Molecular and eco-epidemiology of *Leptospira borgpetersenii* serovar Ballum in wild invasive mammals in a farming environment in New Zealand

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Abstract

Leptospirosis is an important zoonosis in New Zealand where it has historically been associated with livestock. Formerly negligible in human cases notified, *Leptospira borgpetersenii* serovar Ballum—associated with rodents and hedgehogs (*Erinaceus europaeus*)—is now preponderant. The role of wild introduced mammals in the epidemiology of leptospirosis has been overlooked in New Zealand but remains a critical question. In this thesis, we determined the prevalence of *Leptospira* serovars, renal colonisation and seroprevalence in wild mammals and sympatric livestock. During a cross-sectional and a longitudinal survey, house mice (*Mus musculus*), ship rats (*Rattus rattus*) and hedgehogs were trapped in farms with a history of leptospirosis to collect sera and kidneys. Urine and sera from livestock (dairy or beef cattle, sheep) and dogs were also collected on the same farms. Sera were tested by microagglutination test to identify serovars/serogroups that circulate in wildlife for comparison with those circulating in livestock. Urine and kidney samples were used to determine prevalence by qPCR, to isolate circulating leptospires by culture and subject them to whole genome sequencing, in order to determine their phylogenetic relationships and compare them to other sequences locally, nationally and internationally. Capture-mark recapture (CMR) methods were used to investigate the population dynamics of mice naturally infected with Ballum. Finally, the level of lesions and bacterial load in kidneys were assessed visually by histopathology and put in perspective with other results to investigate reservoir dynamics. Direct or indirect presence of Ballum was found in all wild and domestic species investigated. Overall apparent prevalence in mice, rats and hedgehogs was respectively 46%, 95% CI [39, 52%], 44% [26, 62%] and 27% [11, 50%]. It varied greatly between seasons in mice, with a spring peak (83 to 86%) and minimum in autumn (31 to 37%). Mice densities reached up to 56 mice/ha and varied seasonally in the opposite way, resulting in a relatively constant density of infected mice, ranging 3-8 infected mice/ha. An extremely low rate of mutations hindered the investigation of transmission pathways using genomics. However, despite little or no lesions in all species, the bacterial load was markedly higher in mice, suggesting rats and hedgehogs are secondary hosts. Control strategies to mitigate exposure to *Leptospira* in NZ should include wild mammals, and especially mice.

Résumé

La leptospirose est une zoonose grave en Nouvelle Zélande et y a historiquement été associée au bétail. Autrefois négligeable dans les cas humains notifiés, *Leptospira borgpetersenii* serovar Ballum—associé aux rongeurs et aux hérissons (*Erinaceus europaeus*)—est désormais prépondérant. Le rôle des mammifères sauvages envahissants dans l'épidémiologie de la leptospirose a été ignoré en Nouvelle-Zélande mais reste une question critique. Dans cette thèse, nous avons déterminé la prévalence des sérotypes de *Leptospira*, la colonisation rénale et la séroprévalence dans la faune sauvage et le bétail sympatrique. Au cours de deux études transversale et longitudinale, des souris domestiques (*Mus musculus*), des rats noirs (*Rattus rattus*) et des hérissons ont été capturés dans des fermes à haut risque de leptospirose pour collecter du sérum et des reins. De l'urine et du sérum de bétail (bovins laitiers ou allaitants, ovins) et de chiens ont également été collectés dans les mêmes fermes. Les sérums ont été testés par test de microagglutination pour identifier les sérovars/sérogroupes qui circulent dans la faune sauvage et les comparer avec ceux qui circulent dans le bétail. Des échantillons d'urine et de rein ont été utilisés pour déterminer la prévalence par qPCR, pour cultiver et isoler les leptospires circulants et séquencer leur génome, afin de déterminer leurs relations phylogénétiques et de les comparer à d'autres séquences à l'échelle locale, nationale et internationale. Des méthodes de capture-recapture ont été utilisées pour étudier la dynamique des populations de souris naturellement infectées par Ballum. Enfin, le niveau de lésions et la charge bactérienne dans les reins ont été évalués visuellement par histopathologie et mis en perspective avec d'autres résultats pour étudier la dynamique de réservoir. La présence directe ou indirecte de Ballum a été trouvée dans toutes les espèces sauvages et domestiques étudiées. La prévalence apparente globale chez la souris, le rat et le hérisson était respectivement de 46%, 95% IC [39 – 52%], 44% [26 – 62%] et 27% [11 – 50%]. Elle variait fortement d'une saison à l'autre chez la souris, avec un pic printanier (83 à 86%) et un minimum en automne (31 à 37%). Les densités de souris ont atteint jusqu'à 56 souris/ha et ont varié de façon saisonnière dans le sens opposé, résultant en une densité relativement constante de souris infectées, allant de 3 à 8 souris infectées/ha. Un taux de mutations extrêmement faible a entravé l'étude des voies de transmission basée sur la génomique. Cependant, malgré peu ou pas de lésions chez toutes les espèces, la charge bactérienne était nettement plus élevée chez la souris, ce qui suggère que les rats et les hérissons sont des hôtes secondaires. Les stratégies de contrôle pour atténuer l'exposition à *Leptospira* en Nouvelle-Zélande devraient inclure la faune sauvage, et en particulier la souris.

To Yves,

Jean-Baptiste,

and Liam,

my wise,

kind

and mischievous angels

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Several students were involved in what I will call 'satellite projects' that were more or less related to this project (and are listed in Appendix 1): pilot studies (Nanett Fabri, Mark Van de Pol), summer research projects built on this thesis (Oumayma El Attar Sofi, Hedwich Oosterhof), projects using samples taken it the frame of this thesis (Annebel Klaasen, Matthew Edwards), or collaborations on samples taken in the frame of other projects for which I performed some laboratory tests (Gillian Dennis) or on data I helped analyse (Renata Muylaert). Thanks for the broader perspective you offered.

By all account, the PhD is a journey. In addition to all the people already mentioned, I was fortunate to be surrounded by other doctoral (or not) students (or not) that made this journey much more agreeable (dare I say, beerable). I will not make a list as (i) I would certainly forget a name (as has been, still is and will be the case with my supervisors), (ii) this section is already lacking conciseness but would require even more pages to do you justice. So, if you feel you made my life happier during the past three years and more, you are definitely included here. If you don't know, you probably are too. Thanks for the support, agraphia group, kindness, glasses of wine, weekend trips, cakes, cards, meditation sessions, hugs, frogs and geckos, office pranks, games nights, chocolate, early surf sessions, laughter, piano tunes, kittens, energy, conversations, litres of tea and hot chocolate, weirdness, book suggestions, your cooking skills, your baking skills, your everlasting smiles, your encouragements. Thank you.

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And as I began this section with a quote, I will end it with another quote, from Amelia Earhart this time: “The more one does and sees and feels, the more one is able to do, and the more genuine may be one’s appreciation of fundamental things like home, and love, and understanding companionship”.

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Abbreviations

ACC: Accident Compensation Corporation

AIC: Akaike information Criterion

BHQ1: Black Hole Quencher 1, a non-fluorescent chromophore used as 3' quencher in TaqMan™ probes

BLAST: Basic lical alignment search tool

bp: base-pair

BRENDA: bacterial restriction-endonuclease DNA analysis

Ca.: C*irca*

CDS: CoDing Sequence

cgMLST: core genome MultiLocus Sequence Typing

cgST: core genome Sequence Type

CI: Confidence Interval / Credible Interval

CMR: Capture-Mark-Recapture

Ct: Threshold value for qPCR

CT: Clustering Type

DALY: Disability Adjusted Life Years

df: degree of freedom

DFM: Dark Field Microscopy

DoC: Department of Conservation

DNA: Desoxyribonucleic Acid

ELS: European Leptospirosis Society

EMJH: Ellinghausen & McCullough – Johnson & Harris medium

e.g.: *exempli gratia* (for example)

ESR: Institute of Environmental Science and Research Limited

FAM: 6-carboxyfluorescein, a type of fluorophore present in TaqMan™ probes

FAST!: France Aotearoa Science, Technology and !nnovation

g: relative centrifugal force, the acceleration in a centrifuge normalized to Earth's gravity

G: Birmingham gauge, the wire gauge system used to specify the diameter of hypodermic needles

GC content: guanine-cytosine content of a gene or sequence

GE: Genome Equivalent

glmU: glucosamine-1-phosphate acetyl transferase

GPS: Global Positioning System

gyrB: gyrase B

HE: Haematoxylin-Eosin staining

HMM: Hidden Markov Model

i.e.: *id est* (that is)

IgG: Immunoglobulin of class G

IgM: Immunoglobulin of class M

ILS: International Leptospirosis Society

Indel: Insertion-deletion

ℓ, mℓ, μℓ: litre, millilitre, microlitre

LCM: Latent Class Modelling

lipL32: Major *Leptospira* outer membrane lipoprotein 32

LLOD: Lower limit of detection

LPS: LipoPolySaccharide

MAT: Microscopic Agglutination Test

MCMC: Markov Chain Monte-Carlo

^{m}EpiLab: Molecular Epidemiology and Public Health Laboratory

MLST: multilocus sequence typing

MPI: Ministry for Primary Industries

MU: Massey University

MUAEC: Massey University Animal Ethics Committee

NAT: Nucleic Acid Testing

NCBI: National Center for Biotechnology Information

NZ: New Zealand

OIE: World Organization for Animal Health

OR: Odds ratio

PATRIC: PAThosystems Resource Integration Center

PBS: Phosphate buffered saline

PCR: Polymerase Chain Reaction

PI: Post-Infection

PPE: Personal Protective Equipment

PSI: Proportional Similarity Index

QAICc: corrected quasi Akaike information criterion

qPCR: quantitative (real-time) polymerase chain reaction

REA: synonym of BRENDA

RefSeq: the NCBI Reference Sequence database

RFLP: Restriction fragment length polymorphism

RNA: Ribonucleic Acid

RNase: Ribonuclease

rRNA: Ribosomal ribonucleic acid

SD: Standard deviation

SECR: Spatially Explicit Capture-Recapture

SNP: single-nucleotide polymorphism

ST: Sequence Type

STAFF: Sulfamethoxazole, Trimethoprim, Amphotericin B, Fosfomycin, and 5-Fluorouracil

sv, svs: serovar, serovars

UK: United Kingdom

USA: United States of America

VNTR: variable number tandem repeat

vs.: *versus*

WGS: whole genome sequencing

WHO: World Health Organization

WS: Warthin-Starry staining

“Well then, farewell, Æschylus, go and save our city
with noble sentiments, and educate the dunces.
There's plenty of them.”
— *Pluto,* Aristophanes, *Frogs*

Chapter 1

Introduction

Chapter 1. Introduction

Preamble

This thesis is formatted as a thesis by publication. The current chapter is an introduction giving information necessary to understand the context around this thesis that are not addressed in the other chapters. The following five chapters are formatted as independent research projects suitable for publication. Consequently, repetitions occur between chapters.

