was detected. Hoggets with a high Hardjo titre (≥ 1536) were significantly less likely to keep a lamb between docking and weaning than hoggets below this cut-off for both Hardjo and Pomona titres. The results also suggest a reduction of weaning rates in 2-tooths seropositive for Pomona alone and dual Hardjo-Pomona. However, there was no difference in weaning rate observed between vaccinated and control hoggets or 2-tooths. Vaccination was not associated with improved reproduction performance, but was associated with a reduction in loss to follow-up on one farm, although this association may have been observed by chance.

Udder palpation is commonly used in New Zealand to identify the ewes without lambs at tail-docking (Quinlivan et al., 1977). However, it is unclear how reliable this method is to identify losses between tail-docking and weaning. Self-weaning of lambs reaching a high weight or mis-mothering of lambs old enough to survive cannot be excluded and may have caused bias. However, this bias is likely non-differential as it is unlikely to be associated with serostatus or vaccination status.

The only statistically significant association was observed between docking and weaning in hoggets, where animals with Hardjo titres only of ≥ 1536 were less likely to keep a lamb than animals that had lower titres. A difference in weaning rates of up to 22.6 percentage points was observed for this cut-off. No significant association was observed when lower titres were used to define seropositivity, but the point estimate of the OR decreased as the cut-off increased. Since high titres are more commonly observed within a few weeks of exposure, and titres subsequently decrease (Vallée et al., 2015), the finding suggests that, assuming no type I error, reduction in weaning rates was attributable to exposure to Hardjo close to weaning in hoggets. However, the observation was not consistent with the equivalent comparison of weaning rates in 2-tooths, or between vaccinated and control animals, possibly because the prevalence of titres ≥ 1536 was not high enough to allow sufficient statistical power. Furthermore, the previous sampling was at pregnancy scanning, hence it is difficult to infer precisely when exposure happened on this flock. Clinical disease associated with mortality of 9-month-old lambs was reported with *Leptospira interrogans* serovar Hardjo (Schmitz et al., 1981), but the effects of *Leptospira borgpetersenii* serovar Hardjo-bovis on young lambs are unknown.

No statistically significant association between seropositivity for Pomona, either alone or in association with Hardjo, and reproduction performance was detected. However, this serovar was only detected on three farms, hence reducing statistical power. Odds ratios for lamb losses between docking and weaning were consistently low (0.08 to 0.11) when evaluating the exposure of 2-tooths to Pomona alone or both Hardjo and Pomona, but the effect was not significant. This could also be due to a lack of statistical power since only farm B contributed data to the weaning outcome. Serovar Pomona has been reported as being involved in clinical disease and mortality around birth and in young lambs, hence possibly reducing weaning rates (Smith and Armstrong, 1975; Vermunt et al., 1994).

Pomona seropositivity was associated with mid-gestation fetal losses on farm H (Ridler et al., 2015). However, this result could not be observed again when the losses between scanning and tail docking of other farms were added to the model.

Hence, a lamb loss associated with *Leptospira* exposure appeared to occur between docking and weaning with an observed difference of up to 22.6 percentage points. In New Zealand farmed deer (Subharat et al., 2011) vaccination improved mean weaning rate by 6 percentage points and up to 10 percentage points for individual farms when naturally exposed to Hardjo, but did not influence pregnancy rates.

No effect of Hardjo on scanning and docking rates could be demonstrated, despite a high challenge. The absence of effects would support that sheep are a maintenance host for this serovar under the NZ pastoral grazing system, as suggested by Cousins et al. (1989) and Gerritsen et al. (1994) for European conditions. However, the finding stands in contrast to previous observations suggesting an involvement of Hardjo in fetal losses. The demonstration of *L. borgpetersenii* serovar Hardjo genital carriage in sheep (Arent et al., 2013) and of fetal leptospirosis due to serogroups Hebdomadis, Australis, Sejroe, Icterohaemorrhagiae and Pomona (Ellis et al., 1983; Leon-Vizcaino et al., 1987) imply that subfertility and fetal loss due to leptospirosis is biologically plausible.

Despite the absence of significant difference in reproduction performance between vaccinated and control ewes, a complete vaccination program could protect the flock by achieving herd immunity and protect humans against exposure to *Leptospira*. Vaccination was shown to be efficient in reducing urine shedding when the full course of primary and booster vaccination was given before exposure (Chapter 4). It is also possible that the seroprevalence, especially for Pomona, was not high enough to allow for the difference between the vaccinated and control groups to be statistically significant.

Loss to follow-up included mortality, culling for reasons other than reproductive failure, or loss of ear tag. Vaccination was associated with a reduction in loss to follow-up by 10 percentage points between hogget breeding and weaning on farm A. Since this difference was not observed on the other farms exposed to Hardjo, it is still unclear whether this association can be suggested as causal or if it was observed by chance. Furthermore, no association between Hardjo serology and loss to follow-up was recorded on farm A or the other farms (Chapter 3).

It is still unclear what the effects of infection at before weaning or breeding on sheep reproduction are. Hardjo exposure before weaning of the study sheep as lamb was observed on farm F but only a small number of the study sheep were bred as hoggets and not enough reproduction data could be collected. No early exposure to Pomona occurred on the study farms, so its effects on sheep reproduction in early-exposed flocks are still unknown.

6.7. Conclusion

This study used two methods, namely comparison between vaccinated and non-vaccinated ewes, and between seropositive and seronegative ewes, to assess the effect of *Leptospira* Hardjo and Pomona on reproductive performance in sheep. It has shown an overall effect of *Leptospira* infection in dams on reducing lamb survival from docking to weaning after recent infection with Hardjo. However, there was no statistically significant overall reduction in pregnancy and docking rates associated with either Hardjo or Pomona. While these outcomes were more robust for Hardjo, which was present on all farms, they must be regarded with caution for Pomona since farm-level prevalence was low. The results must also be interpreted in relation to the time of exposure to each serovar, since this varied between farms.

Nevertheless, the data suggest that vaccination for achieving herd immunity and preventing human exposure could be cost-effective by reducing post-natal loss of lambs on some farms. More studies are needed to assess the impact of the timing of infection on the reproduction outcome.

6.8. Conflict of interest

The authors report no conflict of interest.

6.9. Acknowledgements

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Federated Farmers, C. Alma Baker Trust, Agmardt, the New Zealand Veterinary Association, MSD Animal Health, Virbac Animal Health, Zoetis, and Massey University Graduate Research School. We want to thank the farmers who volunteered to take part in this study, Neville Haack from ^mEpiLab, Massey University for his assistance with sample collection and laboratory analysis, and all the vets and other persons involved in sample and data collection.

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Chapter 7. General discussion

7.1. Introduction

7.1.1. Aims of this chapter

This chapter aims to discuss the implications of the results of the studies presented in this thesis and putting them in perspective for the industry, veterinarians, farmers and researchers. The implications of the findings will be discussed, particularly concerning the epidemiology of leptospirosis in sheep and which recommendations can be given regarding vaccination. The methodology employed will also be discussed, defended and reviewed to support the validity of the findings. Finally, new research questions will be proposed and suggestions for future work to pursue research on leptospirosis will be given.

7.1.2. Aims of the thesis

The literature review presented in chapter 2 highlighted a scarcity of information about potential production losses linked to Hardjo and/or Pomona exposure of sheep, as well as cost-effectiveness of leptospiral vaccination. An incidence risk of human leptospirosis of 1.2 cases per 100,000 in New Zealand was reported in 2014, and of 1.4/100,000 in 2013 (The Institute of Environmental Science and Research Ltd. 2015) and contact with sheep is a significant risk factor for human infection, especially in meat workers (Dorjee *et al.* 2011; Dreyfus *et al.* 2015). Vaccination of animals, by reducing shedding in urine and the prevalence of carriers, could be a way to reduce human infection but farmers could be reluctant to do this for this purpose alone. However, vaccination protocols for commercially available products require at least 2 injections in the first year and then a yearly booster, which would lead to a significant cost for sheep farmers relative to financial returns. Demonstrating that vaccination was cost-effective by protection against production losses could be a strong incentive to persuade sheep farmers to vaccinate their animals.

The first objective of this thesis was to investigate the potential effects of natural Hardjo and Pomona exposure and leptospiral vaccination on sheep growth and reproduction. For this, a split-flock vaccination trial with vaccination of one third of 2260 ewe lambs on eight commercial farms was implemented. Regular blood samples were collected from non-vaccinated sheep and tested by MAT to assess exposure. Individual weight and pregnancy, docking and weaning data were recorded and compared between vaccinated and non-vaccinated animals, and between different serological statuses of non-vaccinated animals.

Data from the control groups also allowed describing the serological patterns to improve understanding of the on-farm epidemiology of Hardjo and Pomona. Such data had not been reported to date.

In addition, assessing the adequacy of the vaccine protocol was necessary, and this was done by measuring the vaccine effectiveness on *Leptospira* shedding in urine. Urine samples were collected from a random subsample of both vaccinated and unvaccinated sheep on each farm one to two years after the beginning of the study and the samples were analysed by real-time PCR.

7.2. Critical analysis and interpretation of findings, relevance and comparison with current knowledge

7.2.1. Summary of findings

The description of serological patterns showed that all eight farms reached a within-flock Hardjo prevalence of at least 79%. Pomona exposure was demonstrated on three farms, with a within-flock prevalence ranging 21-54%. This high Hardjo exposure allowed for meaningful comparison by serostatus, but the lower prevalence of Pomona may not have provided enough statistical power to detect an effect of this serovar.

This study also gave an insight on the dynamics of infection on farm, with most of the Hardjo exposure occurring in lambs aged 8-17 months, and Pomona exposure in lambs aged 4-14 months. This revealed the period at risk for potential production losses, as this period overlaps with the age of hogget pregnancy. Exposure to Hardjo before weaning was demonstrated on one farm, but no growth effect could be detected. However, this period could be more crucial for growth effects, especially in lambs destined for slaughter, and more data would be needed on the impact of the age of exposure and the effects on growth of exposure to Hardjo and/or Pomona before weaning.

The presence of maternal antibodies was also demonstrated, especially for Hardjo, with a prevalence ranging 3-76% across all eight farms, but the urine shedding results showed that this did not impact on vaccine effectiveness. The research demonstrated an overall vaccine effectiveness of 86.3% on shedding in urine, and also that vaccination of lambs already exposed to Hardjo reduced effectiveness. The effectiveness was also lower on another farm where animals were vaccinated after weaning, but those animals were seronegative for Hardjo and Pomona. This high vaccine effectiveness confirmed that the vaccine protocols used on the study farms were working in spite of not fully following manufacturer's recommendations (two injections four to six weeks apart, with a booster at six months of age if primary vaccination was completed before) with

increased interval between the two injection of primary vaccination and interval to booster, and that any absence of production effect could not be due to vaccine inefficacy.

A large variability in the magnitude and direction of live weight differences between seropositive and seronegative control sheep and between vaccinated and control sheep made any conclusion about the effect of *Leptospira* infection on growth difficult. No difference in pregnancy rates and docking rates were observed between seropositive and seronegative sheep or between vaccinated and control sheep. However, recent Hardjo exposure was associated with a lower weaning rate in hoggets and Pomona exposure was possibly associated with lower weaning rate in 2-tooths, although power was lacking to reach statistical significance. However, no difference in weaning rate was observed between vaccinated and control sheep.

7.2.2. The notion of reservoir species status: is the sheep a reservoir for Hardjo in New Zealand?

Several definitions have been given for the notion of reservoir species. Hathaway (1981) discussed that a reservoir host for *Leptospira*, which would constitute a maintenance population, should require a low infective dose of leptospire, be able to maintain the bacteria in the kidney for a long time relative to the length of the bacteraemic period, and be able to transmit it within the species. Cattle were the only species presented as a reservoir for Hardjo. The results in this thesis and specifically in chapter 3 are compatible with, but do not directly demonstrate, high susceptibility and sheep-to-sheep transmission. More studies, in particular transmission modelling, should be implemented to differentiate sheep-to-sheep transmission and exposure to a common source. The shedding data in chapters 3 and 4 also showed a long carriage time, with animals still shedding up to 14 months after exposure started on the farm. However, more data is needed to quantify more precisely the length of Hardjo shedding in naturally exposed (and possibly regularly re-exposed) sheep.

More recently, Haydon *et al.* (2002) proposed a conceptual framework in which reservoir hosts should be defined relative to a target species, which would be humans in a New Zealand situation. Within this framework, sheep would need to be able to maintain Hardjo infection indefinitely and to infect humans. The data presented in chapter 3, along with the high seroprevalence (Subharat *et al.* 2007; Dreyfus *et al.* 2011; Fang *et al.* 2014) and renal carriage (Dorjee *et al.* 2008; Fang *et al.* 2014) recorded in the past ten years are consistent with maintenance of Hardjo by sheep in New Zealand, but does not provide certain evidence. More robust evidence was given for *L. interrogans* serovar Hardjo by Cousins *et al.* (1989) who could initiate infection and shedding persistent for at least 11 months in a sheep flock without any contact with cattle. However, to the author's knowledge no similar data exist for *L. borgpetersenii*

serovar Hardjo. If Haydon *et al.* (2002) do not propose the absence of pathogenicity as a criteria, the low to absent production effects and clinical disease associate with Hardjo would make contact with human more likely, as less caution will be taken when handling apparently healthy sheep than for example an aborted ewe. According to this framework, it is likely that sheep are part of the reservoir for Hardjo relative to human infection, in association with cattle and deer. In other words, controlling Hardjo in sheep alone would not be efficient in fully stopping Hardjo infection incidence in human without control in other livestock species, but would help reducing it.

Blackmore *et al.* (1982) suggested that the sheep was not a maintenance host for Hardjo, based on an observed seroprevalence (MAT titre ≥ 48) of 18.5% in 928 sheep of different age groups, and the isolation of Hardjo by culture from 3/180 kidneys sampled from two meat plants during a period of six months. The seropositive sheep were from 43 farms, 46% of which had a least one positive sheep. However, older data (Ris 1975) suggested Hardjo could be widespread without causing disease in sheep, and showed intra-flock prevalence of 30-100%. Hence this was already consistent with sheep being a maintenance host for Hardjo. It is possible that the conclusions of Blackmore *et al.* (1982) were made on a sample subjected to selection bias. Furthermore, the test used to detect carriage (culture), could have been less sensitive than current assays (like PCR), thus underestimating the carriage prevalence.

7.2.3. Between-farm variability of the Pomona status in New Zealand

While all farms got exposed to Hardjo, with an invariably high within-farm seroprevalence, only 3/8 got exposed to Pomona, with maximum within-farm prevalence ranging 20.8-54.1%.