Similarly, despite substantial contribution to each chapter, the author of this thesis is not the sole author of the research chapters, which benefited from the inputs and comments of other co-authors. As such, and for the sake of consistency, we use the first-person plural voice throughout this thesis.

Leptospirosis: a ubiquitous disease

Aetiology

Leptospirosis is caused by a pathogenic spirochete belonging to the genus *Leptospira*, and affecting a wide range of vertebrates (Levett, 2001). *Leptospira* are suspected to be saprophytic soil bacteria that evolved into pathogenic strains by adaptation to mammalian hosts (Thibeaux et al., 2018a, Fouts et al., 2016, Xu et al., 2016).

Morphologically leptospires are all similar, with a thin spiral shape and hooked ends and a diameter of 0.1 µm for 6 – 20 µm length range. They are aerobic, very motile Gram negative bacteria (Adler, 2015). The heterogeneity of the lipopolysaccharides (LPS) constituting the outer membrane explains the diversity of serovars (Birtles, 2012). Pathogenic leptospires have optimum growth range of 28 – 30°C and pH 7.2 – 7.6 and are delicate and slow to grow (Adler and de la Pena Moctezuma, 2010). They can survive several months in fresh water under favourable environmental conditions (Levett, 2001, Faine, 1999). For a more detailed review of the organism, see (Adler, 2015, Faine, 1999, Levett, 2001)

The classification and taxonomy of leptospires has greatly evolved since the bacterium *Spirocheta interrogans* was first described as the cause of Weil's disease at the beginning of the 20th century (Adler, 2015), particularly since the emergence of molecular tools. There are currently 23 validated species or genomospecies in the genus *Leptospira* and 42 candidate species awaiting validation by the International Committee on Systematics of Prokaryotes (Thibeaux et al., 2018a, Vincent et al., 2019). The latest study at the time of writing describes two clades, Saprophytes (S) and Pathogens (P), each subdivided into two subclades (S1 and S2 and P1 and P2), where S1 is the former Saprophytic group, S2 a new clade, P1 is the former Pathogenic group and P2 the former Intermediate group (Vincent et al., 2019). It is likely that taxonomic classification schemes will continue to evolve as genomic data become increasingly available.

Alongside this genetic classification, clinical and epidemiological descriptions still often refer to the historical classification of leptospires in two phenotypic species depending on their pathogenic (*Leptospira interrogans sensu lato*) or saprophytic (*L. biflexa s.l.*) nature (Adler, 2015). This classification is based on serological typing and discriminates more than 300 pathogenic serovars (sv), antigenically similar serovars being clustered into serogroups for convenience (Adler, 2015). What determines a serovar is the outer membrane LPS of the bacterium. Variation in the genes related to the LPS does not necessarily follow the strains' evolution: the same serovar can be found in distinct species, or genetically closely related strains can belong to different serovars. As a result, there is no concordance between the genetic and serological classifications and both pathogenic and saprophytic serovars can be found in the same genomospecies (Levett, 2001). For instance, sv Hardjo can be found in both *L. interrogans*—sv Hardjoprajitno—and *L. borgpetersenii*—sv Hardjobovis—(Bulach and Adler, 2018), and as little as one indel (insertion-deletion) on gene *lic12008* (associated with LPS synthesis) is sufficient to explain the difference between *L. interrogans* svs Copenhageni and Icterohaemorrhagiae (Santos et al., 2018).

Several serovar names also refer to serogroup names. For the sake of clarity and conciseness, names employed in this thesis will refer to the associated serovar, unless stated otherwise.

A major zoonosis globally

Leptospirosis is an important zoonotic disease worldwide. Costa, Torgerson and colleagues estimated the morbidity and mortality at 1 million human cases and 60,000 deaths (Costa et al., 2015a), and the burden in terms of DALYs (Disability Adjusted Life Years) at 2.9 million lost per year globally (Torgerson et al., 2015). This places this disease

at the same level as cholera or rabies. While the highest numbers are recorded in Asia and Sub-Saharan Africa, the highest rates are recorded in Oceania (Torgerson et al., 2015, Costa et al., 2015a). Leptospirosis has a clear tropical pattern and the fact that the burden is lower in developed countries probably explains why it is under-studied. This disease is still considered as a neglected disease despite an increase in the number of studies conducted and published (Goarant et al., 2019).

Infection with *Leptospira* causes a broad range of symptoms and the classical description as a haemorrhagic fever only encompasses the most severe cases. The symptoms and signs of disease are protean, and it is often confused with other causes of acute febrile syndrome (*e.g.* dengue, malaria, influenza, chikungunya). Intense headaches and conjunctival suffusions are frequent although not pathognomonic (Adler, 2015). However, the severe cases requiring medical attention are only the tip of the iceberg and the great majority of infections are either mild or subclinical and thus underreported (Levett, 2001). The existence of asymptomatic human carriers has also been described (Ganoza et al., 2010). The description of post-leptospirosis symptoms is recent, and the literature is scarce on the subject, but it unveils an important issue (Goris et al., 2013). One-third of patients diagnosed with leptospirosis experience persistent symptoms (extreme fatigue, myalgia, malaise, headache, and a weak physical condition) several months or even years after onset of disease.

Water-associated exposures are an important risk factor, with floods and heavy rainfall being particularly important in low resource countries, and recreational water activities in developed countries (Mwachui et al., 2015). Exposure to rodents and particularly rats (*Rattus* spp.) or their urine was pinpointed very early after the first descriptions of *Leptospira* (Ido et al., 1918, Adler, 2015), and it is an important risk factor (Kamath et al., 2014, Mwachui et al., 2015). However the relative importance of other species acting as maintenance or as bridge hosts in transmission to humans appears to have been overlooked, and an increasing number of studies pinpoint the risk of contact with cattle and dogs (Guernier et al., 2016, Mwachui et al., 2015, André-Fontaine, 2004, Allan, 2016, Andersen-Ranberg et al., 2016).

Context in New Zealand

A unique island context and natural history

Of all the serovars and species described around the world, only eight serovars, from five serogroups and two species, have been isolated in New Zealand (NZ, Table 1-1).

Those serovars are *Leptospira borgpetersenii* svs Hardjobovis, Ballum, Balcanica and Tarassovi and *L. interrogans* svs Pomona, Copenhageni, Australis and Canicola. The latter two have been isolated on rare occasions from humans only (Midwinter and Fairley, 1999). Evidence suggestive of a novel serovar in the Tarassovi serogroup that is genetically different from *L. borgpetersenii* sv Tarassovi and circulating in cattle, has been described, but this strain has not been isolated at the time of writing (Yupiana, 2019). As in (Nisa et al., 2018, Edwards, 2019), it appears as 'Pasifika' in Table 1-1. Although serological titres against Bratislava and Arborea have been described in pigs and deer respectively, these are believed to be non-specific cross-reactions at the early stage of infection (Bolt, 1990, Subharat et al., 2011b).

Table 1-1 | Endemic serovars found in animals in New Zealand

Genomospecies	Serogroup	Serovar†
Leptospira interrogans	Icterohaemorrhagiae	Copenhageni
	Pomona	Pomona
	Sejroë	Hardjobovis
		Balcanica
Leptospira borgpetersenii	Ballum	Ballum
	Tarassovi	Tarassovi
		'Pasifika'

†Only DNA sequences of 'Pasifika' have been detected at the time of writing, and this new strain requires isolation for complete characterization

Given the richness of *Leptospira* described worldwide, the relative paucity of species and serovars described in NZ could be deemed as unexpected. However, the history and ecological context that surround human activities and the presence of mammal hosts in this country are very specific and could explain this situation.

The handbook of New Zealand mammals (King and Barrett, 2005, with a new edition in preparation) is a very thorough source of information about the natural history of mammals in NZ. A brief overview is given below, and external sources indicated when appropriate.