All recent clinical communications about suspected leptospiral abortion in sheep in New Zealand involved Pomona (West *et al.* 2004; Bruere 2013; Ridler *et al.* 2015). Furthermore, Pomona has been incriminated in clinical disease in lambs (Vermunt *et al.* 1994). The question arose as to why no clearly significant growth or reproduction effect could be associated with Pomona. A reduction of weaning rates was suspected, but possibly suffered from a lack of power. Lack of power could also have been a problem for growth effects. However, the weight difference was between 0 and 1kg, in either direction, suggesting that even with a bigger sample size the effect, if any, would be too small to achieve biological relevance. It could also be a consequence of the selection bias (discussed below), with the farms being endemically infected and a possible bigger effect could occur if the flock was completely naïve prior to exposure. The same remarks can be made about the effects of Pomona on scanning and docking rates.

The suspected reduction of weaning rates associated with Pomona exposure occurred in 2-tooths only. These data came from one farm only. The two other farms

exposed to Pomona were mated as hoggets, hence providing data for the effects in hoggets only. The observation of losses between docking and weaning are biologically plausible given the reports of lamb mortality associated with the presence of Pomona (Vermunt *et al.* 1994), although no clinical manifestations were reported by the farmer. Suspect peri- and postnatal mortality is consistent with observations in deer with Hardjo (Subharat *et al.* 2011). No straightforward explanation as to why the effect would occur in 2-tooths and not in hoggets can be given, especially considering that exposure occurred at the time of hogget pregnancy on all farms. Strain variability could be suspected, with the strain affecting the farm which first mated ewes as 2-tooth being more virulent. Molecular methods would help investigating this hypothesis. Fang (2014) using a multilocus sequence typing method on six isolates collected at a slaughter house from the kidneys of sheep seropositive for Pomona found that the isolates were actually identical to a strain of *L. interrogans* serovar Kennewicki from the Pomona serogroup. Since the implementation of molecular methods in New Zealand is recent, it is not known if Kennewicki is the only representative of the Pomona serogroup in New Zealand, and if not, how much variability in virulence exist between the different serovars and strains.

7.2.4. Maternal antibodies in sheep

So far little has been known about maternally derived antibodies against *Leptospira* in sheep. Andreani *et al.* (1983) measured the maternal antibodies in lambs born from ewes experimentally challenged with *L. interrogans* serovar Hardjo. They had titres ranging 1:1000 to 1:50,000 two days after being fed colostrum and became seronegative (MAT titre <100) after a month. The vaccine manufacturer (“Leptavoid-2”, MSD Animal Health, October 2012, warns that maternal antibodies can interfere with vaccination before six months of age on the product label (http://www.msd-animal-health.co.nz/binaries/Leptavoid_2_Label_tcm51-37062.pdf).

This on-farm study starting at docking, with lambs around one month of age, provided data on the duration of seropositivity in lambs from dams naturally exposed to Hardjo and/or Pomona. Maternal antibodies against Hardjo were detected up to three months of age, but those against Pomona seemed to have disappeared faster. The half-life of Pomona titre in adults after infection was also slightly lower than the half-life for Hardjo. Estimations of agglutinating antibodies duration in human after infection (Dreyfus *et al.* 2015) also showed that Pomona antibodies remain ≥ 48 for a shorter time than Hardjo (10 months vs. 29 months). The observed Hardjo GMT on farms with maternal antibodies ranged 57 to 162, while on the farm with Hardjo exposure in lambs the GMT was 90, suggesting that no cut-off for GMT can help make the distinction between passive and actively derived antibody at the farm level.

The vaccine effectiveness measured by real-time PCR on urine was not reduced by the presence of maternally derived antibodies at a seroprevalence of up to 76%. This indicates that lambs on farm can be vaccinated from tail docking, even in endemically infected flocks, being effective in preventing urine shedding provided that vaccination was completed before exposure. However, to provide complete vaccination guidelines for sheep, information on the effect of dam vaccination on lamb immunity, protection and vaccine interference would be needed. Palit *et al.* (1991) found that calves born from vaccinated dam were protected against experimental Hardjo infection in spite of the presence of maternal antibodies. Ayanegui-Alcerreca (2006) showed that 2-month-old deer calves born from vaccinated hinds had a higher Hardjo and Pomona seroprevalence than calves born from control hinds on one farm, but the GMT was similar between the two groups. The seroprevalence had declined after one month, but the antibodies could persist for up to 9 months in non-infected herds. The author also did not find any evidence of interference with vaccination, as measured by serology but not by shedding in urine.

It is also unknown whether maternal antibodies are protective for lambs. The absence of interference with vaccination and the presence of maternal antibodies and real infection of lambs at the same time on one farm suggest that they may not be protective. Another explanation would be that some lambs on this farm did not receive an adequate amount of antibodies in the colostrum. In a controlled trial of calves born from dams vaccinated against Pomona and challenged at 1 and 4 weeks of age (McDonald and Rudge 1957), 0/10 and 1/15 calves, respectively, showed signs of infection, suggesting that, at least for a short period after birth, these antibodies can be protective against infection.

7.2.5. Cost effectiveness of vaccination in sheep

No effect of seropositivity or vaccination could be demonstrated on sheep growth. The only possible effect observed, albeit marginally non-significant ($p < 0.1$), was that vaccinated hoggets at the time of weaning of their lambs were lighter by 1.8kg than non-vaccinated hoggets on one farm. It is possible that this difference was simply due to sampling variation. This may also be revealing the biological cost of the vaccination, where animals use some resources to build immunity. Similar weight losses due to vaccination are not uncommon and were already observed by Goodwin-Ray *et al.* (2008) in a vaccination trial for pasteurellosis. This was however, not observed on the other farms and occurred a year after vaccination, making this hypothesis less plausible. Another possible explanation for this weight difference on this farm may be related to the weaning losses associated with Hardjo. On this farm, the weaning rate was 87% (45/52) in the vaccinated and 79% (90/114) in the control hoggets ($p = 0.34$, Pearson's chi-square). It is possible that hoggets that lost their lambs were able to use the energy for gaining weight instead of producing milk, hence being heavier. Since these animals

were over-represented in the control group, the control group could have appeared heavier. This farm had the highest observed difference of weaning rates between vaccinated and control sheep, which would explain why the weight difference was not observed on the other farms. Furthermore, on the same farm, the highest difference in weaning rates between sheep seropositive for Hardjo and sheep seronegative was reported (22.6 percentage points). This supports the hypothesis that the higher weight in control sheep is actually an indirect effect of the reproduction losses.

Losses from docking to weaning seem to be the main production losses associated with leptospiral infection. The maximal observed reduction in weaning rates due to seropositivity (MAT titre ≥ 48) was 21 per 100 hoggets for Hardjo and 40 per 100 hoggets for Pomona, though this was not significant. Looking at the cost-effectiveness of vaccination at the industry level should take into account the possibility of avoiding human cases, especially in meat workers. Vaccination of sheep would reduce the risk for people in contact with sheep, presumably mainly meat workers, farmers and veterinarians. Meat workers at one plant were shown to be exposed to 3 to 54 carcasses per day depending on the work position and whether it was a period at risk such as after floods (Dorjee *et al.* 2011). Dreyfus *et al.* (2014) found a seroprevalence of 4 to 28% for Hardjo and 5 to 16% for Pomona in workers in 4 sheep plants, while Sanhueza *et al.* (2015a) found a seroprevalence of 0% for Hardjo and 2.6% for Pomona among 178 farmers, all farming types included. Sanhueza *et al.* (2015b) found a seroprevalence of 2.2% for Hardjo and 2.5% for Pomona from 277 veterinarians with greater risk to those dealing more with cats and dogs and those home slaughtering cattle or pigs. Dreyfus *et al.* (2015) found, in sheep plant workers, an annual risk of clinical manifestations of 0.78% and reported that among the new infections, 47% had influenza-like symptoms requiring an average of 4.4 days off work, which suggest that vaccination of sheep would also have a significant economic impact on human health. However, this remains to be quantified. This work is currently in progress.

7.3. Chosen methodology and effect on conclusions

7.3.1. Selection bias and marginal effects

The eight study farms were selected with little to no random process, possibly introducing a selection bias. Farms A to G were selected from participants to a cross-sectional study on Johne's disease and leptospirosis for which farms were selected at random (Verdugo *et al.* 2010; Dreyfus *et al.* 2011), based on number of breeding ewes and cattle and the known presence of Hardjo and/or Pomona. All the farmers pre-selected from the database agreed to participate in the study. Farm H volunteered for the study a year later, after observing production losses that were suspected to be related to Pomona (Ridler *et al.* 2015).

Any introduced selection bias would likely have had little effect on internal validity, as an effect would arise only if the bias was differential, i.e. if the bias was different for the compared groups (Elwood 2007) p82. Here, since the vaccinated animals were selected at random within farms, and the exposure occurred naturally, the selection bias was the same for vaccinated and control sheep and for the seropositive and seronegative sheep, which means that the estimates found in the studies presented in this thesis are likely accurate for this study population.

The effect of potential selection bias on external validity is likely more important than the effect on internal validity. The selection process did not provide a sample of farms that was representative of New Zealand sheep farm. These farms could have some specificity: large farms are over-represented, which could be associated with specific management practice. Even if no farmer declined to participate after being contacted, farmers aware of leptospirosis and the associated risk are possibly over-represented, which could have led to a selection bias towards farms endemic and/or more at risk for leptospirosis. Even if the target population is farms with Hardjo and/or Pomona exposure, the study farms could have a specific exposure profile. However, it would have been difficult to randomly select farms from farms exposed to Hardjo and/or Pomona without relying on the farmer's acceptance and participation, and the risk of not having enough exposure on the study animals during the study period would have been bigger. The serological patterns described in chapter 3 would not be possible to generalize to all sheep farms in New Zealand, but it provides a first idea on the exposure patterns, especially for Hardjo considering the consistency of pattern across 7 out of 8 study farms. The extrapolation of the production effects to other sheep farms is also challenging, as other unmeasured management factors also likely act as confounders or as effect modifiers, such as feeding or general flock health. Hence, it cannot be excluded that on farms where sheep are under more stress, for example less feed available or concomitant disease, significant production losses happen.

Furthermore, the data was collected over one production cycle only, with animals experiencing a maximum of two annual weather patterns for the animals mated as 2-tooth. The summer 2013-2013, during which lambs from hoggets were weaned and preceding the breeding period of 2-tooth, was recorded as the driest in 40 years in New Zealand (Porteous and Mullan 2013). Hence, the significant association between the presence of high Hardjo titres, likely due to recent exposure, and lamb loss between docking and weaning during this summer may not be revealing the "normal" situation, where the animals would not be submitted to the same stress. A longitudinal monitoring of flocks over several years and several birth cohorts, on farms selected at random from farms exposed to Hardjo and/or Pomona, could help quantifying the magnitude of the selection bias, validate these results by replication, or refute them.

If increasing sample size does not correct non-random sample selection, the small number of farms selected, in addition to increasing uncertainty, could also have exacerbated potential selection bias because it failed to cover the whole variability spectrum of the eligible farms. On the other hand, if the sample size was bigger, there would have been a risk of being more precise around a biased estimate. The number of farms that could be visited regularly enough for the study was limited by the time and human resources available for this project.

The structure of the data involved repeated measures on sheep and clustering within farms. The presence of this potentially important selection bias made the use of a random effect for farm to account for this clustering less desirable, as they would assume that the sample of flocks represent a population, since they are modelled assuming they come from a general population using a random process (Gelman and Hill 2006; Dohoo *et al.* 2009). The same goes for marginal models (or population average models) such as GEEs that would be interpreted as the effects on an average farm, which does not make sense considering the absence of random selection. Furthermore, the use of fixed effects allowed the use of interactions to quantify the change of effects between farms.

7.3.2. Split-herd trial: comparison of design with a similar work in deer

A previous PhD work (Subharat 2010) implemented similar studies as those presented in chapters 5 and 6 to detect an effect of vaccination on farmed deer naturally exposed to Hardjo and/or Pomona, also using a split-herd vaccination trial design. The main differences in design between the two works are presented in

Table 7-1.

Table 7-1 Differences in design between Subharat (2010) in red deer and the current work in sheep

Study design item	Current work	Subharat (2010)
Number of farms and average number of animals per farm	8 farms 169-324 per farm	5 farms (growth), 6 farms (reproduction) 60-120 per farm (growth) 40-84 per farm (reproduction)

Ratio vaccinated:control	1:2 for study animals, grazed with non-study animals	1:1 for study animals, grazed with non-study animals
Age at enrolment	Docking Closest possible to birth, around months old (weaning on one farm) High prevalence of maternal antibodies at d0	3-month-old (growth) Rising 2-year-old (reproduction) Likely no maternal antibodies
Outcome measured by sex of animals	Females only, same animals for growth and reproduction	Males for growth Females for reproduction
Infection status at d0	Uncontrolled Looked at when the study started; resulted in vaccination of infected animals on one farm	Controlled Administration of antibiotics (streptomycin) to clear any possible pre-existing infection and renal carriage to mimic infection of naïve herd
Timing of infection	Uncontrolled Age of exposure was what would be naturally occurring on farms	Controlled Study animals first isolated and treated then joined with untreated flock at 5-month-old (growth study) or after weaning (reproduction)

The studies used different strategies to ensure non-infected animals were vaccinated. Subharat (2010) ensured the animals were free of leptospire by treating them with antibiotics. This was logistically not possible for this study, which included many more animals, which means the cost would have been higher. The large-scale use of antibiotics is also questionable and may not have been accepted by the farmer. Hence, enrolling animals as young as possible was the alternative. This allowed reproducing a vaccination schedule that would likely be the one used by the farmers and test its effectiveness, taking in account the possible presence of maternal antibodies and the risk of pre-existing infection.

Furthermore, the design used by (Subharat 2010) allowed control on the timing of infection, which could have an impact on the effects on production. In cattle, it was suggested that abortion linked with Pomona occurred mainly in the last trimester, while abortion due to Hardjo could occur at any stage (Ellis 1994). In addition, growth effects would have the most economic impact if they occurred on slaughter lambs, so exposure on lambs young enough would be necessary to quantify these effects.

Hence, if the sheep were exposed before or after the period at risk for the considered serovar, no effect on production would be observed. Controlling the timing of exposure in a similar way was logistically difficult in this study, but the chosen design allowed for a more accurate quantification of the production effects at the population level, since the occurrence of early or late exposure was not modified.

7.3.3. Reduction of type I and type II errors

In observational studies such as those presented here, the researcher may have little control on the quality of the data available. Hence, precautions were taken at the analysis stage to increase the robustness of the production effect results, by reducing both type I (rejecting the null hypothesis when it was true, or declaring a result significant when it was not) and type II (failing to reject the null hypothesis or failing to detect a significant effect) errors at the analysis stage.