NZ was one of the last lands on Earth colonized by humans and had remained isolated from other land masses for several million years. No terrestrial mammals were present before human settlers imported them. Only two living species of bats and at least three other species now extinct had been present in the country before the first Māori settlers arrived circa 1200 AD (Hand et al., 2018). Māori brought with them the kurī (Polynesian dog) and the kiore (*Rattus exulans*). Europeans re-discovered this distant land in the 18th century and imported in successive waves all the other mammals currently present in the country. Most were willingly introduced: pigs, cattle, sheep,

goats for farming; horses for transport; cats and dogs as pets; hedgehogs (*Erinaceus europaeus*) to remind them of their native land; rabbits (*Oryctolagus cuniculus*), hare (*Lepus europaeus*), different species of deer or the Himalayan tahr (*Hemitragus jemlahicus*) for game; brush-tailed possums (*Trichosurus vulpecula*) for fur; different species of wallabies; stoats (*Mustela erminae*), weasels (*M. nivalis*) and ferrets (*M. putorius furo*) in the vain hope they would control the animals that they had intentionally—possums, rabbits—or unintentionally introduced—house mice (*Mus musculus)*, ship rats (*R. rattus)*, brown rats (*R. norvegicus*) and had become invasive.

The situation for wild introduced mammals in New Zealand is unusual. Unlike in other places in their distribution, house mice are not only commensal to humans but can be found in pastures and forest habitats. While hedgehogs are protected in Europe and appear to be declining in abundance, they are thriving here to the detriment of many endemic species of snails, insects and native birds. Mustelids and feral cats have no other wild carnivores to compete with.

NZ acclimatisation societies had their peak of activity in the late 19th-early 20th century and not only imported several species of mammals, but also of birds and plants, with severe impact on endemic species. Between the arrival of Māori and 1840 (signature of the Treaty of Waitangi that made NZ a British colony) it is estimated that 6.7 million hectares of forest were cleared, and 8 million between 1840 and 2000 (Dawson, 2007). Most of these lands have been used to create pastures and farming land. The country has thus undergone drastic modifications in a relatively short amount of time that affect the ecology of all species present.

Human cases on the rise

Leptospirosis is the most common occupational zoonosis nationally (Victoriano et al., 2009). Contrary to other countries, water activities or the presence of rodents have not been deemed important risk factors. People living in rural communities and agricultural workers, especially dairy farmers and meat workers represent high-risk groups for contracting leptospirosis (Victoriano et al., 2009, Cowie and Bell, 2012, Dreyfus et al., 2015b, Thornley et al., 2002). Leptospirosis is a notifiable disease in NZ, and surveillance is conducted by the Institute of Environmental Science and Research (ESR).

The most recent estimated annual cost of human leptospirosis due to time absent from work and treatment is 4.42 million US$, 95% probability interval [2.04, 8.62] for notified cases (Sanhueza et al., 2019). Nonetheless, these figures overlook indirect costs as well as personal costs to workers, families and businesses, especially burdening the

people suffering from post-leptospirosis symptoms who could represent 30% of the acute cases (Goris et al., 2013). The particularly important rate of underreporting—that Sanhueza (2019) estimated as high as 22-fold compared to notified cases—should also be taken into account.

In the 1970s, Hardjobovis and Pomona represented 99% of notified human cases (Mackintosh, 1981) and 80 to 90% of these were dairy farm workers (Mackintosh et al., 1982, Blackmore and Schollum, 1982). In contrast to numerous other countries, rodents and wildlife were considered of minor importance in leptospirosis epidemiology in NZ (Hathaway and Blackmore, 1981b, Hathaway, 1978). It is believed that the dramatic decrease in the number of cases since the 1980s (Figure 1-1) is linked to the implementation of vaccination in dairy cattle and pigs, along with increased awareness and use of personal protective equipment (PPE; Marshall and Chereshsky, 1996).

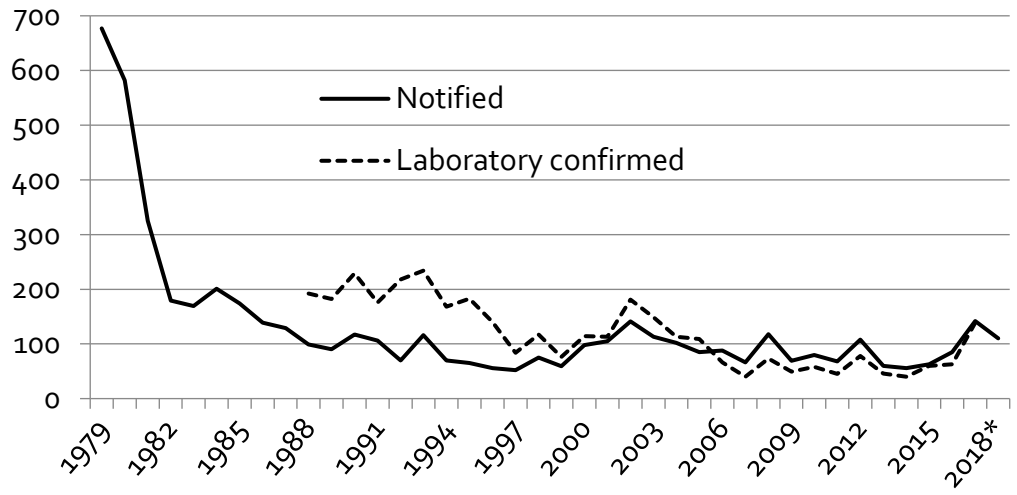

Figure 1-1 | Temporal evolution of notified human cases of leptospirosis in New Zealand *(Source ESR data, *provisional)*

The epidemiology of human cases has evolved during the last decades (Thornley et al., 2002). After the dramatic decrease in numbers in the 1980s, the number of cases has remained stable the last decade and reached a historical minimum between 2007 and 2014. While serovars Hardjobovis and Pomona accounted for more than 90% of the cases in the early 2000s (Crump et al., 2001), Ballum is now the predominant serovar after Hardjobovis (Figure 1-2).

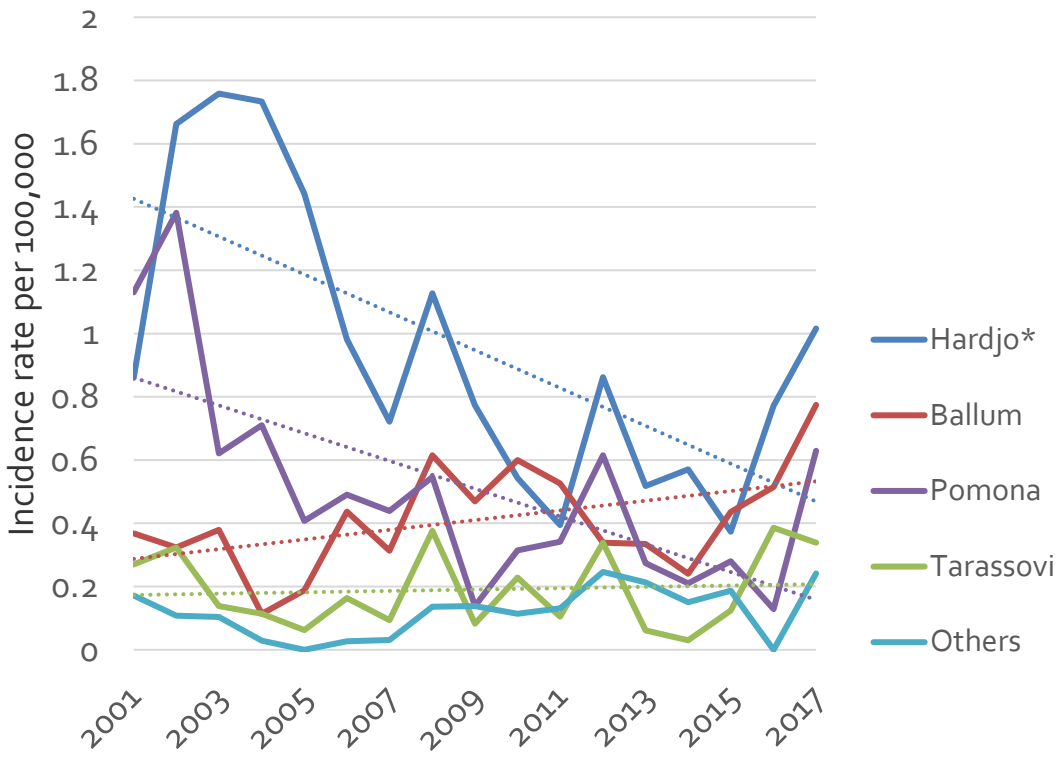

Figure 1-2 | Annual human incidence rate of leptospirosis by attributed serovar in New Zealand *(extrapolated from ESR data). The dotted line represents the linear trendline for each serovar. *Hardjo and Balcanica are indistinguishable serologically by MAT.*

In a study on *Leptospira* exposure in beef cattle, sheep and deer farmers conducted in 2013, Sanhueza found Pomona and Ballum as the most prevalent serovars (respectively 2.6 and 2.1%), with titres for Ballum in the range 96-384 while titres for Pomona and Copenhageni ranged from 24 to 96, and no seropositivity for Hardjobovis (Sanhueza et al., 2017).

An increase in the incidence of cases linked to serovar Ballum had been noted by Thornley in the 1990s (2002) and it is a growing concern. In 2016 two District Health Boards (DHBs, Waikato and Northland) alerted an increase in the number of leptospirosis notifications. In both regions, serovar Ballum predominated (personal communication, Dr Richard Wall and Dr Virginia McLaughlin, Medical officers of Health).

The occupational pattern also appears to have changed, with an increasing proportion of cases notified in occupations not traditionally expected to be at high-risk (Figure 1-3).

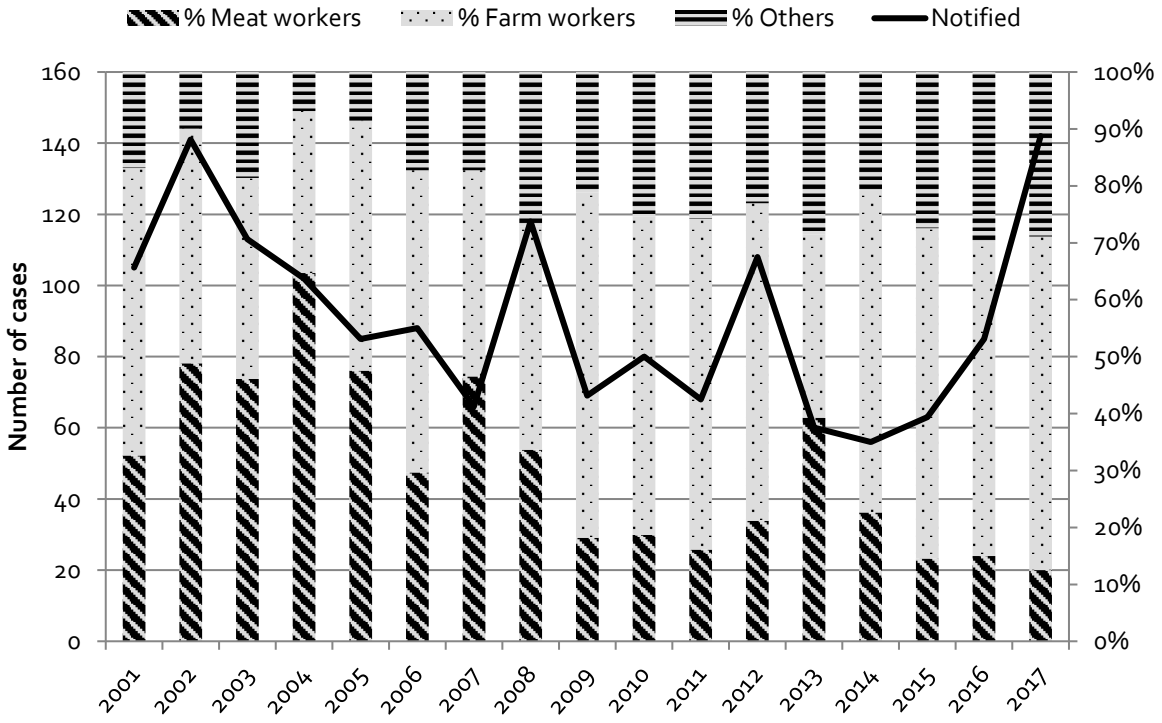

Figure 1-3 | Number of cases and proportion of high-risk occupations in notified cases of leptospirosis in New Zealand *(Source ESR data)*

Situation in the domestic reservoir

Aside from risk of disease for farm and abattoir workers, leptospirosis is a well-recognised cause of abortion in livestock (Ridler et al., 2015, Sanhueza et al., 2013) with thus an important impact in the agricultural sector, both in terms of public health and production loss. Direct costs in the agricultural sector (mainly attributed to vaccination costs and loss of income due to animal morbidity and mortality) are estimated to be 20 million NZ$, 95% CI [15.7, 25.2] annually (Sanhueza, 2016). A majority of dairy cattle and pigs are vaccinated, but vaccination is not as frequent in deer and beef and sheep farms. Vaccines available in NZ are bivalent (Hardjobovis, Pomona) or trivalent (Copenhageni in addition) for ruminants, bivalent for pigs (Pomona, Tarassovi), and monovalent (Icterohaemorrhagiae) for dogs.

Vallée (2016) presents in a detailed table a summary of the published cross-sectional studies of *Leptospira* seroprevalence in New Zealand pastoral livestock during the last decade, and Yupiana recently performed a systematic review of leptospirosis in dairy herds (Yupiana, 2019). Hardjobovis is the most prevalent serovar (seroprevalence range 5-54%), in agreement with the notion of cattle, and more recently sheep and deer, acting as maintenance hosts for this serovar (Marshall and Manktelow, 2002, Wilson et al., 1998).

The presence of Tarassovi in livestock is a growing concern, especially since Yupiana et al. reported vaccinated dairy cattle are shedding leptospires with serological results associated to this serovar (Yupiana et al., 2019a).

Leptospira infection is also described in dogs (Harland, 2015) and in cats (Shophet, 1979a). Whereas dogs are sensitive and develop a clinical leptospirosis, cats are more resistant and do not necessarily develop a detectable titre of antibodies following infection (Shophet, 1979b). Vaccination against leptospirosis is recommended for working dogs (Cave et al., 2016) but the vaccines available are targeting Icterohaemorrhagiae and Copenhageni and the use of livestock vaccines 'off-label' seems common (Cave et al., 2014). Although serovars included in available vaccines like Hardjobovis are the most frequently encountered in dogs, there is a recent call to test for a broader range of serovars to better diagnose cases of leptospirosis due to other serovars (Thompson, 2018).

There is therefore a relative understanding and control of the situation in the domestic reservoir, but an increasing need in terms of public health to gain a better understanding of the sylvatic reservoir, in a context where the eco-epidemiology of leptospirosis is likely to be affected by NZ natural history.

Reservoir dynamics – Theoretical concepts

Eco-epidemiology

In the word eco-epidemiology used in the title of this thesis, eco- refers to ecology, the science dealing with all the relations of all living beings to all their environment (Taylor, 1936). Epidemiology is classically defined as the study of diseases and health factors in a population (Moutou et al., 1991). The sub-discipline eco-epidemiology thus integrates interactions between various hosts, pathogens and their environment. The use and acceptance of the term eco-epidemiology is not universal (the French direct translation *éco-épidémiologie* seems more used than its English counterpart), and the word can be used in slightly different ways (March and Susser, 2006), or the notion replaced by other similar terms, such as disease ecology or community epidemiology (Ostfeld et al., 2008, Johnson et al., 2015). However, we argue that disease ecology may be an ill-based term, as the cut-off between diseased and healthy hosts can sometimes be blurred, especially when maintenance hosts are involved.

Traditional dichotomy Reservoir / Accidental hosts

Traditionally the study of infectious diseases has been based on studies of single-host single-pathogen systems and complex systems were either oversimplified or neglected. In epidemiological literature, the dichotomy 'reservoir' / 'accidental hosts' of *Leptospira* has traditionally been used to describe host-pathogen interactions (Babudieri, 1958, Leighton and Kuiken, 2001, Heath and Johnson, 1994). Historically 'reservoir hosts' have been defined as animals "in which the infection persists without causing severe signs of disease" (Heath and Johnson, 1994, p. 1519). Those 'reservoir hosts' were considered to be at a balance point in their relationship with their serovar and to show no or only subclinical signs of disease (Babudieri, 1958). Alternatively, *Leptospira* would spillover to non-reservoir, or 'accidental hosts', not so well adapted to the serovar, and more likely to experience morbidity or mortality as a result of infection.

This dichotomy is, however, not satisfactorily fitting the reality and has sometimes been confusing. For instance, although maintained by dogs, *L. interrogans* sv Canicola causes acute forms of canine leptospirosis (André-Fontaine, 2006) and although not reservoirs for strains belonging to serogroups Pomona and Icterohaemorrhagiae, apparently healthy dogs are suspected to be a source of infection by those serovars for their owners (Gay et al., 2014, Harland, 2015). Infected spillover hosts or 'accidental hosts' can present a continuum of symptoms, from an asymptomatic infection to a fatal leptospirosis (Hathaway, 1981c). They can thus be casualties, carriers, transmitters, sentinels of infection—or a combination thereof. For this reason, we will not refer to accidental hosts in the remainder of this thesis and will use the term 'reservoir' to encompass the whole maintenance system—environmental compartment included—and prefer the term of maintenance host for a specific population of hosts.

More recent conceptual frameworks

The last two decades, the uptake of ecological methods in epidemiology (eco-epidemiology or disease ecology) gave a new impetus in the study of more complex systems and numerous conceptual frameworks were published. Key concepts such as reservoir, maintenance and target hosts were revisited and refined, and new concepts were developed such as maintenance communities or populations, bridge or liaison hosts, spillover and spillback.

Definitions of concepts used in the context of this thesis are briefly given below, but more detailed information can be found in the references cited as well as in (Webster et al., 2016, Haydon et al., 2002, and Ashford, 2003). In a specific ecosystem, the host diversity and community composition determine the dynamics of transmission between

species, and different transmission systems can be defined (Figure 1-4). The **target population** is the population or subpopulation of concern to the observer (Viana et al., 2014), often humans in the case of zoonotic diseases. Rather than considering the source of infection for this target host as a whole, Caron et al. (Caron et al., 2015) differentiate a transmission function from the maintenance function. **Maintenance host populations** are populations able to maintain the pathogen permanently, regardless of the symptoms they may present. Sometimes, the pathogen persists indefinitely in several connected populations of non-maintenance hosts, or a mix of maintenance and non-maintenance (or secondary) hosts, leading to the need to define a **maintenance host complex**. The **bridge host** is a "non-maintenance host population able to transmit a pathogen from a maintenance host/complex to the target population, otherwise not or [only] loosely connected to the maintenance complex" (Caron et al., 2015, p. 3). It can link maintenance and target populations in the spatial, temporal or behavioural dimensions (Caron et al., 2015). In the case of leptospirosis, where bacteria can survive in and be acquired from the **environmental compartment**, that also has to be taken into account. In the remainder of this thesis, we will rely on the above different host definitions.