7.3.3.1. Multiple comparison adjustments to reduce type I error

Analysis of growth effects involved 31 pairwise comparisons for the vaccination model and 53 pairwise comparisons for the serological status model with cut-point at 48. Hence, the probability of finding at least one significant weight difference just by chance (with a significance threshold of 0.05) would be 80% and 93%, respectively, for each model. Hence, adjusting for multiple comparisons was necessary to avoid type I errors. The method chosen, the false discovery rate (Benjamini and Hochberg 1995), discussed in chapter 5, is very easy to implement as it is done post-analysis using only the set of unadjusted p-values, ranked in increasing order. The only “disadvantage” of the method is the change in the interpretation of the p-value. However, the new interpretation (no more than 5% of the weight differences that are declared significant are actually type I error) may be more intuitive and more relevant to the interpretation of the findings, when the researcher looks at the probability of a result to be a “false discovery”. This method should be encouraged when adjustment for multiple comparisons is needed, in replacement for conservative methods such as Bonferroni adjustment (Glickman *et al.* 2014).

7.3.3.2. Multiple imputation to reduce type II error in observational studies

Missing data arise commonly in observational studies like this one, especially when occurring on commercial farms, with no specific directive given to the farmer on management of the flock, and for a study lasting up to 2.5 years. The missing data were partially accounted for in the analytical studies of chapter 6, but the missingness due to loss to follow-up (animal disappearing completely instead of once) was not accounted as this would be part of the normal procedure on farms. The data missing due to a single

missed sampling was accounted for by multiple imputation in chapter 6, where the weight was a covariate, but not in chapter 5 where the weight was the outcome. A recent simulation study (Dohoo and Emanuelson 2015) showed that multiple imputation for missing outcome was of little impact on the results, as measured by the proportion of bias, the mean SE of estimates and the proportion of estimates that differ from the true value by no more than 20%.

The use of multiple imputation was efficient and straightforward using the “mice” package in R, while providing different options for the imputation method (van Buuren 2012), but did require some learning about the different options and concepts behind missingness patterns and multiple imputation methods. It avoided the use of complete case analysis, the usual approach and default method in statistical packages, but which decrease the power of the study. The mating weights were assumed to be missing at random, a situation for which multiple imputation has been shown to reduce bias and increase power when compared to complete case analysis (Dohoo 2015). Hence, the effort required for implementing the method resulted in more robust estimates of the reproduction effects, but were not worth implementing for the analysis of growth effects since the missing values were for the outcome. The use of methods to account for missingness, at least for predictors in analytical studies, should be encouraged.

7.4. Conclusions and suggestions for future work

The results observed in the work presented in this thesis provided some answers on the on-farm Hardjo and Pomona serological patterns of hoggets and 2-tooths, on the vaccine effectiveness with respect to shedding in urine, on the effect of exposure on production and on the cost-effectiveness of vaccination. However, it also led to new questions that can be suggested as future research.

The total burden of leptospirosis for New Zealand remains to be estimated. It should take into account the effect on animal production including beef cattle but also animal welfare. Furthermore, it should aggregate data on human health, with most of the data already available thanks to the work of Dreyfus (2013) and Sanhueza (work in progress). This would allow estimation of cost-efficiency of livestock vaccination to be more accurate, for each species, as it will depend on the origin of human exposure. Vaccination of dairy cattle along with awareness campaign was shown to significantly reduce incidence in humans (Marshall 1987).

The determinants of the observed peak of exposure should be understood. Is there a real seasonality? Would this pattern be reproduced during several years and across a more representative sample of farms? Is this high peak compatible with sheep to sheep transmission? Disease transmission modelling using the data in this thesis is in

preparation, in association with the University of Lancaster, to try to identify possible common source exposure such as wildlife. Furthermore, little recent data on the prevalence of the different serovars in wildlife in New Zealand is currently available. Hence, investigating the different serovars and strains present in New Zealand wildlife, including in animals around farms, would help understand the epidemiology of *Leptospira* in this country.

Growth losses would have the most economic impact if they occurred on slaughter lambs. However, only one farm was exposed to Hardjo and none to Pomona before weaning. To be complete, the analysis of production effects should include estimation of the effects on lambs exposed before weaning. This may, however, be difficult to implement if this situation is rare on commercial farms. However, how often early or late exposure occurs would be relevant information in itself, as no data is available on it. Furthermore, extending this study to several production cycles could give information on possible effects of chronic infection. Leptospirosis is associated with renal carriage and lesions in the host species (Faine *et al.* 1999), it is thus possible that these lesions would have an impact on production but not straight after infection.

Some farm to farm variability, in both production effects and exposure, especially for Pomona, was observed on this sample of farms. However, as discussed above, this sample of farms was not representative of all sheep farms in New Zealand. Some data on the exposure profile of Pomona on farms in New Zealand, conducted on a representative sample, would be necessary, including for estimation of production effects at the industry level. Furthermore, the variability in the effects should be explored using molecular methods such as the multilocus sequence typing used by Fang (2014) or whole genome sequencing (Collins-Emerson *et al.* 2015) to assess between farm variability of strains. Such work, including whole genome sequencing, is currently underway at Massey University and a preliminary study showed that the New Zealand strains of Hardjo-bovis and Pomona were genetically distinct from reference strains (Collins-Emerson *et al.* 2015) In addition, comparing strains between farms, within species, would inform possible interspecies transmission.

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Appendix

Appendix 1: Link to online repository containing the raw data used for this thesis

<https://www.dropbox.com/sh/ni7ahltoq0st80f/AACewV1CyW5qJBkXxoJXyI2ea?dl=0>

Appendix 2: Published article: Serological patterns, antibody half-life and shedding in urine of *Leptospira* spp. in naturally exposed sheep

Vallée E, Heuer C, Collins-Emerson JM, Benschop J, Wilson PR. Serological patterns, antibody half-life and shedding in urine of *Leptospira* spp. in naturally exposed sheep. *New Zealand Veterinary Journal* 63, 301-12, doi:10.1080/00480169.2015.1049668, 2015

Clinical Communication

Factors associated with fetal losses in ewe lambs on a New Zealand sheep farm

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Abstract

CASE HISTORY: As part of a production study of ewe lambs on a large farm in the Waikato region of New Zealand in 2011, pregnancy diagnosis was undertaken twice by trans-abdominal ultrasonography at 68–103 and 97–132 days of gestation. At the second pregnancy diagnosis 257/3,790 (6.8%) ewe lambs had evidence of non-viable fetuses or absence of a pregnancy that was present at the previous pregnancy diagnosis (fetal loss).

LABORATORY FINDINGS: Serum antibody titres for *Leptospira interrogans* serovar Pomona appeared generally higher in 10 ewe lambs with fetal loss compared with 10 that were still pregnant. Histopathological investigation was not able to confirm that fetal loss was associated with leptospiral infection.

EPIDEMIOLOGICAL INVESTIGATION: In the 2012-born cohort of ewe lambs 443 were vaccinated with a bivalent leptospirosis vaccine and 882 unvaccinated. Serum was collected from 124 non-vaccinated ewe lambs between January and December 2013 for measurement of antibodies to *Leptospira* serovar Pomona and *L. borgpetersenii* serovar Hardjo-bovis using a microscopic agglutination test (MAT). Less than 5% of these ewe lambs were seropositive until May, but by August 85% and 48% of animals were seropositive to *Leptospira* serovars Hardjo-bovis and Pomona, respectively.

Fetal loss in non-vaccinated ewe lambs was 78/882 (9%) compared with 23/443 (5%) in vaccinated ewe lambs. Combined data from the 2011- and 2012-born ewe lambs (n=5,115) were analysed using a logistic regression model and fetal loss as the dependent variable. In the final model fetal loss was associated with pre-mating bodyweight (p=0.003), weight change from pre-mating to initial pregnancy diagnosis (p<0.001), year born and leptospirosis vaccination status (p=0.013). Amongst the serologically monitored ewe lambs, there were associations between fetal loss and being

seropositive to *Leptospira* serovar Pomona using titre cut-points of 1:48 and 1:768 (p<0.001).

CLINICAL RELEVANCE: Low pre-mating weight and/or low weight gain from mating to pregnancy diagnosis was associated with increased fetal loss, emphasising the importance of ewe lambs achieving target pre-mating weights and liveweight gains during pregnancy. Infection with *Leptospira* serovar Pomona was associated with fetal loss in the 2012-born cohort and the possibility of infection with this serovar should be considered when investigating cases of fetal loss.

KEY WORDS: *Fetal loss, pregnancy loss, leptospirosis, sheep, reproduction, liveweight*

Introduction

Ewe lambs (7–9 months of age) have the potential to be successfully bred provided they reach adequate bodyweight and body condition score (BCS) and are appropriately managed (Kenyon *et al.* 2014). However, fetal losses will clearly have a negative impact on the profitability of breeding ewe lambs. In this paper fetal loss is defined as either the *in-utero* presence of non-viable fetuses identified during trans-abdominal ultrasonography, or when ewe lambs were confirmed pregnant at initial pregnancy diagnosis but were not pregnant at a subsequent pregnancy diagnosis.

It has been reported that fetal loss is greater in ewe lambs than in adult ewes (Mulvaney 2011), although the normal level of fetal loss in the former age group has not been defined. Fetal loss affecting up to 38% of ewe lambs has been reported in research studies (West *et al.* 2004; Howe *et al.* 2008, 2012; Mulvaney *et al.* 2010) and anecdotally reported by farmers, veterinarians and pregnancy scanning operators in New Zealand.

Abortion in sheep in New Zealand is almost always due to infectious causes, in particular *Toxoplasma gondii*, *Campylobacter fetus fetus*, *Salmonella* Brandenburg, and less frequently other pathogens (West 2002). The majority of these agents cause late-term abortion with expulsion of a fetus, with the exception of *T. gondii* which can cause pregnancy loss at any stage of gestation.

In contrast, the majority of fetal loss reported in New Zealand occurred in ewe lambs that were vaccinated against *T. gondii* and *C. fetus fetus* (West *et al.* 2004; Howe *et al.* 2012; Marshall

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2014). *Neospora caninum* has been implicated in some cases of fetal loss in maiden ewes in New Zealand (Howe *et al.* 2008, 2012) although the role of *N. caninum* in ovine fetal loss is still unclear (Howe *et al.* 2012). In other cases, no known pathogens have been identified (West *et al.* 2004; Howe *et al.* 2012; Marshall 2014).

Studies investigating the effects of liveweight gain during pregnancy on fetal loss appear inconsistent (Kenyon *et al.* 2014). Wallace *et al.* (1996, 1997) reported fetal loss in well-grown ewe lambs housed indoors and fed concentrate diets to achieve very high liveweight gains (234–323 g/day) during pregnancy. Pregnant ewe lambs are unlikely to achieve liveweight gains of this magnitude under pastoral conditions in New Zealand, but increased levels of fetal loss have not been reported in rapidly growing ewe lambs in New Zealand (Morris *et al.* 2005; Kenyon *et al.* 2008; Mulvaney *et al.* 2010). In contrast Sakata (2011), in a preliminary report, found that ewe lambs that experienced fetal loss had slower growth rates from 1 to 83 days of pregnancy compared with those that maintained their pregnancy.

In a non-peer-reviewed report, West *et al.* (2004) suggested *Leptospira interrogans* serovar Pomona was a possible cause of fetal loss in one of five flocks of maiden ewe lambs and 2-year-old ewes. *Leptospira* spp. have occasionally been diagnosed as a cause of ovine abortion outside New Zealand (Ellis *et al.* 1983; Leon-Vizcaino *et al.* 1987). Exposure to *Leptospira* spp., as evidenced by antibodies to *L. borgpetersenii* serovar Hardjo-bovis and *Leptospira* serovar Pomona, is common in New Zealand sheep. One cross-sectional study reported flock seroprevalences of 91 and 74%, and individual seroprevalences in adult sheep of 43 and 13% for *Leptospira* serovars Hardjo-bovis and Pomona, respectively (Dreyfus *et al.* 2011). Seroprevalence amongst <1-year-old lambs killed through a slaughterhouse was lower than that found amongst adult ewes, with seroprevalences of approximately 2 and 0.3% to *Leptospira* serovars Hardjo-bovis and Pomona respectively in 3–4-month-old lambs, and 14 and 3%, respectively in 8–11-month-old lambs (Dorjee *et al.* 2008).

This clinical communication reports on an investigation into fetal loss in ewe lambs on a commercial New Zealand sheep farm. The associations with bodyweight, BCS, fetal number and antibody titres to *Leptospira* spp. were investigated.

Case history

As part of a production study, all the 2011-born cohort of replacement ewe lambs (n=4,340) on a large farm in the Waikato region of New Zealand were individually identified. Production parameters including liveweight and BCS were recorded prior to mating and at an initial and a subsequent pregnancy diagnosis. BCS was recorded on a scale of 1 (emaciated) to 5 (grossly fat) with increments of 0.5 (Jefferies 1961). The ewe lambs were managed under standard New Zealand pastoral conditions and were vaccinated against clostridial organisms, *T. gondii*, *C. fetus fetus* and *C. jejuni*. All replacement ewe lambs on the farm were run with entire rams at a ratio of 1:50. Pregnancy diagnosis was undertaken twice (Table 1) by trans-abdominal ultrasonography. The outcome of the initial pregnancy diagnosis was defined as non-pregnant, single (one fetus) or multiple (two or more fetuses). Pregnancy percentage (ewe lambs pregnant/ewe lambs

Table 1. Summary of mating dates and pregnancy scanning dates for two cohorts of ewe lambs on a commercial New Zealand sheep farm.

Parameter	Cohort of ewe lambs	
	2011-born	2012-born
Mating start	11 April 2012	5 April 2013
Mating end	16 May 2012	16 May 2013
Length of mating period	35 days	41 days
Date of initial pregnancy diagnosis	23 July 2012	1 July 2013
Gestation range at initial pregnancy diagnosis	68–103 days	46–87 days
Date of second pregnancy diagnosis	21 August 2012	13 August 2013
Gestation range at second pregnancy diagnosis	97–132 days	89–130 days

mated × 100) and scanning percentage (fetuses identified/ewe lambs mated × 100) were 3,823/4,340 (88%) and 5,852/4,340 (135%), respectively. Ewe lambs that were non-pregnant at initial pregnancy diagnosis were culled.

At the second pregnancy diagnosis, 257 of the remaining 3,790 ewe lambs (6.8%) had either the presence of a non-viable fetus *in-utero* (no heartbeat and showing signs of degeneration), or a ewe lamb that had been pregnant at the initial pregnancy diagnosis was no longer pregnant (fetal loss). As a consequence, serum samples were collected by jugular venepuncture from 10 ewe lambs with fetal loss and from 10 ewe lambs that still had a viable pregnancy.