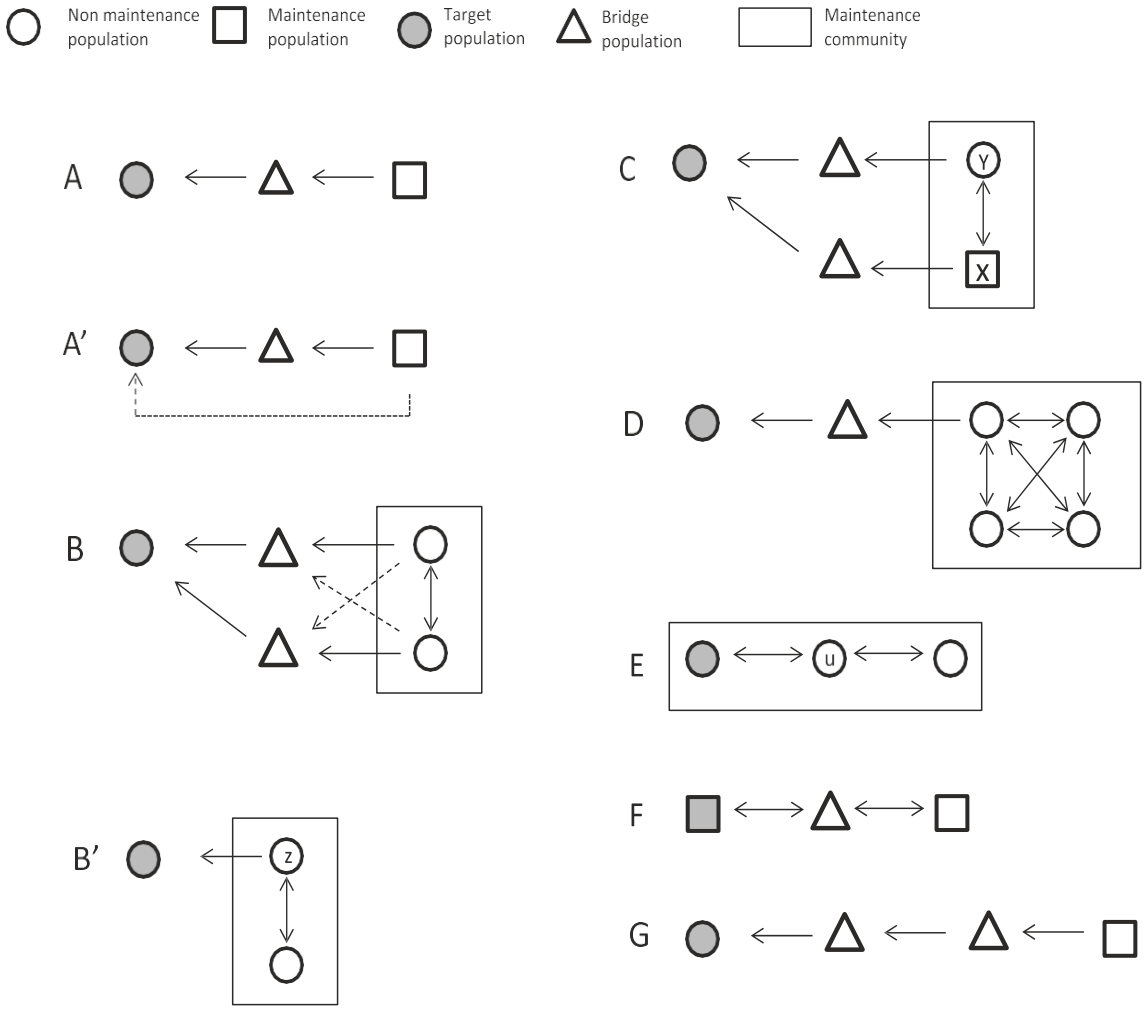

Figure 1-4 | Definition of different target-bridge-maintenance systems *(reproduced from Caron* et al. *(2015), licensed under CC-BY 4.0, with the kind permission of the authors). A represents the simplest maintenance-bridge-target system. In A', the maintenance and target populations are less connected (frequency/intensity of infectious contacts) than between the maintenance-bridge-target populations. In B, mitigation strategies aimed at one bridge host cannot fully control pathogen transmission to the target host because of the alternative bridge host's pathway. If both maintenance populations were in contact with both bridge hosts (i.e. if dashed arrows exist), controlling contacts between the target population and bridge hosts should be simpler than other control options. In B', according to our definition, Z is not considered as a bridge population as it belongs to the maintenance community. In C, stopping contacts between the maintenance population and the target population by acting on one of the two bridge hosts would not be enough to stop transmission, which can still occur through the second bridge host. D is a special case of B, understanding the complexity of the maintenance community is not necessary to control the pathogen transmission risk to the target population, which can be achieved through the control of arrows connecting the bridge host. In E, none of the host populations can sustain the infection by itself and according to our definition, u is not considered as a bridge population as it belongs to the maintenance community. In F, the bridge host connects the target population with another maintenance host creating a system with a maintenance meta-population, which could change the epidemiological dynamics of the system and the probability of success of intervention strategies (e.g. vaccination coverage to achieve control of the infection in the target population). G is a special case where two bridge hosts are necessary to achieve the transmission function. Good knowledge of the ecological interactions in the ecosystem will be necessary to identify such complex interactions between bridge hosts.*

Structure of this thesis and overarching goal

The overall aim of this thesis was to investigate whether wild mammals are an important source of *Leptospira* infection for livestock and humans. Although investigations of human aspects of the disease are not present in this thesis, the overarching goal of this work is to provide knowledge on the animal compartment to, *in fine*, protect people.

The current introduction gives an updated overview of the taxonomy and importance of the aetiological agent of leptospirosis, the ecological context in New Zealand, and gives an overview of theoretical concepts that will be developed in the following chapters.

Chapter 2 provides a review of the literature focusing on the eco-epidemiology of *L. borgpetersenii* sv Ballum, and presents available knowledge on its maintenance hosts and its potential to spill over to livestock.

To study the epidemiology of Ballum, it was necessary to understand the reservoir dynamics in a multi-host multi-pathogen system. Chapter 3 is a cross-sectional case study in two farms. Wildlife and sympatric domestic animals (sheep, cattle and dogs) were sampled in two contrasting systems in March-April 2017, a dairy farm and a beef and sheep farm. It allowed a screening of the wildlife species present on farm, an assessment of their abundance, and an investigation of the serovars of *Leptospira* circulating in wildlife and domestic animals sharing the same biotope. This study led to the evidence-based selection of mice, ship rats and hedgehogs as a model system for the reservoir dynamics of Ballum.

Chapter 4 is a longitudinal study focusing on the spatio-temporal dynamics of *Leptospira* infection in the population of mice in the same dairy farm as in Chapter 3. In addition to the sampling session described in Chapter 3, three other sampling sessions were implemented using capture-mark recapture methods to estimate densities and densities of infected animals.

In Chapter 5, different sequencing techniques, with different discriminating power, are applied to samples obtained in the sampling sessions described previously, and used for the study of *Leptospira* diversity and population structure. The usefulness and limitations of these methods to differentiate infecting serovars and investigate the within-strain genetic diversity of *Leptospira* are discussed.

Chapter 6 builds on results of Chapters 3, 4 and 5 to investigate further the reservoir dynamics of Ballum in a community of maintenance hosts, using histopathology as an additional tool.

In Chapter 7, the knowledge gaps identified in Chapters 1 and 2 and the results of the studies presented in the subsequent chapters are puts in perspective and discussed from different stakeholders' perspectives.

This overall structure is summarized in Figure 1-5 below.

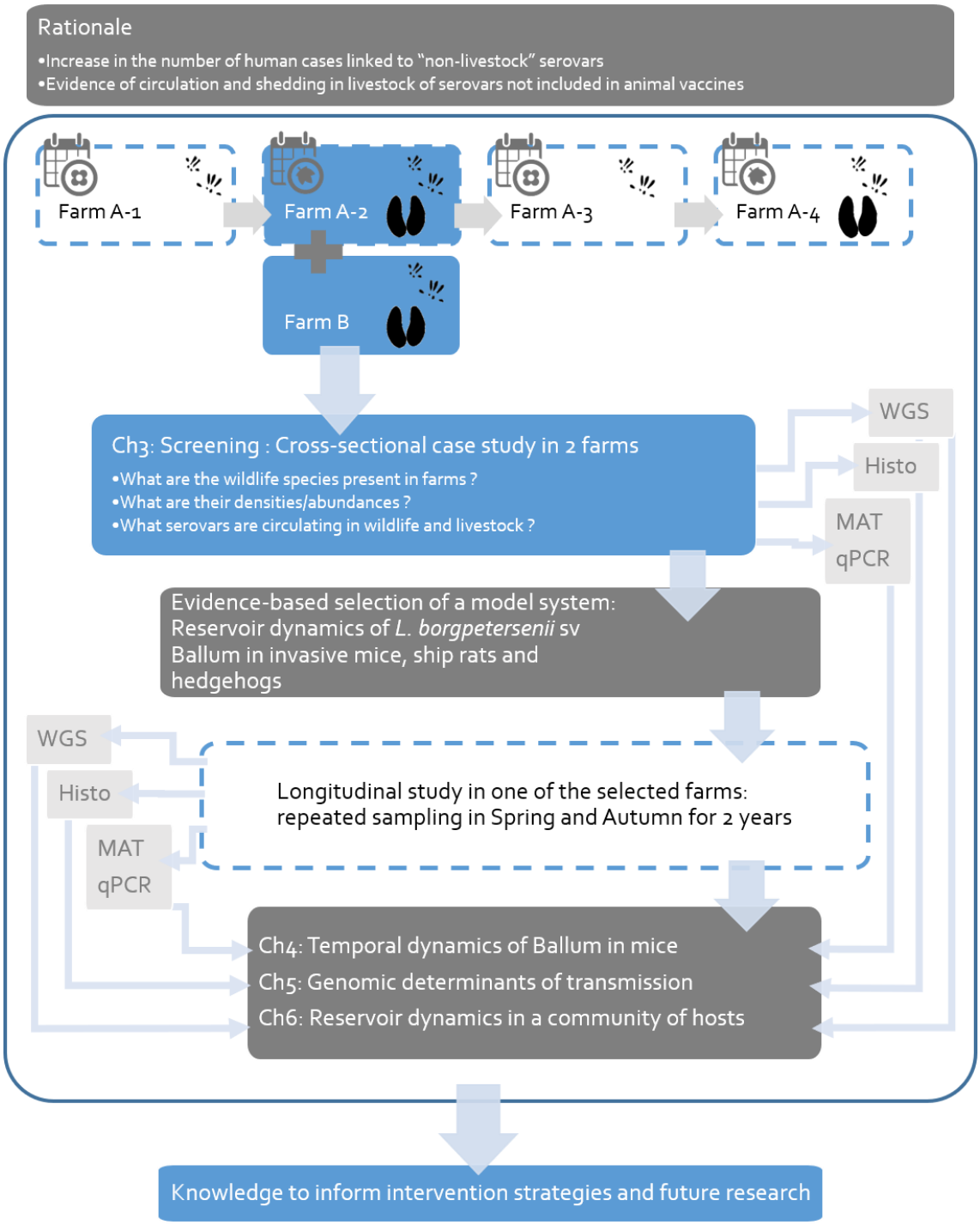

Figure 1-5 | Flowchart of the general organisation of this thesis. *The mice toeprints and cattle hoofprints represent sampling of wild species and livestock, and the flower and maple leaf icons represent spring and autumn seasons*

"Dicebat Bernardus Carnotensis nos esse quasi nanos gigantum humeris insidentes, ut possimus plura eis et remotiora videre non utique proprii visus acumine aut eminentia corporis, sed quia in altum subvehimur et extollimur magnitudine gigantea."
— John of Salisbury, *Metalogicon*

Chapter 2

Of mice, cattle, and men: review of the eco-epidemiology of *Leptospira borgpetersenii* serovar Ballum in an island setting

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Chapter 2.
Literature review

Abstract

Rodents and wildlife, unlike in numerous countries worldwide, were previously identified as a minor public health concern for leptospirosis in New Zealand (NZ). Here, this zoonosis is clearly related to occupation, with two-third of cases being farm or abattoir workers. Livestock species independently maintain serovars Hardjo and Pomona, both included in livestock vaccines. Livestock vaccination is thus a mainstay of *Leptospira* control in NZ. However, the increasing importance of *L. borgpetersenii* serovar Ballum, that now causes a third of notified human cases, suggests wildlife may be an overlooked source of infection. The role of livestock in the epidemiology of Ballum is unclear but recent findings indicate livestock could be bridge hosts for humans or part of the maintenance community.

We drew from two disease ecology frameworks to study the determinants of Ballum transmission and spillover. We collated published studies and preliminary findings of ongoing studies pertaining to (a) the infection dynamics of Ballum in potential maintenance hosts, including their distribution, densities, prevalence (MAT or culture) and pathogenesis, (b) *Leptospira* shedding and survival in the environment, (c) the competency of cattle as a potential bridge species, and (d) exposure (risk) factors for two potential spillover hosts, namely cattle (bridge host) and (e) humans (target hosts).

Our literature review identified all known and suspected wild maintenance hosts of Ballum in NZ, and narrowed the focus on introduced rodents (*Mus musculus, Rattus rattus, R. norvegicus*), and hedgehogs (*Erinaceus europaeus*). Their roles remain poorly defined and have not been thoroughly investigated in NZ for more than 35 years. These species are distributed throughout NZ, and present on pastures. For cattle, experimental and natural infections suggest a low pathogenicity and the possibility to shed Ballum, but information on survival in the environment is scarce and risk factors not well defined.

Our approach identifies gaps in the knowledge of Ballum and highlights the potential spillover to cattle for this serovar. Further studies will be important to ascertain the role that wild species and cattle may play in its transmission to humans, to

understand its survival in the environment, and to inform more effective control strategies.

Introduction

Leptospira are suspected to be saprophytic soil bacteria that evolved into pathogenic strains by adaptation to mammalian hosts, and genomic tools are beginning to unravel the diversity of species in this genus (Thibeaux et al., 2018a, Vincent et al., 2019). Of the more than 300 *Leptospira* serovars and 65 species or candidate species described around the world, only eight serovars, from five serogroups and two species, have been isolated in New Zealand (NZ). Those serovars are *Leptospira borgpetersenii* serovars (sv) Hardjobovis, Ballum, Balcanica and Tarassovi and *L. interrogans* sv Pomona, Copenhageni, Australis and Canicola. The two last have been isolated from humans only (Midwinter and Fairley, 1999).

In NZ, leptospirosis was initially described as the "dairy farm fever" (Christmas et al., 1974). It remains nowadays clearly related to occupation, with more than two-thirds of cases being farm or abattoir workers (ESR 2017). In the 1970s, Hardjobovis and Pomona represented 99% of notified human cases (Mackintosh, 1981) and 80 to 90% of these were dairy farm workers (Mackintosh et al., 1982, Blackmore and Schollum, 1982). Indeed, livestock species were found to maintain Hardjobovis and Pomona independently. As observed by Hathaway (1978), in the same NZ farm environment livestock would harbour and maintain Hardjobovis or Pomona, brush-tailed possum serovar Balcanica, and rodents and hedgehogs serovar Ballum. Livestock titres to serovars other than Hardjobovis or Pomona were attributed to cross-reactivity and spillover events were considered as rare, sporadic events (Hathaway, 1978, Hellstrom, 1978). To describe this host specificity the term 'nidality' or 'ecological niche' has been used (Blackmore and Hathaway, 1980, Hathaway, 1981c, Ferris et al., 1961).

As a result, and in contrast to numerous other countries, rodents and wildlife were then considered of minor importance in leptospirosis epidemiology in NZ (Hathaway and Blackmore, 1981b, Hathaway, 1978). Vaccination of dairy cattle and pigs using vaccines against serovars Hardjo and Pomona reduced the exposure levels in farm and meat workers and helped decrease the incidence of leptospirosis (Marshall and Chereshsky, 1996). Livestock vaccination remains a mainstay of *Leptospira* control nowadays (Yupiana, 2019).

However, the increasing importance of Ballum (Thornley et al., 2002) as a cause of up to a third of notified human cases (ESR 2017, 2019), suggests that wildlife may be an

overlooked source of infection. Farmers are among notified Ballum cases, yet the role of livestock in the epidemiology of Ballum is unclear (Cowie and Bell, 2012).

This chapter reviews knowledge available on this serovar in wildlife, and further explores the possibility for this serovar to spillover to domestic hosts.

Scope of this review and methodology

The natural history of NZ mammals and the presence of a limited number of *Leptospira* serovars offers an opportunity to focus on the eco-epidemiology of a specific serovar, without the pitfalls of more complex systems that exist elsewhere. Except otherwise stated, the scope of this review is limited to literature on the eco-epidemiology of *L. borgpetersenii* sv Ballum relevant to a NZ context.

We drew from two disease ecology frameworks (Plowright et al., 2017, Caron et al., 2015) to inform the choice of components for inclusion in the review and to adjust the searching terms to match each of them. These components corresponded to the different barriers that need to be crossed for a pathogen to spillover to a new host, based on the hypothesis that cattle act as bridge hosts (Figure 2-1).

Barriers pertained to:

- (a) the presence and abundance of maintenance hosts;
- (b) the infection dynamics of Ballum in maintenance hosts, including prevalence and pathogenesis;
- (c) Ballum shedding and survival in the environment;
- (d) the exposure and competency of cattle as a potential bridge species; and
- (e) the exposure (risk factors) for humans (target hosts).

Although genetic, physiological and immunological attributes of the spillover hosts are also important determinants of transmission (Plowright et al., 2017), these were considered beyond the scope of this work.

These barriers spanned subjects and disciplines (ecology, veterinary medicine, epidemiology, microbiology, human medicine). The breadth of the subject and the limited time available to conduct this review precluded the use of a systematic approach.