Laboratory findings

Sera were tested at a commercial veterinary diagnostic laboratory (Gribbles Veterinary, Auckland) for antibodies to *Leptospira* serovar Pomona using a microscopic agglutination test (MAT). In brief, this involved agglutination of live leptospira with specific antibodies (if present) and the reaction read under a dark-field microscope and graded from 0 to 100% agglutination. Samples were tested at doubling dilutions from 1:50 to ≥1:1600 and the last dilution that still showed 50% agglutination was reported as the titre. Titres ≥1:200 were considered an indicator of present or past infection with the *Leptospira* serovar tested for. The presence of antibodies to *N. Caninum* was tested using an ELISA (IDEXX Chekit *Neospora caninum* antibody ELISA, IDEXX Laboratories Inc, Maine, USA). In addition, four ewe lambs with non-viable fetuses observed *in-utero* were subject to euthanasia and their intact uteri removed and submitted to the same laboratory for histology of maternal and fetal tissues and bacteriology of fetal stomach contents.

All 20 serum samples were negative for antibodies to *N. caninum*, no bacteria were isolated from fetal stomach contents and there were no histological lesions in either fetal or maternal tissues consistent with a known infectious cause of fetal loss. Serum antibody titres for *Leptospira* serovar Pomona appeared generally higher in the ewe lambs with fetal loss compared with those that were still pregnant; eight of 10 ewe lambs with fetal loss had MAT titres ≥1:1600, one had a titre of 1:400 and one had no titre, whereas six of 10 ewe lambs that were still pregnant had titres <1:200, two had titres of 1:200 and one each had a titre of 1:800 and ≥1:1600. However, using histology it was not possible

to confirm that *Leptospira* serovar Pomona was responsible for the fetal loss in the four ewe lambs that had been subject to euthanasia.

Less than 0.5% of aborted fetuses were observed subsequent to the second pregnancy diagnosis but no investigation of these was undertaken. The farmer did not consider the number of aborted fetuses to be greater than what would normally be observed each year.

Epidemiological investigation

The following year a vaccination and serological study was initiated in the 2012-born cohort of ewe lambs ($n=1,797$) to further investigate the potential involvement of *Leptospira* serovar Pomona in fetal losses. Again all ewe lambs were individually identified. This study was approved by the Massey University Animal Ethics Committee (Palmerston North, NZ).

One third of the ewe lambs ($n=443$) from this cohort were vaccinated with a bivalent leptospirosis vaccine conferring protection against *Leptospira* serovars Pomona and Hardjo-bovis (Leptavoid 2, MSD Animal Health, Upper Hutt, NZ). A sensitiser vaccination was given on 8 January 2013, at which time the animals were 4–5 months of age, and a booster vaccination was given 5 weeks later on 12 February 2013.

A subset of 124 non-vaccinated ewe lambs were given an additional ear-tag and serum was collected from these by jugular venepuncture on 8 January, 12 February, 5 April, 16 May, 1 July, 13 August and 19 December 2013 for measurement of serum antibodies to *Leptospira* serovars Hardjo-bovis and Pomona using a MAT performed at the Hopkirk Research Institute (Palmerston North, NZ). The MAT was conducted based on the method of Faine (1982). In brief, two-fold dilutions of each serum sample were made using 0.9% saline, from 1:24 to 1:3,072. The samples were incubated for 1.5–4 hours with live cultures of either *Leptospira* serovar Hardjo-bovis or serovar Pomona. Each dilution was examined under a dark-field microscope for the presence of agglutination or lysis. The titre was recorded as the highest dilution at which $\geq 50\%$ of leptospires were agglutinated, and a titre of $\geq 1:48$ was considered positive. The percentage of ewe lambs that were seropositive for both serovars remained under 5% until May 2013. By August, 85% and 48% of this cohort were seropositive to *Leptospira* serovars Hardjo-bovis and Pomona, respectively, and by December 97% and 54%, respectively, were seropositive (Figure 1).

The ewe lambs were managed in a similar way to the 2011-born cohort described above and had similar mating and pregnancy diagnosis dates (Table 1). Similar production data (liveweights, BCS, fetal number) were recorded. The pregnancy percentage and scanning percentage for the 2012-born cohort were 1,352/1,797 (75%) and 1,832/1,797 (102%) respectively. Fetal loss in non-vaccinated ewe lambs was 78/882 (9%) compared with 23/443 (5%) in vaccinated ewe lambs. The mean liveweight gain between pre-mating and initial pregnancy diagnosis for both the 2011- and 2012-born cohorts combined was 40 g per day.

Combined data from the 2011- and 2012-born cohorts of ewe lambs that were pregnant at initial pregnancy diagnosis ($n=5,115$) were analysed using a logistic regression model and

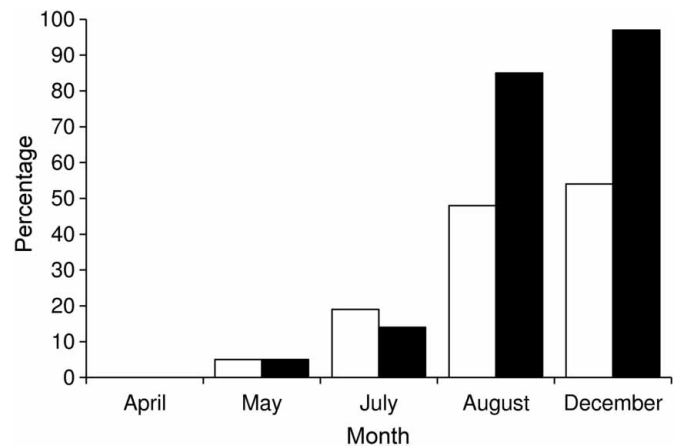


Figure 1. Percentage of 124 ewe lambs that were seropositive to *Leptospira borgpetersenii* serovar Hardjo-bovis (black bars) and *L. interrogans* serovar Pomona (white bars) on a commercial New Zealand sheep farm, when blood sampled between the months of April and December 2013. Samples were considered positive with titres $\geq 1:48$ using a microscopic agglutination test. Note that not all animals were sampled in August.

fetal loss (binary) as the output variable. Initially liveweight and BCS at pre-mating and again at initial pregnancy diagnosis, liveweight change between pre-mating and initial pregnancy diagnosis, fetal number (one or two), year born and leptospirosis vaccination status were included as explanatory variables. Non-significant variables were eliminated by manual backward elimination. These analyses were carried out using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

The final model included pre-mating bodyweight, weight change from pre-mating to initial pregnancy diagnosis, year born and leptospirosis vaccination status. From this model, both pre-mating bodyweight and weight change from pre-mating until initial pregnancy diagnosis were associated with fetal loss (Table 2), independent of year born or leptospirosis vaccination status. This equated to 1 SD increase in mean pre-mating liveweight (4.8 kg) being associated with a 1.3% decrease in fetal loss. One SD difference in mean weight change from pre-mating to initial pregnancy diagnosis (2.8 kg) was associated with a 2% decrease in fetal loss (Figure 2). The model-predicted difference in fetal loss between vaccinated and non-vaccinated ewe lambs, adjusted for

Table 2. Estimated OR, with 95%CI, for fetal loss in ewe lambs on a commercial New Zealand sheep farm from a logistic regression model, assessing the associations with leptospirosis vaccination, year born (2011 or 2012), pre-mating liveweight, and weight change between pre-mating and initial pregnancy diagnosis.

Variable	OR	95% CI		P-value ^a
		Lower	Upper	
2012 vaccinated	Reference			
2011 non-vaccinated	1.50	0.93	2.43	0.588
2012 non-vaccinated	1.89	1.14	3.13	0.013
Pre-mating liveweight (kg)	0.96 ^b	0.99	0.93	0.003
Weight change (kg)	0.89 ^c	0.85	0.92	<0.001

^a Significance of association with fetal loss.

^b OR for fetal loss for every 1 kg increase in pre-mating liveweight.

^c OR for fetal loss for every 1 kg increase in weight change.

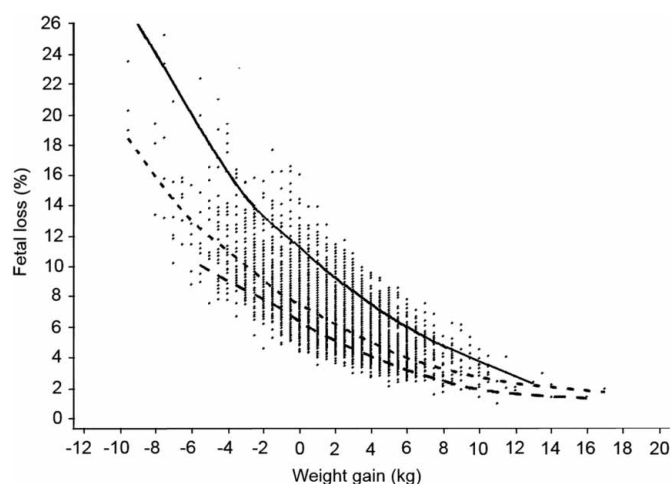


Figure 2. Estimated percentage of fetal loss in ewe lambs on a commercial New Zealand sheep farm with weight change between pre-mating and initial pregnancy diagnosis, for lambs that were vaccinated for leptospirosis (---) in 2012, or not vaccinated in 2012 (—) or 2011 (- -).

bodyweight, was 3.6 (95% CI=1.5–12.7)%. There was no association between fetal loss and either BCS or fetal number.

For the 124 animals that were blood sampled, a logistic regression model was developed to evaluate the association between fetal loss and being seropositive for *Leptospira* serovar Pomona and/or serovar Hardjo-bovis at first pregnancy diagnosis. Serological titre cut-points of 1:48 and 1:768 were used in separate models. The models included explanatory variables of individual serovar Hardjo-bovis and serovar Pomona status (positive/negative) at the initial pregnancy diagnosis, an interaction term for serovar Hardjo-bovis by serovar Pomona status and liveweight at initial pregnancy diagnosis. These analyses were carried out using R v3.0.3 (R Development Core Team, 2009; R Foundation for Statistical Computing, Vienna, Austria).

From the models there were associations between fetal loss and being seropositive to *Leptospira* serovar Pomona at cut-points of 1:48 and 1:768 (Table 3). There was no association between being seropositive for *Leptospira* serovar Hardjo-bovis and fetal loss at either cut-point. The interaction of serovar Hardjo-bovis by serovar Pomona was not significant in the model using a titre cut-point of 1:48. No ewe lamb was positive for both serovar Hardjo-bovis and serovar Pomona using a cut-point of 1:768.

Table 3. Estimated OR, with 95%CI, for fetal loss in 124 ewe lambs on a commercial New Zealand sheep farm from a logistic regression model, assessing the associations with being seropositive to *Leptospira borgpetersenii* serovar Hardjo-bovis (serovar Hardjo-bovis) or *L. interrogans* serovar Pomona (serovar Pomona) using a microscopic agglutination test with cut-points of 1:48 or 1:768.

Variable	OR	95% CI		P-value ^a
		Lower	Upper	
Serovar Hardjo-bovis titre \geq 1:48	2.74	0.33	16.75	0.290
Serovar Pomona titre \geq 1:48	13.77	3.38	72.41	<0.001
Serovar Pomona titre \geq 1:768	27.14	4.65	194.15	<0.001

^a Significance of association with fetal loss.

Discussion

In this investigation approximately 7% of ewe lambs experienced fetal loss in the period of mid to late gestation. The design of the study did not allow for determining early loss (before the initial pregnancy diagnosis) or late loss (after the second pregnancy diagnosis) and it is possible that additional fetal losses may have occurred during these times. Fetal loss was more common amongst ewe lambs whose growth rates during early-mid pregnancy were lower than the flock average. This finding is consistent with that of Sakata (2011) and suggests that poor weight gain or weight loss in early-mid pregnancy may be detrimental to maintenance of pregnancy in ewe lambs. In this investigation the weight gain of the ewe lambs between mating and pregnancy diagnosis was lower (40 g/day) than that recommended for ewe lambs under New Zealand pastoral conditions (100–150 g/day) during this period (Kenyon *et al.* 2014). Fetal loss was also more common amongst ewe lambs with pre-mating liveweights that were lower than the flock average.

Amongst the ewe lambs that were blood sampled in the 2012-born cohort, those with positive (\geq 1:48) or high (\geq 1:1748) *Leptospira* serovar Pomona titres at the initial pregnancy diagnosis were more likely to subsequently suffer fetal loss compared with those that were seronegative at this time. Unfortunately blood samples were not collected at the subsequent pregnancy diagnosis from those ewe lambs who suffered fetal loss. However, in bovine studies it has been reported that serum antibody titres take around one week to develop whereas fetal death and/or abortion occurred 17–29 days after artificial infection (Morter *et al.* 1958; Murphy and Jensen 1969) making it biologically plausible that these ewe lambs could have a high *Leptospira* serovar Pomona titre with a live fetus *in-utero* at the initial pregnancy diagnosis and then have the fetus/s subsequently die due to infection over the following 1–3 weeks.

The possible role of *Leptospira* serovar Pomona in fetal losses in the 2012 cohort was also evidenced by unvaccinated ewe lambs being more likely to have fetal loss than those that were vaccinated. Nevertheless, given that nearly half of the monitored ewe lambs seroconverted to *Leptospira* serovar Pomona between May and August (during gestation), the magnitude of fetal losses in unvaccinated ewe lambs were still relatively low compared with some other reports (West *et al.* 2004; Howe *et al.* 2012).

It is unclear whether *Leptospira* serovar Pomona was involved in fetal losses in the 2011-born cohort. The serum samples collected at the second pregnancy diagnosis from ewe lambs with fetal loss and from those that were still pregnant provided insufficient evidence to confirm a diagnosis. Confirming a diagnosis of leptospirosis can be difficult for veterinarians investigating fetal loss in ewe lambs at the time of, or subsequent to, cases occurring. Confirmation would ideally involve PCR testing of leptospiral DNA within fetal fluids, tissues or amniotic fluid. Unfortunately, the quality of samples available during a fetal loss investigation is frequently too poor for effective leptospirosis testing to proceed because of autolysis and desiccation of the tissues (F. Hill,¹ pers. comm.).

The lack of association between ewe lambs being seropositive for *Leptospira* serovar Hardjo-bovis and fetal loss confirms the likely

¹F. Hill, Gribbles Veterinary, Palmerston North, NZ.

role of sheep as a maintenance host for this serovar (Cousins *et al.* 1989; Gerritsen *et al.* 1994), where only subclinical or no effects are expected. In contrast, they could be considered an accidental host for *Leptospira* serovar Pomona, where more severe effects might occur (Hathaway 1981).

In summary, in this flock low pre-mating weight and/or low weight gain from mating to pregnancy diagnosis was associated with increased fetal loss, emphasising the importance of ewe lambs achieving target pre-mating weights and liveweight gains during pregnancy. Infection with *Leptospira* serovar Pomona was associated with fetal loss in the 2012-born cohort and the possibility of infection with this serovar should be considered when investigating cases of fetal loss.

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Appendix 3: Published article: Factors associated with fetal losses in ewe lambs on a New Zealand sheep farm

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Scientific Article

Serological patterns, antibody half-life and shedding in urine of *Leptospira* spp. in naturally exposed sheepE Vallée*[§], C Heuer[†], JM Collins-Emerson[‡], J Benschop[‡] and PR Wilson*

Abstract

AIMS: To determine within-farm prevalence, longitudinal pattern of exposure measured by serology, antibody titre longevity and point prevalence of shedding in urine of *Leptospira borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona in naturally infected sheep on a sample of commercial farms in New Zealand.

METHODS: On eight commercial sheep farms, between September 2011 and January 2014, blood samples were collected from 115–217 ewe lambs on each farm, at intervals of 2–11 months. They were analysed by microscopic agglutination test (MAT) for antibodies to *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona, using a titre cut-point of 48. Urine from 98 animals was tested by quantitative PCR (qPCR). The half-life of antibodies was estimated in 185 sheep for serovar Hardjo and 21 for Pomona, and the seroprevalence and mean titre of animals lost to follow-up was compared with those remaining in the study.

RESULTS: Within-flock seroprevalence for serovar Hardjo reached a maximum at 17–22 months of age, ranging from 79 to 100%. Seroprevalence for serovar Pomona rose above 10% on three farms and increased to 21–54% by 4–14 months. Seroconversions occurred mainly from late autumn to early summer at 7–15 months of age. Seroprevalences ranging from 3 to 76% for serovar Hardjo and 0.5 to 15% for serovar Pomona were observed up to 3 months of age, likely due to maternally derived immunity. The half-life of antibody in response to infection was estimated to be 6.7 (95% CI=5.8–7.9) months for serovar Hardjo and 6.3 (95% CI=4.8–9.0) months for Pomona. The prevalence of sheep with urine positive for leptospire on qPCR on each farm ranged from 11 to 88%. All but one of the qPCR-positive animals were seropositive for serovar Hardjo. On two farms where Pomona exposure was observed, animals that were lost to follow-up

had a higher geometric mean titre for serovar Pomona than those remaining in the study.

CONCLUSIONS: This study demonstrated seasonal exposure from autumn to early summer in young sheep, a wide range of within-flock serological and shedding prevalence, and gives an estimation of the half-life of MAT titres in sheep. More extensive data are needed to fully understand the epidemiology of leptospirosis in sheep flocks across New Zealand and, along with economic analysis, to justify and design cost-effective and efficient control measures to protect human and animal health.

KEY WORDS: *Leptospirosis*, sheep, epidemiology, seroprevalence, Hardjo, Pomona, antibody, half-life, PCR, shedding

Introduction

Leptospira borgpetersenii serovar Hardjo and *L. interrogans* serovar Pomona are the two most common serovars in New Zealand livestock. Serovar Hardjo is maintained by sheep (Cousins *et al.* 1989; Gerritsen *et al.* 1994) and cattle (Ellis *et al.* 1981; Faine *et al.* 1999). Pigs are conventionally regarded as the maintenance hosts for serovar Pomona, but sheep (Morse *et al.* 1957; Hodges 1974) and cattle (Carter *et al.* 1982; Kingscote and Wilson 1986) are commonly infected and shed the bacteria.

A large-scale cross-sectional study conducted in 2010 over the North and South Islands in New Zealand, using a titre cut-point of ≥ 48 , showed that 91 and 74% of sheep flocks had at least one adult animal that was seropositive to serovars Hardjo and Pomona, respectively, and 43 and 14% of the sheep had a titre ≥ 48 for serovars Hardjo and Pomona, respectively (Dreyfus 2013). This contrasts with previous cross-sectional studies, using the same titre cut point, conducted on lambs and ewes slaughtered at a North Island meat plant that reported a farm prevalence of 46 and 51%, and a sheep seroprevalence of 19 and 4% for serovars Hardjo and Pomona, respectively (Blackmore *et al.* 1982). Similarly Dorjee *et al.* (2008) reported a farm prevalence of 33 and 4% and a sheep prevalence of 5 and 1% for serovars Hardjo and Pomona, respectively, in mostly lambs from different regions of both islands. Both farm and animal seroprevalence increased with the age of the animal. While serovar Hardjo

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GMT	Geometric mean titre
ICC	Intra-class correlation
MAT	Microscopic agglutination test
qPCR	Quantitative PCR

is usually reported to be more prevalent than Pomona, Fang *et al.* (2014a) reported similar sheep-level prevalence for both serovars (37% for serovar Hardjo, 35% for Pomona and 16% dual seroprevalence).

Within infected farms, the seroprevalence for both serovars Hardjo and Pomona is variable. Prevalence <50% was usually reported (Blackmore *et al.* 1982; Dorjee *et al.* 2008; Dreyfus 2013), but more recently Fang *et al.* (2014a) found a within-farm seroprevalence ranging from 40 to 95% for serovars Hardjo and/or Pomona. On farms known to be experiencing an outbreak, up to 84% of mixed-age ewes were found seropositive for serovar Hardjo, and 22% for Pomona (Blackmore *et al.* 1982).

Fang *et al.* (2014a), using a quantitative PCR (qPCR) assay, reported 31% of sheep excreting leptospires in urine, and a within-farm shedding prevalence ranging 5–69%. Those authors also showed that sheep with serovar Hardjo titres ≥ 48 were 12.5 times more likely to be shedding leptospires than seronegative sheep. A previous study using culture failed to reveal the presence of bacteria in urine of sheep (Blackmore *et al.* 1982).

Little is known about the epidemiology of leptospirosis in sheep flocks, as acknowledged by Martins and Lilenbaum (2014). Some studies report clinical disease and deaths in lambs as young as 2–4 months due to serovar Pomona (Hartley 1952; Vermunt *et al.* 1994; Dorjee *et al.* 2005). Another study showed high seroprevalence for serovar Hardjo in hoggets and ewes but no titres in lambs (Blackmore *et al.* 1982). Those authors demonstrated the risk period for infection to be between 9 and 24 months. Little information is available on maternally derived antibody protection, or on the age at which lambs become susceptible to *Leptospira* spp. infection.

Hence, other than cross-sectional serological studies of serovars Hardjo and Pomona, along with reports of clinical occurrences, little is known about leptospirosis or its epidemiology and production effects on commercial sheep farms in New Zealand. This paper presents descriptive data from a longitudinal study of production effects of serovars Hardjo and Pomona in sheep flocks to improve understanding of the epidemiology of these serovars in this species. The objectives were to determine the within-farm prevalence and longitudinal exposure pattern of serovars Hardjo and Pomona using serology, to evaluate the titre decay rate, to characterise *Leptospira* spp. shedding in urine, and to evaluate a possible bias in seroprevalence and mean titre due to animals lost to follow-up, in naturally infected sheep on New Zealand commercial farms.

Material and methods

Study design

This study was an opportunistic analysis based on data from animals from a field trial of the potential effects of serovars Hardjo and Pomona on sheep growth and reproduction involving vaccination of a subset of sheep, as described by Vallée *et al.* (2014). That study was conducted from 2011 to 2013 on eight commercial farms, with sentinel animals sampled on between five and eight occasions for up to 28 months to assess exposure to serovars Hardjo and Pomona. Data presented in this paper were from non-vaccinated control animals only, with lambs being animals <1-year-old and hoggets being animals 1–2-years-old.

This study was approved by the Massey University Animal Ethics Committee (Palmerston North, NZ).

Farms and animals

Six farms were located in the North Island and two in the South Island of New Zealand. Farm descriptions, as provided by the farmers, are presented in Table 1. All the animals were farmed under typical New Zealand commercial farming conditions, and grazed on pasture all year round.

Farms A–G were selected in June 2011 from a previous study on Johne's disease and leptospirosis (Dreyfus 2013) in which they were seropositive for serovars Hardjo and/or Pomona, and where the farmer agreed to take part in this study, primarily investigating the effect of leptospirosis on growth and reproduction. Farm H was selected in 2012 based on its recent history of sub-optimal hogget reproductive performance and the known presence of serovars Hardjo and Pomona on the farm.

Between 115 and 217 ewe lambs on Farms A–G in 2011 were randomly selected at tail docking in spring, at a median age of 2–6 weeks, and identified by ear tag with a unique number. The study period, September 2011 to January 2014, included summer 2012–2013 which was recorded as the driest in 40 years, as measured by the soil moisture deficit, between mid-October and mid-April (Porteous and Mullan 2013). In February 2013, another 198 hoggets were enrolled on Farm H at weaning, with a median age of 23 weeks.

Blood and urine collection

Animals were blood sampled on the dates shown in Table 2. Blood was collected by jugular venipuncture into blood collection tubes without anticoagulant. Blood samples were transported on ice to the ^mEpiLab, Massey University (Palmerston North, NZ) where they were stored at 4°C until processing, usually within 24 hours of collection. The samples were centrifuged at 1400g for 10 minutes. The sera were diluted at 1:6 with 0.9% saline and stored at –20°C on microplates until the microscopic agglutination test (MAT) was performed, 1 week to 18 months after blood collection.

Urine from between four and 16 animals per farm, for a total of 98 randomly selected animals, was collected in a sterile 60 mL container labelled with the animal identifier between 41 and 90 weeks after the beginning of the study. Urination was stimulated by placing a hand over the nostrils until urine was voided. Urine samples were transported on ice and stored at 4°C until processing. The samples were processed and the DNA was extracted within 24 hours of collection.

Microscopic agglutination test

Serum samples were tested in the EpiLab. The MAT was conducted as described by Fang *et al.* (2014b), based on the method reported in Adler *et al.* (1982). Briefly, two-fold dilutions in 0.9% saline of each serum sample were created, from 1:24–1:3,072. The samples were then incubated for 1.5–4 hours with live cultures of the tested serovar. Each dilution of each sample was then checked under a dark-field microscope for the presence of agglutination or lysis. Standard *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona antisera were obtained from the WHO/FAO/OIE leptospirosis reference laboratory, Brisbane, Australia. The *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona used for the test were hamster passaged strains that were provided by Schering-Plough, Wellington, New Zealand. The test was considered

Table 1. Description of the eight farms used in a study to determine serological patterns, antibody half-life and shedding in urine of *Leptospira* spp. in naturally exposed sheep.

Farm	District	Species	Breeding ewes (n)	Sheep breed	Lambing period	Area (ha)	Topography
A	Manawatu	Sheep, cattle	6,000	Romney/composite	01 Sep. to 20 Oct. 2011	1,900	Rolling to medium/steep hills
B	Tararua	Sheep, cattle	8,500	Perendale/composite	15 Sep. to 03 Nov. 2011	2,016	Steep hills
C	Wairoa	Sheep, cattle	4,700	Romney Coopworth cross	01 Sep. to 20 Oct. 2011	1,050	Flat to steep hills
D	Central Hawke's Bay	Sheep, cattle	4,500	Romney Texel cross	20 Aug. to 05 Oct. 2011	1,416	Flat to rolling hills
E	Hurunui, Canterbury	Sheep, cattle, deer	10,400	Romney	15 Sep. to 20 Oct. 2011	6,130	Flat to high country
F	Hurunui Canterbury	Sheep, cattle, deer	10,000	Lincoln Merino cross	15 Sep. to 20 Oct. 2011	2,973	Flat to rolling hills
G	Manawatu	Sheep, cattle, deer	1,050	Romney	10 Sep. to 30 Oct. 2011	408	Easy to moderate hills
H	Waikato	Sheep, cattle,	7,800	Composite Romney Finn Coopworth	15 Aug. to 30 Sep. 2012	2,500	Easy to steep hills

positive for the highest dilution where 50% or more of the leptospire were agglutinated or lysed.

Quantitative PCR

Urine samples were centrifuged at 10,600g for 20 minutes, and sediment re-suspended in 200 µL of phosphate buffered saline, after discarding the supernatant. The DNA was then extracted using the QIAamp DNA Mini Kit (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand) as per manufacturer's instructions, for a final volume of 200 µL of DNA template solution. The samples were then kept at -20°C until the qPCR was run.

The qPCR used was adapted from Subharat *et al.* (2011) and Fang *et al.* (2014b) and was shown to have a detection limit of 10³ cells/mL for *L. borgpetersenii* serovar Hardjo artificially inoculated in deer urine. The qPCR reaction solution was made of 5 pM of primers targeting the *gyrB* gene (Slack *et al.* 2006), of sequence 5'-TGAGCCAAGAAGAAACAAGCTACA-3' (2For) and 5'-MATGGTTCCRCCTTCCGAAGA-3' (504Rev), 2.4 µM of SYTO9 (Invitrogen Corp., Carlsbad, CA, USA), 12.5 µL of a commercial mastermix (Roche LightCycler 480 Probes Master 04707494001, Roche Diagnostics GmbH, Mannheim, Germany), 2 µL of the DNA preparation and double distilled water for a total volume of 25 µL.

The qPCR was run on a Rotor-Gene Q (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand). An initial denaturation of 10 minutes at 95°C was followed by 40 cycles, consisting of 10 seconds of denaturation at 95°C, 20 seconds of annealing at 63°C and 10 seconds of elongation at 72°C. The melting temperature was measured by monitoring the fluorescence on the green channel, every 0.2°C from 78–90°C. A positive control consisting of either DNA extracted from a live culture of a strain of *L. interrogans* serovar Copenhageni isolated from sheep in New Zealand or DNA extracted from sheep urine inoculated with a live culture of a New Zealand strain of *L. borgpetersenii* serovar Hardjo, was used for each PCR run as well as a negative control of double distilled water. Confirmation of positive samples was determined by comparing the melting temperature with the positive control.

Statistical analysis

Log titre and geometric mean titre

If X was the MAT titre of an individual animal, the log titre Y was calculated as follows:

$$Y = \log_2 \left(\frac{X}{12} \right) \quad (1)$$

The MAT titre was obtained from the log titre by using the relationship:

$$X = 2^Y \times 12 \quad (2)$$

The geometric mean titre (GMT) was calculated as follows, using all animals with a MAT titre of 24 and above:

$$\text{GMT} = 2^{\bar{Y}} \times 12 \quad (3)$$

where \bar{Y} was the mean log-level titre of lambs at one sampling of a farm. Animals for which no antibodies were detected were excluded from the calculation of the GMT. These formulae ensured that a round log titre corresponded to a tested dilution (1 for 1:24, 2 for 1:48 etc.). The GMT was calculated for each sampling date, stratified by farm. When sheep were selected on two occasions the serological results from both occasions were pooled, and the GMT was calculated as indicated. The 95% CI for the GMT was calculated at the log level, and then the lower and upper boundaries were back-transformed.