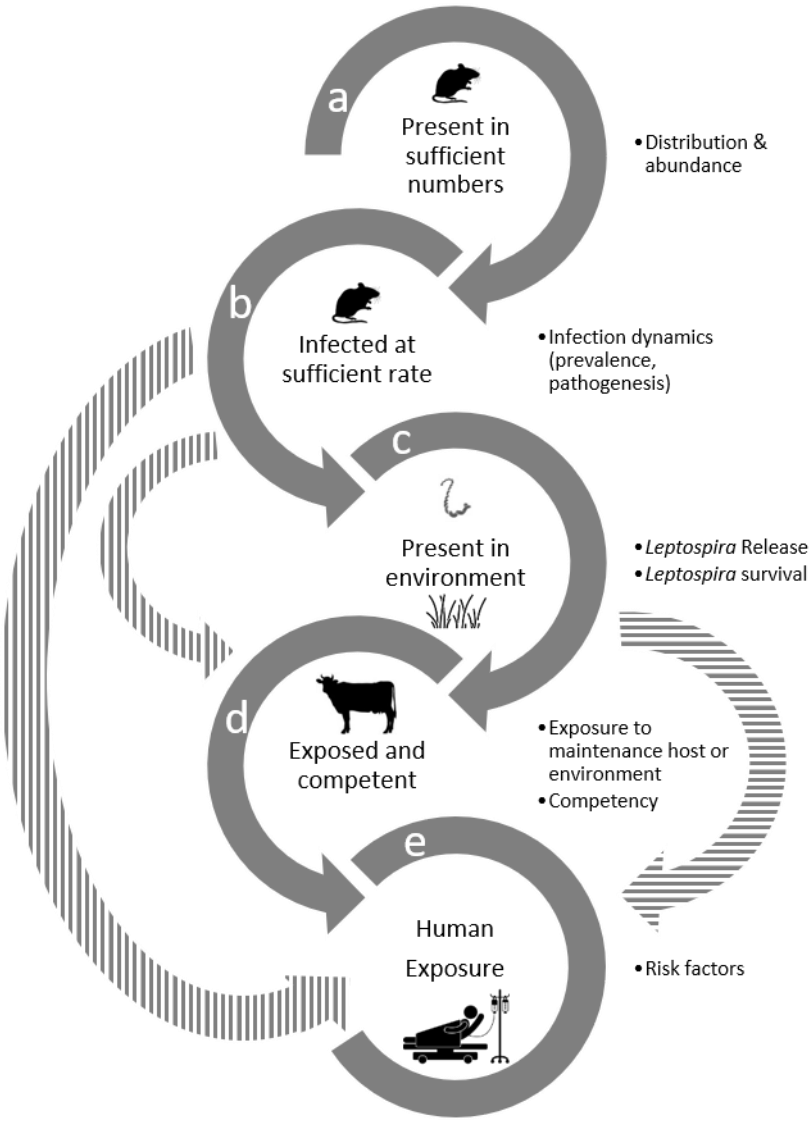

Figure 2-1 | Theoretical barriers to *Leptospira* spillover from wildlife to humans and component included in the literature search. *This diagramme assumes wild mammals are maintenance hosts, cattle act as bridge hosts and humans are target hosts.*

After the history of Ballum was looked over, a first search of the literature on wildlife as a source of *Leptospira* infection in NZ was conducted (Moinet et al., 2017, see also Appendix 2) and this informed a reduced list of maintenance hosts to include in the review (see below). As dairy cattle are in frequent contact with people compared to most other livestock species, we also limited the notion of bridge host to this livestock species and production system. When no or little information was available for the serovar Ballum, we searched for the species *L. borgpetersenii* or alternatively the genus *Leptospira*.

The online databases used were Scopus, Web of Science, and SciQuest. The specific keywords and search algorithms used in Web of Science are detailed in Table 2-1. For the initial search (#Wildlife AND #Lepto AND #NZ), searches were also performed

on the references cited in the articles retrieved. Google Scholar, the Massey University Library database (Discover) and the New Zealand database NZResearch.org.nz were also searched for additional literature on *Leptospira* in NZ (including grey literature), and the paper archives of the Leptospirosis Reference Centre at the Amsterdam University Medical Centre (spanning 1915-1990) for the keywords 'New Zealand', 'hedgehog' or 'Ballum'.

Because New Zealand mice and rabbits are specific breeds of experimental animals widely used in biomedical research, initial searches for rodent densities retrieved thousands of irrelevant articles. For this component, the subject area was refined, and the search limited to articles published after 2004 (Table 2-1). Searching occurred between January 2018 and September 2018.

Table 2-1 | Electronic search strategy used as for Web Of Science with terms used in the initial search and for each theoretical barrier to *Leptospira borgpetersenii* serovar Ballum spillover from wildlife to humans included in the literature review (a to e, see Figure 2-1) and detail of search strings for each term.

	Initial search or Barrier	**Terms**
	Initial search	#Wildlife AND #Lepto AND #NZ
a	Distribution and abundance	#Hedgehog AND #NZ AND #Density #Rodents AND #NZ AND #Density Refined By: WEB OF SCIENCE CATEGORIES: (ECOLOGY OR ZOOLOGY OR BIODIVERSITY CONSERVATION OR ENVIRONMENTAL SCIENCES) IC Timespan=2004-2020
b	Infection dynamics	#Wildlife AND #Lepto AND #NZ †
c	Survival	Ballum AND #Survival / #Lepto AND #Survival
d	Cattle exposure	#Cattle AND Ballum AND #NZ / #Cattle AND #Lepto AND #NZ
e	Human exposure	#Human AND Ballum AND #NZ / #Human AND #Lepto AND #NZ AND #Risks

Terms	**Search strings**
#Lepto	Leptospir* OR "Weil disease" OR "Weil's disease" OR "dairy farm fever" NOT Leptospirill*
#NZ	New-Zealand OR "New Zealand" OR Aotearoa
#Wildlife	#Wild OR #Possum OR #Mustelid OR #Ruminant OR #Wildboar OR #Rodents OR #Hedgehog
#Wild	Wild OR wildlife OR free-ranging OR "free ranging" OR feral
#Density	distribution OR range OR density OR densities OR abundance* OR "population dynamics"
#Hedgehog	hedgehog OR "*Erinaceus europaeus*"
#Rodents	rodent OR rodents OR rat OR rats OR *Rattus* OR mice OR "*Mus musculus*" OR mouse OR murine OR kiore
#Ruminants	deer OR *Cervus* OR *Axis* OR *Alces* OR moose OR chamois OR *Rupicapra* OR *Odocoileus* OR *Dama* OR Tahr OR *Hemitragus* OR "feral goat" OR "feral sheep"
#Mustelid	*Mustela* OR stoat* OR ferret* OR weasel*
#Possum	possum OR *Trichosurus*
#Wildboar	("*Sus scrofa*" AND (wild OR feral)) OR"wild pig" OR "feral pig" OR "wild boar"
#Survival	Survival OR environment* OR outside OR biofilm* OR "resistance form" OR disseminat* OR virulence
#Cattle	cattle OR cow OR calf OR calves OR bovin*
#Human	"public health" OR human OR occupation* OR hospital OR worker OR farmer OR tourist OR forestry
#Risks	risk OR "risk factor" OR "risks factors" OR exposure

† *Results of Initial search*

First descriptions of Ballum

The type strain for serovar Ballum (Mus 127) was first isolated from a field-house mouse (*Mus musculus spicilegus*) on a Danish farm in 1943 (Borg Petersen, 1944). The range of the species currently described as *Mus spicilegus*, the steppe mouse, is however limited to countries around the Black Sea (Greece to Ukraine and southwestern Russia) and it is likely that the host in that report instead likely belonged to the subspecies *domesticus* (Schwarz and Schwarz, 1943). Schüffner and Bohlander had previously isolated another strain (S-102) from a white mouse in their laboratory colony in Amsterdam in 1941, but wartime conditions limited further investigations (Wolff et al., 1949).

The serogroup Ballum includes *L. borgpetersenii* svs Ballum, Castellonis, Arborea, Kenya, Guangdong and Soccoestomes, as well as *L. santarosai* sv Peru and, the more recently described *L. mayottensis* sv Kenya (Brenner et al., 1999, Hamond et al., 2015, Bourhy et al., 2014).

Early serosurveys conducted on *Leptospira* in animals and humans in NZ did not cover this new serogroup (Ballum) in their testing panel (Kirschner and Gray, 1951, Blakelock and Allen, 1956, Webster, 1957, Shortridge, 1960, Daniel, 1966, Daniel, 1967). Smith was the first, in 1963, to include it in the initial screening phase of his macroscopic agglutination test panel, pooled with Canicola and Icterohaemorrhagiae (Smith, 1964). Of 98 sera from hedgehogs (*Erinaceus europaeus*) sampled in Hamilton, Upper Hutt, Dunedin and Auckland, three were reactive to this antigen pool, all from the Hamilton area, and two failed to agglutinate with the specific Icterohaemorrhagiae and Canicola antigens. Smith did not have Ballum specific antigens available for further investigation. Eight guinea pigs were inoculated with a mix of kidney and urine from 12 hedgehogs from the Hamilton area. Although 2/8 died, "apparently of leptospirosis", he did not isolate any *Leptospira* from their blood, urine or kidneys, nor from the urine cultures of 16 additional hedgehogs (Smith, 1964, p. 105).

The first isolation of Ballum was performed in 1967 from two sick dairy farm workers (Till, 1968 in Hellstrom, 1978) and from the urine of healthy calves six years later (Ris et al., 1973). Ballum was included in the Microscopic Agglutination Test (MAT) panel of De Lisle et al. (1975) and none of the 26 possum sera tested were positive for this serovar. It was not until 1976 that Blackmore et al. (1976) published the first isolates of Ballum from wildlife, a brown rat (*Rattus norvegicus*) and a rabbit (*Oryctolagus cuniculus*) trapped in the environment of an artificial breeding centre for cattle. Bovine and ovine kidneys were also sampled in the centre and organisms were seen by dark-

field microscopy (DFM) in some. However, isolation of those organisms was not successful.

Known and suspected wild maintenance hosts of *Leptospira* spp. in NZ

Virtually all species of wild terrestrial mammals introduced to NZ have previously been investigated for exposure to *Leptospira* spp. As a result, only the brush-tailed possum (*Trichosurus vulpecula*), and rats, mice and hedgehogs were considered of interest, as a maintenance host of *L. borgpetersenii* sv Balcanica for the possum, and Ballum for the others.

Other NZ wild species have typically been considered as insignificant hosts for the maintenance and propagation of domestic animal or human leptospirosis and have not been thoroughly studied. Studied species for which an absence of serological titres to *Leptospira* spp. has been reported in NZ (Anonymous, 1983, Daniel, 1967, Hathaway and Blackmore, 1981b, Hathaway et al., 1981) include feral pig (*Sus scrofa*), hare (*Lepus europaeus*), stoat (*Mustela erminea*), ferret (*Mustela putorious furo*), weasel (*Mustela nivalis*), Sika deer (*Cervus nippon*), Fallow deer (*Dama dama*), white-tailed deer (*Odocoileus virginianus borealis*), Chamois (*Rupicapra rupicapra*), and Himalayan tahr (*Hemitragus jemlahicus*).