Seroprevalence

Seroprevalence was calculated for each farm on every sampling date, for serovars Hardjo only, Pomona only, and both serovars, as the proportion of animals sampled on that day that had a titre ≥ 48 , the cut-point currently recommended for serovar Hardjo in sheep (Blackmore *et al.* 1982).

Titre pattern with age

The log titre, as defined in equation (1), was modelled separately for each serovar as a function of the median age of the sheep in months, using a zero-inflated Poisson regression (Hilbe 2007). Animals with dual Hardjo-Pomona titres were included in both

Table 2. Date of blood sampling, approximate age, number of sheep sampled on eight farms and total number seropositive (microscopic agglutination test titre ≥ 48) for *Leptospira borgpetersenii* serovar Hardjo and number positive for serovar Hardjo and *L. interrogans* serovar Pomona, and geometric mean titre (GMT).

Farm	Date	Approx. age (weeks) ^a	N sampled ^b	Number seropositive (%)		GMT (95% CI)	
				Serovar Hardjo	Serovars Hardjo & Pomona		
A	01 Oct. 2011	1	194	125 (64.4)	3 (1.5)	162 (134–197)	
	15 Dec. 2011	12	182	9 (4.9)	0 (0)	29 (26–33)	
	02 Aug. 2012	45	171	32 (18.7)	4 (2.3)	856 (525–1395)	
	01 Nov. 2012	58	106	83 (78.3)	0 (0)	308 (257–369)	
	03 Apr. 2013	79	108	85 (78.7)	3 (2.8)	434 (370–509)	
B	22 Nov. 2011	6	210	27 (12.9)	0 (0)	69 (51–93)	
	17 Jan. 2012	14	192	0 (0)	0 (0)	NA	
	30 May 2012	33	176	9 (5.1)	3 (1.7)	946 (365–2447)	
	05 Dec. 2012	60	141	137 (97.2)	59 (41.8)	408 (353–471)	
	16 Apr. 2013	79	136	130 (95.6)	58 (42.6)	301 (257–352)	
	30 Jul. 2013	94	139	114 (82.0)	57 (41.0)	160 (134–192)	
	04 Dec. 2013	112	122	115 (94.3)	56 (45.9)	221 (187–261)	
	14 Jan. 2014	118	98	87 (88.8)	41 (41.8)	252 (198–320)	
	C	17 Oct. 2011	3	207	122 (58.9)	22 (10.6)	71 (62–81)
		01 Dec. 2011	10	205	94 (45.9)	0 (0)	58 (50–67)
26 Apr. 2012		31	188	0 (0)	0 (0)	NA	
06 & 23 Nov. 2012		58	172	169 (98.3)	32 (18.6)	293 (264–326)	
D	28 Mar. 2013	79	77	77 (100)	16 (20.8)	436 (372–510)	
	30 Sep. & 06 Oct. 2011	6	181	5 (2.8)	0 (0)	41 (26–64)	
	24 Nov. 2011	14	164	2 (1.2)	0 (0)	36 (21–63)	
	27 Mar. 2012	31	161	2 (1.2)	0 (0)	152 (25–933)	
	09 Aug. 2012	51	158	12 (7.6)	0 (0)	1935 (1315–2847)	
E	18 Dec. 2012	69	111	97 (87.4)	2 (1.8)	572 (459–712)	
	25 Oct. 2011	3	217	165 (76.0)	1 (0.5)	109 (94–125)	
	19 Dec. 2011	11	207	35 (16.9)	0 (0)	34 (31–38)	
	02 May 2012	30	197	2 (1.0)	0 (0)	272 (9–8107)	
	04 Apr. 2013	79	134	130 (97.0)	6 (4.5)	344 (295–400)	
F	18 Jul. 2013	94	136	132 (97.1)	5 (3.7)	400 (347–462)	
	28 Oct. 2011	4	190	35 (18.4)	0 (0)	90 (65–125)	
	20 Dec. 2011	11	169	18 (10.7)	0 (0)	553 (318–962)	
	03 May 2012	31	163	33 (20.2)	2 (1.2)	484 (338–692)	
	20 Nov. & 07 Dec. 2012	59	156	109 (69.9)	2 (1.3)	409 (345–483)	
G	24 Jan. 2013	69	52	52 (100)	5 (9.6)	239 (208–274)	
	19 Oct. & 07 Nov. 2011	2	115	26 (22.6)	0 (0)	57 (45–73)	
	12 Dec. 2011	10	114	17 (14.9)	0 (0)	52 (39–70)	
	10 Apr. 2012	27	111	0 (0)	0 (0)	NA	
	01 Aug. 2012	43	111	3 (2.7)	0 (0)	110 (23–531)	
H	14 & 19 Nov. 2012	58	92	76 (82.6)	1 (1.1)	306 (231–406)	
	05 Feb. 2013	70	68	65 (95.6)	0 (0)	534 (453–631)	
	12 Feb. 2013	23	198	3 (1.0)	0 (0)	48 (48–48)	
	20 Mar. 2013	28	177	0 (0)	0 (0)	24 (NA)	
	16 May 2013	36	169	5 (3.0)	0 (0)	59 (31–111)	
	01 Jul. 2013	42	173	24 (13.9)	5 (2.9)	620 (343–1122)	
	18 Dec. 2013	67	109	107 (98.2)	59 (54.1)	386 (324–461)	

NA=not applicable.

^a Based on the mid-point lambing date.

^b Where sheep were selected on two occasions, the total number of lambs selected is given.

models. Animals with no detectable antibodies were assigned a log-titre of 0. The binary component modelled the probability of a sheep to be exposed to Hardjo or Pomona and the count part the expected log titre of a sheep given that it was exposed. The same set of predictors was used for both these elements of the model. A quadratic term for age was added to adjust for the

non-linear relationship with the log titre. Farm was added as a fixed-effect to account for clustering within farm and an interaction, farm by age, was added.

The final model was checked by fitting a zero-inflated negative binomial regression to detect possible presence of additional

over-dispersion. The intra-class correlation (ICC) of the residuals within sheep was calculated from a one-way ANOVA as described by Shrout and Fleiss (1979) to confirm the absence of further correlation in the data.

The log titre for each farm was then predicted as a function of age from <1 to 28 months and bootstrap 95% CI of the predictions were calculated using 1,000 replications. Loess curves were fitted with the smoothing function being the log titre predicted from the zero-inflated Poisson model as a function of age, with a span of 0.7 for individual farm plots and 0.75 for all farms.

Titre decay and antibody half-life

To model the titre decay, animals aged >3 months that had a titre of 768–1,536 for serovars Hardjo or Pomona were selected, with the assumption that sampling happened close to the date of the actual peak titre. Animals from which no data were available after the assumed peak were removed from the analysis. The time was set to zero on the day of the observed maximum titre, and subsequent samplings were calculated as the time in months since the maximum titre. The dependent variable log titre for serovars Hardjo or Pomona, calculated using equation (1), was regressed on time. The possible violation of independence was evaluated by looking at the effect of adding farm as a fixed-effect and/or sheep as a random-effect on the regression coefficient and model fit.

The half-life of the antibodies in months, assuming an exponential decrease, was then calculated as the inverse of the linear regression coefficient for time, which was the monthly rate of antibody decay. The shape of the decay curve indicated the approximate duration of MAT antibodies.

Leptospiral shedding in urine

Shedding prevalence of *Leptospira* spp. was calculated on each Farm A–H and the GMT was calculated within each farm for serovars Hardjo and Pomona for animals that were positive or negative as determined using the qPCR assay.

To examine the relationship between titre and probability of urine shedding, a logistic regression model was developed with the qPCR result (positive/negative) as the binary dependent variable, and a categorical variable for serological status (negative or positive for serovar Hardjo only or positive for serovars Hardjo and Pomona, with no animal being positive for serovar Pomona only), and the median flock age at the time of sampling in months as explanatory variables. A random effect was added to account for clustering within farm. Titre cut-offs for serological status from 48–1,536 were tested to determine the best association between MAT titre and shedding, and the model with the best fit as measured by the lowest Akaike information criterion was selected. When the presence of leptospirosis on a farm could not be confirmed by the observation of seroconverting animals the risk of false positive qPCR results was increased, so the qPCR results for this flock on this date were not included in the model.

Animals lost to follow-up

As the number of animals present at each sampling date varied greatly, the effect of the loss to follow-up on seroprevalence and mean titre was assessed by comparing seroprevalence at a titre of 48 and GMT between animals that remained in the study, animals that were culled, and animals that were missing for an unknown reason on the following sampling date. Animals missing for unknown reasons included unreported culling and deaths. Seroprevalence was compared using Fisher's exact test,

stratified by farm and date. The GMT was compared using a two-sided *t*-test at the log level, stratified by farm and date.

All analyses were conducted in R v3.0.3 (R Development Core Team, 2014; R Foundation for Statistical Computing, Vienna, Austria).

Results

Seroprevalence and mean titres

Animals seropositive for *L. borgpetersenii* serovar Hardjo were present on Farms A–G at ages 1–6 weeks, at the beginning of the study, as shown in Table 2. Animals seropositive for *L. interrogans* serovar Pomona were present on Farms A–E and G at ages 1–6 weeks, as shown in Table 3.

Hardjo seroprevalence, either alone or as a co-infection with Pomona, subsequently decreased less than 5% at the second (2–3 months of age) or third sampling (6–8 months of age) on all but Farm F where the seroprevalence for Hardjo remained above 10% for at least 14 months, the end of the study (Table 2). From the fourth sampling onwards, all farms had a high Hardjo seroprevalence, reaching >80%, including animals also seropositive for serovar Pomona. The GMT for serovar Hardjo ranged from 41–162 at the first sampling and decreased to <60 at the second sampling on all but Farm F where it increased to 553. The observed maximum GMT for serovar Hardjo ranged from 400–1,935. Among the 769 animals that remained present throughout the study, between zero and 15 animals per farm, for a total of 35 sheep across all farms, never had titres ≥ 48 for serovar Hardjo after exposure started on the farm.

The seroprevalence for Pomona, including dual seroprevalence, rose above 10% on three farms (B, C and H), observed in animals aged 8–14 months, and the maximum prevalence ranged from 20.8–54.1% including animals positive for Hardjo and Pomona (Table 3). The GMT for Pomona ranged from 24–96 at the first sampling. The observed maximum GMT for serovar Pomona on those farms ranged from 166–768. Among the 769 animals that were present throughout the study, between 42 and 126 animals per farm, for a total of 587 sheep across all farms, never had titres ≥ 48 for serovar Pomona after exposure started on the farm.

Titre pattern with age

The predicted mean log titres for serovar Hardjo with increasing age of animals within each farm are presented in Figure 1. The shapes of the curves are similar across farms. On all farms the mean predicted titre decreased between 5 and 10 months of age, but subsequently reached a maximum at around 15 months.

The predicted mean log titre for serovar Hardjo for the first 2 years of life for sheep across all farms is presented in Figure 2. The mean titre decreased from spring through summer to early autumn, then increased, indicating seroconversion, mainly through winter and spring. The predicted mean titre reached a maximum at 17–22 months of age, and then started to decrease until the last observation at 28 months of age.

The predicted mean log titres for serovar Pomona with increasing age within each farm are presented in Figure 3. The predicted titres showed different patterns across farms, with a marked increase only on Farms B, C and H. The increase in predicted

Table 3. Approximate age, number of sheep sampled on eight farms, total number seropositive (microscopic agglutination test titre ≥ 48) for *Leptospira interrogans* serovar Pomona, including those seropositive for serovars Pomona and Hardjo, and geometric mean titre (GMT).

Farm	Approx. age (weeks) ^a	N. sampled	Number seropositive for serovar Pomona (%)	GMT (95% CI)
A	1	194	4 (2.1)	73 (29–183)
	12	182	0 (0)	NA
	45	171	10 (5.8)	41 (35–48)
	58	106	0 (0)	NA
	79	108	3 (2.8)	32 (25–43)
B	6	210	2 (1.0)	34 (23–50)
	14	192	4 (2.1)	38 (29–51)
	33	176	23 (13.1)	665 (384–1150)
	60	141	60 (42.6)	104 (85–127)
	79	136	60 (44.1)	82 (68–100)
	94	139	71 (51.1)	100 (82–121)
	112	122	60 (49.2)	71 (60–84)
C	118	98	46 (50.0)	76 (61–96)
	3	207	32 (15.5)	55 (44–69)
	10	205	0 (0)	24 (NA)
	31	188	1 (0.5)	26 (22–32)
	58	172	32 (18.6)	166 (105–262)
D	79	77	16 (20.8)	127 (75–214)
	6	181	2 (0.8)	24 (24–24)
	14	164	1 (0.6)	192 (NA)
	31	161	3 (1.9)	48 (NA)
	51	158	9 (5.7)	51 (40–66)
E	69	111	2 (1.8)	76 (15–390)
	3	217	1 (0.5)	96 (NA)
	11	207	0 (0)	NA
	30	197	0 (0)	NA
	79	134	6 (4.5)	30 (25–35)
F	94	136	5 (3.7)	36 (27–49)
	4	190	0 (0)	NA
	11	169	0 (0)	NA
	31	163	7 (4.3)	52 (38–71)
	59	156	6 (3.8)	30 (25–37)
G	69	52	5 (9.6)	54 (41–70)
	2	115	5 (4.3)	63 (29–139)
	10	114	0 (0)	NA
	27	111	1 (0.9)	96 (6–1453)
	43	111	1 (0.9)	38 (19–75)
H	58	92	2 (2.2)	68 (54–87)
	70	68	0 (0)	24 (NA)
	23	198	8 (2.7)	35 (27–46)
	28	177	1 (0.6)	768 (NA)
	36	169	5 (3.0)	43 (43–81)
	42	173	32 (18.5)	368 (271–499)
	67	109	59 (54.1)	254 (185–348)

NA=not applicable.

^a See Table 2 for dates of sampling.

mean titre started at varying ages between farms; around 5 months on Farm B, and 10 months on Farms C and H.

The predicted mean titre for serovar Pomona for the first 2 years of life for the sheep across all farms is presented in Figure 4. No clear maximum or subsequent decline was observed in the overall smoothed prediction.

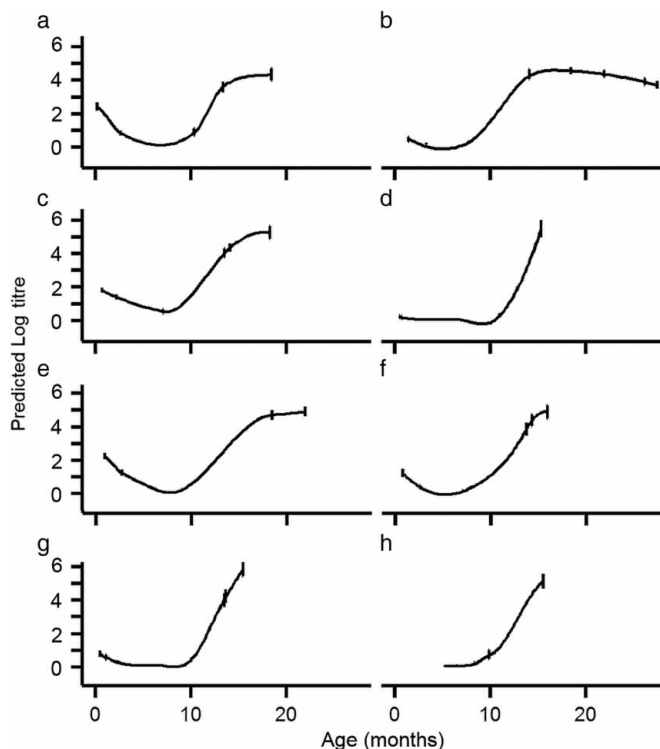


Figure 1. Predicted mean log titre for *Leptospira borgpetersenii* serovar Hardjo as a function of sheep age on eight different farms (a–h), with Loess smoothing.

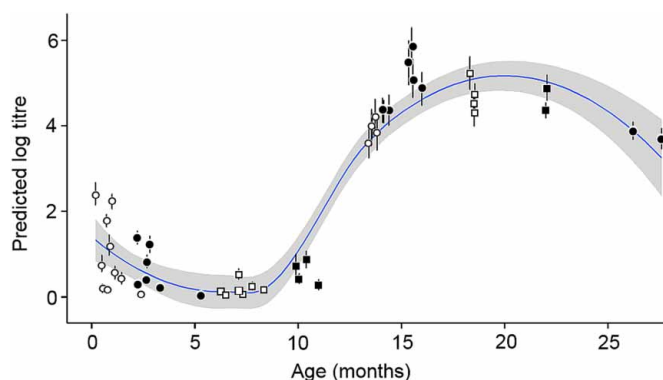


Figure 2. Predicted mean log titre for *Leptospira borgpetersenii* serovar Hardjo including all farms as a function of sheep age, with Loess smoothing and bootstrap 95% CI around predicted values. Season is shown as spring ○; summer ●; autumn □ and winter ■.

The ICC of the residuals within sheep showed that no further correlation was present for either serovar Hardjo or Pomona models.

Titre decay and antibody half-life

There were 185 sheep from Farms A–H with titres 768 or 1,536 for serovar Hardjo, and 21 sheep from Farms B, C and H with titres 768–1,536 for serovar Pomona. Between one and four serology results per animal were available over periods of 1–14 months after the peak titre for serovar Hardjo. One to five serology results were available over periods of 2–20 months after the peak titre for serovar Pomona. Adding farm as a fixed-effect in the model did not improve the fit and did not change the regression coefficient, thus showing no evidence of clustering or confounding by farm. The models were thus kept as simple linear regressions with only time (months) as the variable of

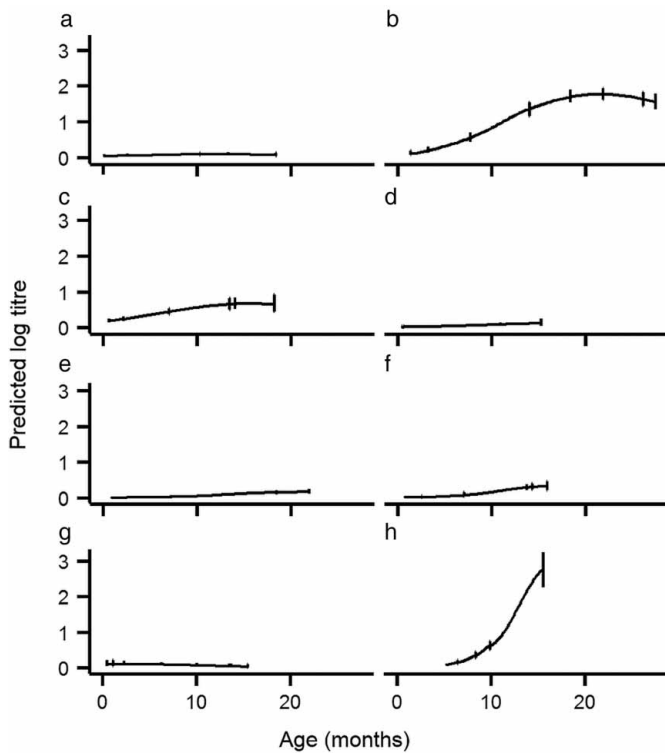


Figure 3. Predicted mean log titre for *Leptospira interrogans* serovar Pomona as a function of sheep age on eight different farms (a–h), with Loess smoothing.

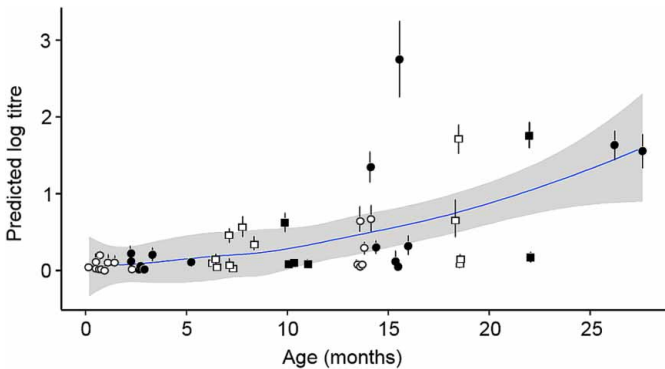


Figure 4. Predicted mean log titre for *Leptospira interrogans* serovar Pomona including all farms as a function of sheep age, with Loess smoothing and bootstrap 95% CI around predicted values. Season is shown as spring ○; summer ●; autumn □ and winter ■.

interest. The log titre for serovar Hardjo declined by 0.15 each month for the 14-month period and for Pomona by 0.16 for the 20-month period, assuming a constant decrease. The corresponding estimated half-life was 6.7 (95% CI=5.8–7.9) months for serovar Hardjo antibodies and 6.3 (95% CI=4.8–9.0) months for Pomona antibodies. Hence, the antibody duration depended on half-life and titre value at peak (Figure 5).

Between zero and nine animals per farm, for a total of 18 sheep across all farms, had a titre <48 for serovar Hardjo at the final sampling but had a titre ≥48 that could not be attributed to maternally derived antibodies at least once. The maximum titre of these animals ranged 48–3,072. Between one and nine animals per farm, for a total of 35 sheep across all farms, had a titre <48 for serovar Pomona at the final visit but had a titre ≥48 that could not be attributed to maternally derived antibodies at least once. The maximum titre of these animals ranged 48–768.

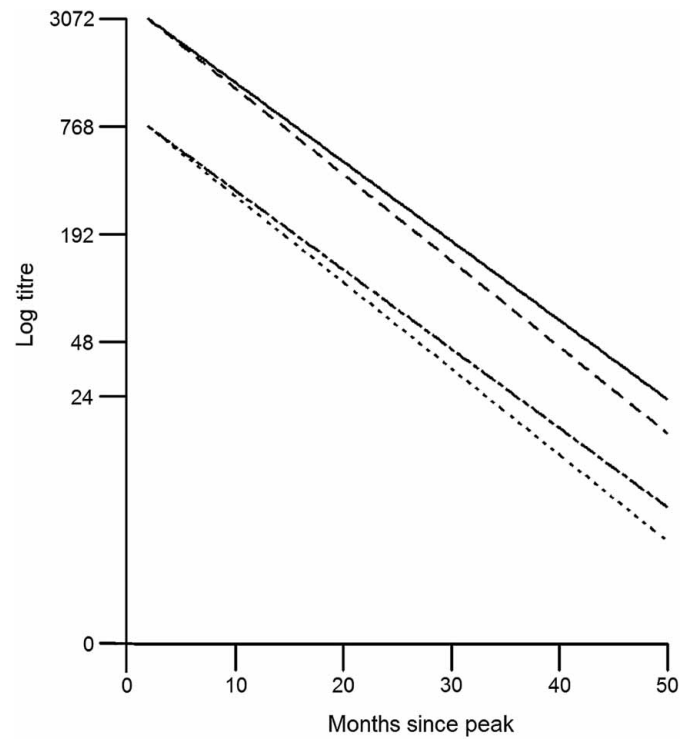


Figure 5. Predicted linear regressions of log titre for *Leptospira borgpetersenii* serovar Hardjo (plain line with peak at 3,072 and dot-dash line with peak at 768) and *L. interrogans* serovar Pomona (dashed line with peak at 3,072 and dotted line with peak at 768), as a function of time in months after a peak in naturally infected sheep.

Shedding in urine

Urine from 17 animals from Farms D and G was collected before the observation of seroconversion on the farm and were thus not included in the analysis. All 17 were qPCR-negative for *Leptospira* spp., and the GMT for serovars Hardjo and Pomona was zero on both farms. Results for the prevalence of leptospiral shedding in urine for the remaining farms are presented in Table 4. On farms where seroconversion was observed, 50/81 urine samples were qPCR-positive. The within-farm shedding prevalence ranged from 11 to 88% (Table 4).

Sheep that were positive for shedding of leptospires in urine were observed between 0 and 8 months after exposure was observed on the farm. Exposure was defined as the date when seroprevalence rose above 10% after having decreased to less than 5%, apart from Farm F where the animals were considered to have been exposed from the beginning of the study.

The results of the logistic regression model examining the relationship between titre and probability of urine shedding are presented in Table 5. Prevalence of urine shedding was best explained when a titre of 48 was used as the lower limit for the definition of a seropositive sample. Animals with a titre ≥48 for serovar Hardjo only, or both serovars Hardjo and Pomona, were more likely to be qPCR positive than seronegative animals, with OR=52.8 (95% CI=7.5–1108.6) and OR=21.0 (95% CI=2.0–538.5), respectively.

Animals lost to follow-up

Among the 1,523 ewe lambs enrolled, 769 were still present at the end of the study, 304 had been reported as culled by the farmer, and 450 were missing for unknown reasons. The prevalence of lambs that were seropositive for serovar Hardjo, and the GMT for serovars Hardjo and Pomona, on farms where there was a

Table 4. Date and number of sheep urine-sampled on eight farms, number positive for *Leptospira* spp. using quantitative PCR (qPCR), and number seropositive (microscopic agglutination test (MAT) titre ≥ 48) with geometric mean titre (GMT) for *Leptospira borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona, by qPCR status.

Farm	Sampling date	N sampled	qPCR +ve	Exposure interval ^a	Serovar Hardjo				Serovar Pomona			
					qPCR +ve		qPCR -ve		qPCR +ve		qPCR -ve	
					MAT +ve	GMT (95% CI)	MAT +ve	GMT (95% CI)	MAT +ve	GMT (95% CI)	MAT +ve	GMT (95% CI)
A	01-Nov.-12	8	7	3	7/7	384 (212–695)	1/1	768	0/7	0	0/1	0
B	30-Jul.-13	9	2	14	2/2	96 (25–373)	6/7	242 (154–380)	0/2	24	3/7	610 (246–1508)
C	06-Nov.-12	4	3	0	3/3	242 (154–380)	1/1	48	0/3	0	0/1	0
E	18-Jul.-13	9	1	4	1/1	192	8/8	384 (246–599)	0/1	0	0/8	24
F	20-Nov.-12	12	5	6	4/5	146 (53–402)	1/7	96	0/5	0	0/7	24 (24–24)
G	14-Nov.-12	16	14	0	14/14	848 (555–1296)	1/2	192 (NA)	0/14	0	0/2	0
H ^b	13-Aug.-13	15	13	1	9/9	1536 (926–2548)	1/2	1536 (NA)	7/9	517 (121–2199)	0/2	24
H ^b	18-Dec.-13	8	5	5	3/3	768 (351–1683)	3/3	610 (388–959)	1/3	1536	2/3	272 (9–8107)
Total		81	50	0–14	43/44	552 (394–773)	22/31	328 (230–469)	8/44	312 (84–1160)	5/31	121 (36–401)

NA= not applicable; +ve=positive; -ve=negative.

^a Months since the prevalence rose above 10% for either serovars Hardjo or Pomona on the farm.

^b Not all animals were blood sampled.

Table 5. Coefficients for the final mixed logistic regression analysis estimating probability of leptospiral shedding in the urine of sheep (determined using quantitative PCR), with the explanatory variables age and serological status (determined using microscopic agglutination test titres ≥ 48) for *Leptospira borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona, and farm as a random effect.

Variable	β	SE of β	P value ^a
Intercept	3.19	1.60	0.047
Age (months)	-0.39	0.09	<0.001
Seropositive Hardjo alone	3.97	1.17	<0.001
Seropositive Hardjo and Pomona	3.04	1.54	0.022
Farm	Variance=0		

^a Significance of association.

difference between animals that were retained in the study and those lost to follow-up are presented in Table 6.

No difference in seroprevalence for serovar Pomona was observed on any farm between lost to follow-up animals and those remaining in the study. No difference in GMT for Pomona was observed between retained and lost to follow-up sheep on Farms A, D, E, F and G, which were those where there was no increase in predicted mean titres with age. No difference in GMT for Pomona was observed on Farm H, where there was an increase in predicted mean titres with age (Figure 3).

There was no difference in seroprevalence and GMT for Hardjo and Pomona between the animals known to have been culled for impaired productivity and the animals that remained in the study.

Table 6. Number and prevalence of lambs that were seropositive (microscopic agglutination test titre ≥ 48) for *Leptospira borgpetersenii* serovar Hardjo, and geometric mean titre (GMT) for serovar Hardjo and serovar Pomona, on farms where there was a difference between animals that were subsequently retained in the study (Remained) and lost to follow-up (Lost).

Farm	Date when loss to follow-up recorded	Remained	Lost	P value ^a
Hardjo seroprevalence				
F	Nov.–Dec. 2012	66/105 (63%)	45/53 (85%)	0.005
G	Dec. 2012	1/3 (33%)	65/68 (96%)	0.01
H	Jul. 2013	4/15 (27%)	9/106 (8%)	0.06
Hardjo GMT				
A	Aug. 2012	6.6	2	<0.001
C	Nov. 2012	4.8	4.5	0.09
F	Nov. 2012	5.4	4.9	0.07
Pomona GMT				
B	May 2012	4.7	7	0.006
	Jul. 2013	2.9	3.9	0.04
	Dec. 2013	2.5	3	0.007
C	Dec. 2011	1	2.2	<0.001
	Nov. 2012	3.8	4.8	0.08

^a Significance of Fisher's exact test or t-test.

Discussion

Our results provide what is, to our knowledge, the first detailed study of the within-farm prevalence and longitudinal exposure

pattern to *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona on a sample of sheep farms in New Zealand.

Monitoring of MAT titres in sheep for up to 27 months revealed a pattern of Hardjo seroconversion that was similar on all farms. The animals were first exposed when 8–17 months old, which seasonally coincided with a period from the end of winter to early summer. This timing concurs with that reported by Dorjee *et al.* (2008) showing an increase in seroprevalence between July and November. It confirms that hoggets are generally more likely to be Hardjo seropositive than lambs, as previously shown by Dorjee *et al.* (2005). However, while that seems to be the usual pattern, variation did exist, as early Hardjo infection of 3-month-old lambs was observed on Farm F. Exposure to Pomona started at a similar age (4–14 months), but titres increased at a lower rate and steadily over the entire observation period of about 2 years.

Seasonality of infection could be related to the ecology of *Leptospira* spp. and a wetter climate in winter and spring. It has been demonstrated that winter provides good conditions for survival of serovar Pomona in soil and environmental transmission (Hellstrom and Marshall 1978). However, the different exposure patterns for serovars Hardjo *vs.* Pomona suggest that ecological determinants (e.g. survival, shedding, host preference) may be serovar-specific. Maternal antibodies for serovar Pomona were observed on only one farm and on six farms for Hardjo. The within-farm seroprevalence rose above 10% on only three farms for Pomona but reached 80% on all farms for Hardjo, contributing to evidence of a different ecology between these two serovars.

Co-grazing of hoggets in their first gestation with other reservoir species can also be suggested as a risk factor for exposure, as has been observed for deer (Subharat *et al.* 2012b). Conflicting evidence exists on the effect of co-grazing sheep with other species on seroprevalence of Hardjo. In Brazil, Genovez *et al.* (2011) showed that grazing sheep with cattle increased the risk of being positive for Hardjo, and in the United Kingdom Hathaway *et al.* (1984) suggested that sheep flocks were more likely to be Hardjo-positive when cattle density increased, although that relationship was not confirmed statistically. On the other hand, evidence for transmission across species was not observed in a multi-species prevalence study in New Zealand by Dreyfus (2013). However, the seroprevalence observed in that study was high (97% of seropositive sheep flocks), thus limiting the power of the study to find associations with risk factors.

The within-farm prevalence of serovar Hardjo rose from <5% to 80–100% over a 10-month period, suggesting a high infection pressure, probably exposure to a common source of infection such as contaminated water, and not only animal to animal transmission. The within-farm prevalence was higher in our study than observed by others in New Zealand, mostly less than 40% (Blackmore *et al.* 1982; Dorjee *et al.* 2008; Dreyfus 2013), but consistent with the upper values of within-line prevalence found in New Zealand by Fang *et al.* (2014a). However, those studies were conducted on fewer animals of a different age, including mixed-age sheep which could have seroconverted and reverted to non-detectable titres over time.

At the first sampling 13–76% of the 2–6-week-old lambs had MAT titres ≥ 48 for serovar Hardjo. Apart from Farm F, seroprevalence on all farms subsequently dropped to under 5%, suggesting that these early titres were due to maternal antibodies. These likely maternal antibody titres were observed for up to

3 months in some lambs, hence longer than suggested by previous studies (Andreani *et al.* 1983), but less than the 6 months observed in cattle (Hellstrom 1978). It is still unclear whether these apparently maternally derived antibodies are protective against infection and whether the presence of newly acquired agglutinating antibodies is directly correlated with protection against infection, as has been suggested in cattle (Zuerner *et al.* 2011). The observed trend on farms also suggests that from spring to autumn, lambs were either protected or not exposed. The rapid seroconversion in late autumn to early summer suggests that either flock immunity was poor or exposure suddenly increased during this period.

The pattern of seroconversion on Farm F was different to that on other farms, with the first Hardjo seroconversions observed between tail docking in spring and weaning of 3-month-old lambs in summer, as shown by a higher GMT at weaning than on the other farms. However, the seroprevalence at that time was low (10%) and was less than 20% in the following autumn. Hence, most seroconversion occurred at the same period as on the other farms, between late autumn and early summer. As farms in this study were not selected randomly, they may not be fully representative of New Zealand commercial sheep farms, and more work would be necessary to know how frequently early exposure similar to that observed in Farm F happens. Further, this study was largely conducted in 2011–2012, hence most farms experienced only one complete yearly weather pattern. The exceptionally dry summer in early 2013 could also have affected re-exposure of the animals and influenced the seroprevalence observed at the last samplings.

A similar seasonal trend for first exposure was also present for serovar Pomona. Seroconversion was observed only on three farms, and exposure started from autumn to spring. The seroprevalence in lambs aged ≤ 4 months was <5% apart from one farm where it reached 15% before declining to under 5%. To our knowledge, little has been published on the seroprevalence of serovar Pomona in different age groups of sheep or on maternally derived immunity for this serovar.

The within-farm seroprevalence for Pomona was between 20 and 50%, where present, and mostly as a dual Hardjo-Pomona infection. Seroprevalence was less than for Hardjo, and varied between farms as observed in earlier studies (Blackmore *et al.* 1982; Dorjee *et al.* 2008; Dreyfus 2013).

Leptospira serovars Hardjo and Pomona, which belong to different serogroups, are not known to cross-react in the MAT, which was shown to have an overall serogroup specificity of 75% in the convalescent phase, with high variability between serogroups (Levett 2003). Among the six serovars known to be endemic in New Zealand (Marshall and Manktelow 2002), only Hardjo and *Leptospira interrogans* serovar Balcanica can potentially cross-react (Hathaway and Marshall 1980), but the latter is adapted to brush-tail possums (*Trichosurus vulpecula*) and is expected to cause only sporadic infection in sheep (Mackintosh *et al.* 1981). Cross-reaction between serogroups and paradoxical reactions are also possible during the acute phase (Levett 2001), but the low number of animals with a titre ≥ 48 that became negative by the end of the study suggests that this situation was rare or absent. Hence, several observations of the same animal being seropositive for both Hardjo and Pomona likely shows dual infection.

No clear cut-off currently exists to differentiate acute infection from past infection, or maternal antibodies from infection in

lambs. A cut-off of ≥ 48 has been suggested to detect past exposure (Blackmore *et al.* 1982). In this study, using this cut-off in lambs would have led to misclassification as infected on all farms, due to maternal antibodies. However, the GMT for serovar Hardjo was < 96 on all farms except Farm A at the first sampling. On the second sampling the GMT was still < 96 on all farms except Farm F, where seroconversion was happening. On some farms some lambs still had maternal antibodies for serovar Hardjo at 3 months of age. This shows that no clear cut-off can be defined at an individual level, but use of flock-level data can give evidence of maternal immunity rather than exposure of lambs.

The mean predicted titre for serovar Hardjo peaked at around 20 months-of-age, and the observed GMT peak varied between 400 and 1,935. This peak occurred later than the maximum observed by Blackmore *et al.* (1982) who found a higher GMT in lambs < 12 months of age than in hoggets. Observed titres to serovar Pomona showed no consistent pattern with increasing age after the initial exposure, due to lower seroconversion rates and not all farms being infected with Pomona. The mean predicted titre by farm did not show any age-related peak either. The observed GMT peak for Pomona varied between 166 and 665. The mean titre was lower for Pomona than for Hardjo, as observed earlier (Blackmore *et al.* 1982; Dreyfus 2013). The observed peak mean Pomona titre was coincidental with the observation of seroconversion, suggesting that the peak titre occurs rapidly after exposure. Fang (2014) reported that the peak titre occurred within 2 weeks after experimental infection with Hardjo or Pomona.

The age at exposure was consistent with the model of Hathaway (1981) for *Leptospira* spp. circulation in maintenance hosts, with young animals being infected before joining the breeding flock and thus potentially achieving protective immunity; that is before the risk period for reproductive loss, but still during the risk period for suspected reduced growth in lambs exposed to Pomona (Webster and Reynolds 1955). However, hogget mating is becoming more popular in New Zealand, putting hoggets at risk of suspected reproductive sub-performance due to exposure to serovars Hardjo and Pomona during their first pregnancy (Ellis *et al.* 1983; Leon-Vizcaino *et al.* 1987; West *et al.* 2004).

Differences in GMT and seroprevalence for serovar Hardjo between animals retained in the study and animals lost to follow-up occurred occasionally, but the size of the difference was not consistent and this was observed when sample sizes were low. Hence, these differences were likely due to a statistical type I error. It is thus unlikely that missing animals affected the estimates of seroprevalence or GMT for Hardjo. However, animals lost to follow-up possibly caused an underestimate of the seroprevalence, GMT and transmission for Pomona, as they had a higher GMT for this serovar on several occasions on two farms. This result suggests that Pomona may be associated with increased culling or mortality rates in lambs and hoggets, though this needs to be substantiated.

The within-farm urinary shedding prevalence ranged from 11–88%. Two of the nine animals sampled on one farm were still shedding 8 months after titres ≥ 48 for serovars Hardjo and Pomona were first observed, confirming that leptospiral shedding in sheep can be persistent. All but one qPCR-positive animals were seropositive for Hardjo and none was seropositive for

Pomona only. This strongly supports the presence of Hardjo shedding but no inference can be made about Pomona shedding based on qPCR and MAT without confirmation of serovar by culture. The shedding prevalence was higher than the zero prevalence in urine and 20% (3/15) in kidneys from one of 54 lines observed 30 years earlier (Blackmore *et al.* 1982), or than the 21% of kidney culture rate in seropositive sheep reported more recently by Dorjee *et al.* (2008). Those two studies used culture and dark field microscopy so the difference could be, at least partially, due to the fact that PCR detects DNA material while culture requires live organisms, and *Leptospira* spp. are known for being difficult to grow and sensitive to transport and contamination (Smith *et al.* 1994). Prevalence of urine shedding was also higher than the 33% observed by Fang *et al.* (2014a) who used the same qPCR assay as the present study but animals in that study were from different age groups, including mixed-age ewes. Those authors observed a seroprevalence of 57%. The lowest shedding prevalence in the current study was observed on the two farms (B and E) sampled during the second winter of the study, suggesting that shedding may be more frequent in lambs or young hoggets than in older animals.

In this study one animal with a positive qPCR result did not have detectable antibodies for serovar Hardjo or Pomona. Dorjee *et al.* (2008) and Fang *et al.* (2014a) found that 5/499 (1%) and 2/71 (3%) of seronegative sheep were culture- or qPCR-positive, respectively. This indicates that sheep can transmit leptospires without having a MAT titre ≥ 48 . Shedding without detectable titres for Hardjo has also been observed in cattle and deer (Subharat *et al.* 2012a) which are recognised maintenance hosts for this serovar.

Sheep with a titre ≥ 48 for serovar Hardjo were more likely to have a positive qPCR result. This supports the findings of Fang *et al.* (2014a) that the risk of shedding in animals seropositive for Hardjo was 12.5 times the risk of seronegative animals. The utility of MAT for the prediction of shedding is primarily at flock level, hence our findings support those of Fang (2014) that MAT is a robust tool at flock level for the evaluation of the risk of infection posed to humans. In recent years human infection has been associated with sheep handling, especially at abattoirs (Dorjee *et al.* 2011; Dreyfus *et al.* 2014). The high seroprevalence and urinary shedding observed in the present study confirms bacterial circulation among sheep and that these animals can be a source of infection for humans.

The lack of specificity of the qPCR in identifying the infecting serogroup means that the shedding sheep could actually be shedding one or several serovar(s) other than Hardjo or Pomona. However, considering the high seroprevalence of Hardjo, revealing a high exposure, it is very likely that the majority of qPCR positive sheep were shedding Hardjo. Our estimate of shedding prevalence is also likely to be conservative, as no specific precaution was taken to account for the possible presence of PCR inhibitors in urine causing false negatives. Nevertheless further MAT screening using a panel of other serovars is required to fully validate the range of serovars in the New Zealand sheep population.

This study provides, to our knowledge, the first estimation of half-life of agglutinating antibodies against serovars Hardjo and Pomona in naturally exposed sheep. A half-life of 6.7 months for Hardjo and 6.3 months for Pomona would mean that for a sheep with a peak titre of 3,072, it would take 47 months to become seronegative (titre < 48) for Hardjo or 44 months for

Pomona, and for a sheep with a peak titre of 384, it would take 27 months for Hardjo, or 25 months for Pomona, to be negative. However, on farms where both serovars were detected, the GMT was higher for serovar Hardjo than for Pomona, suggesting that the peak titre would be lower for Pomona and thus the antibodies would be detected for a shorter time than for Hardjo. Comparing these results with Fang (2014), who showed that some sheep experimentally challenged with Pomona became seronegative 42 days after infection, reveals that the likely continuous re-exposure of sheep on farm, as supported by the shedding results, may play an important role in the maintenance of MAT titres. Other published work reported positive MAT titres for >3 months in sheep after experimental infection with serovars Hardjo or Pomona and >4 months in sheep naturally exposed to Hardjo (Hodges 1974; Andreani et al. 1983; Gerritsen et al. 1994).

Clinical disease in sheep has rarely been reported in recent studies (Dreyfus 2013), but loss to follow-up for unknown reasons was associated with a higher GMT for Pomona on two farms in the present study. The frequency of exposure of young lambs to serovar Pomona before weaning on New Zealand commercial sheep farms is unknown, but this serovar has been associated with clinical disease and mortality (Vermunt et al. 1994). *L. interrogans* serovar Hardjo type Hardjoprajitno has been suspected in abortions and perinatal loss in sheep (Ellis et al. 1983), but the precise effects of *L. borgpetersenii* serovar Hardjo type Hardjobovis and *L. interrogans* serovar Pomona on sheep production in New Zealand and the associated economic losses still remain to be quantified.

This study provides insight into some aspects of the epidemiology of *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Pomona on commercial sheep flocks in New Zealand, in particular age of exposure, within-farm prevalence over time, duration of MAT titres, shedding and the association between MAT antibodies and shedding. However, additional studies across more farms and several years are needed to assess the effect of weather patterns and how consistent exposure is over time. Shorter sampling intervals around the periods of high risk of exposure between birth and 1 year of age are needed to fully understand the seroconversion pattern, assess the frequency of early exposure of lambs 4-months-old or younger and antibody dynamics. Shedding data with short sampling interval are also needed to better estimate the risk of infection for both animals and humans.

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