On occasion MAT titres were found in wallabies (probably dama wallabies, *Macropus eugenii*) against Hardjobovis and Ballum; in feral cats (*Felis catus*) against Pomona and Ballum; in feral goats (*Capra hircus*) against Pomona, Hardjobovis, Balcanica and Ballum; in hunted deer (*Cervus elaphus*) against Pomona and Copenhageni; and more recently in NZ fur seal (*Arctocephalus forsteri*) against Canicola, Hardjobovis and Pomona and in NZ sea lions (*Phocarctos hookeri*) against Pomona (Anonymous, 1983, Inglis, 1984, Daniel, 1966, Mackereth et al., 2005, Mist, 1984, Roe et al., 2010, Schollum and Blackmore, 1981). Those results were interpreted as sporadic infections through contact with known maintenance species of these serovars (livestock, rodents or other wild species).

The literature available was synthesized in Table 2-2. All publications relied on serological methods (agglutination, complement fixation, MAT) and only some on culture of the bacteria from blood, urine or kidney, and subsequent typing by serotyping, CAAT (cross-agglutination absorption test), or BRENDA (bacterial restriction-endonuclease DNA analysis). Some studies assumed that leptospires isolated or observed under DFM belonged to a certain serovar without actually typing them (e.g. Shortridge,

1960, Kirschner and Gray, 1951). Except for two pilot studies carried out prior to this work (Fabri, 2016, van de Pol, 2016), no PCR methods were used. It is important to note that for several studies, the number of animals tested was limited. Also, Ballum was not included in the test panel in several studies (in grey in Table 2-2) and very few studies were conducted outside of the North Island. Given that some wild or feral species were not tested, or tested in very limited numbers, it is therefore possible that some of them harbouring Ballum (or other serovars) were not detected.

Most of the available information on Ballum infection in NZ wildlife comes from two concomitant research projects conducted in the late 1970s (Brockie, 1977, Brockie and Till, 1977, Hathaway, 1978). The first project led by Brockie initially focused on hedgehogs, and after isolating serovar Ballum from the kidneys of five healthy female hedgehogs caught in dairy farms throughout the North Island, suggested this species was a major reservoir for this serovar in NZ (Brockie and Till, 1977). Brockie also trapped mice and brown rats harbouring Ballum in refuse dumps in the North Island (Brockie, 1977). The second project carried out by Hathaway and supervised by Blackmore and Marshall focused on possums and Balcanica (Hathaway et al., 1978, Marshall et al., 1976), but they also conducted a survey investigating *Leptospira* in several species (Hathaway et al., 1981) and another focused on ship rats and brown rats in refuse dumps and farming environments (Hathaway, 1978). They found that contrary to ship rats, Ballum culture prevalence was density-dependent for brown rats. It was concluded that ship rats were maintenance hosts while brown rats were able to maintain Ballum only in high-density populations (Hathaway and Blackmore, 1981a). However, the study did not ascertain the presence or absence of mice in the refuse dumps where those rats were shot, and mice could have acted as the primary maintenance host for this serovar.

Worldwide, Ballum has been isolated in a variety of geographical places and hosts, often rodents of the *Muridae* or *Cricetidae* families (e.g. Brown and Gorman, 1960), but also *Didelphidae* (common and Virginia opossums *Didelphis marsupialis and D. virginiana*), various carnivores (McKeever et al., 1958) or snakes (hog-nosed snake Heterodon platyrhinus Yager et al., 1953, Ferris et al., 1961). This serovar is common in Europe and the Americas but is less frequently reported in Asia (Zhang et al., 2012). Wild or laboratory house mice were proposed as the main reservoir of this serovar (Babudieri, 1955, Bharti et al., 2003), probably because of their cosmopolitan and commensal nature, and because of the frequency of isolations of Ballum from this species. Isolation was occasionally successful from brown and ship rats, for instance in Portugal (Fraga de Azevedo et al., 1951), Britain (Michna and Ellis, 1974), Puerto Rico, British Columbia (Yager et al., 1953), Hawaii (Higa and Fujinaka, 1976), Italy (Farina and Babudieri, 1957) but they are more commonly associated with serovars from the Icterohaemorrhagiae

serogroup (Babudieri, 1955, Bharti et al., 2003). Hedgehogs are more commonly associated with serovars within Australis serogroup in Europe (Ayral et al., 2016, Wolff and Bohlander, 1965), but there is mention of one strain of Ballum (Kipod 88) isolated from the kidneys and brain of a hedgehog in an urban area in Israel (Tel-Aviv) in 1957 (Van der Hoeden, 1958). Except for this strain, Ballum has only been isolated from hedgehogs in NZ.

The remainder of this chapter focuses on the known maintenance hosts of Ballum in NZ: the mouse, ship rat, brown rat and hedgehog.

Table 2-2| Summary of published studies investigating *Leptospira* and especially *L. borgpetersenii* serovar Ballum in wild species in New Zealand.

Seroprevalence and culture prevalence are indicated for Ballum only. Studies where Ballum was not included in the serology panel appear in grey.

Reference	α	Place	Habitat†	Sp§	Test‡	Cut-off	B*	H*	P*	T*	C*	B'*	O*	Sero +ve	#S	Sero prev	Cult +ve	#C	Cult prev
(Kirschner and Gray, 1951)	κ	Auckland, Christchurch, Dunedin	Urban	Rn	MAT, AT	80	n	n	n	n	y	n	y	\	53	\	0	53	0%
(Kirschner and Gray, 1951)	κ	Auckland, Christchurch, Dunedin	Urban	Rr	MAT, AT	80	n	n	n	n	y	n	y	\	47	\	0	47	0%
(Blakelock and Allen, 1956)	κ	Wellington	Urban	Rn	AT	\	n	n	y	y	y	n	y	\	62	\	\	\	\
(Blakelock and Allen, 1956)	κ	Wellington	Urban	Rr	AT	\	n	n	y	y	y	n	y	\	121	\	\	\	\
(Webster, 1957)	ε, κ	NZ – NS	Farm	Ee	AT	\	n	n	y	n	n	n	n	\	2	\	0	2	0%
(Shortridge, 1960)	π	Pukekohe district	NS	"Rat"	NS	NS	.	.	.	.	y	.	.	\	25	\	\	\	\
(Smith, 1964)	κ	Hamilton, Upper Hutt, Dunedin, Auckland	Urb, Suburb	Ee	AT	\	p	n	y	y	y	n	y	3	98	3%	0	28	0%
(Smith, 1965)	κ	NZ – NS	Urb, Suburb	Ee	NS	NS	.	.	.	.	.	.	.	.	98	\	0	11	0%
(Smith, 1965)	κ	NZ – NS	NS	"Deer"	NS	NS	.	.	.	.	.	.	.	0	15	0%	\	\	\
(Daniel, 1966)	κ	Kaingaroa Forest, North Island	Forest	Ce	MAT	200	n	n	y	y	y	n	y	\	109	\	\	\	\
(Daniel, 1967)	κ	NZ	Forest	Dd	MAT	200	n	n	y	y	y	n	y	\	88	\	\	\	\
(Daniel, 1967)	κ	NZ	Forest	Ov	MAT	200	n	n	y	y	y	n	y	\	40	\	\	\	\
(Daniel, 1967)	κ	NZ	Forest	Cn	MAT	200	n	n	y	y	y	n	y	\	42	\	\	\	\
(Daniel, 1967)	κ	NZ	Forest	Hj	MAT	200	n	n	y	y	y	n	y	\	93	\	\	\	\
(Daniel, 1967)	κ	NZ	Forest	Ru	MAT	200	n	n	y	y	y	n	y	\	14	\	\	\	\
(Daniel, 1967)	κ	NZ	Forest	Ch	MAT	200	n	n	y	y	y	n	y	\	150	\	\	\	\
(Daniel, 1967)	κ	NZ	Forest	Ss	MAT	200	n	n	y	y	y	n	y	\	36	\	\	\	\
(de Lisle et al., 1975)	κ	Whanganui district	Farm	Tv	MAT	200	y	y	y	n	y	n	.	0	26	0%	0	NS	0%

Reference	α	Place	Habitat†	Sp§	Test‡	Cut-off	B*	H*	P*	T*	C*	B'*	O*	Sero +ve	#S	Sero prev	Cult +ve	#C	Cult prev
(Brockie, 1975b)	κ	North Island	NS	Tv	MAT	100	.	y	.	.	.	.	.	\	146	\	\	146	\
(Marshall et al., 1976)	NS	NZ	NS	Tv	\	\	\	\	\	\	\	\	\	\	\	\	1	1	\
(Blackmore et al., 1976)	κ	Bulls, Manawatū	Rural	Rn	MAT	20	y	y	y	n	y	n	n	1	2	50%	1	2	50%
(Blackmore et al., 1976)	κ	Bulls, Manawatū	Rural	Tv	MAT	20	y	y	y	n	y	n	n	0	2	0%	0	2	0%
(Blackmore et al., 1976)	κ	Bulls, Manawatū	Rural	Le	MAT	20	y	y	y	n	y	n	n	0	3	0%	0	3	0%
(Blackmore et al., 1976)	κ	Bulls, Manawatū	Rural	Ee	MAT	20	y	y	y	n	y	n	n	0	4	0%	0	4	0%
(Blackmore et al., 1976)	κ	Bulls, Manawatū	Rural	Oc	MAT	20	y	y	y	n	y	n	n	1	11	9%	1	11	9%
(Brockie and Till, 1977)	κ	North Island	Farm	Ee	MAT	100	y	y	y	y	y	n	y	28	78	36%	5	78	6%
(Brockie, 1977)	κ	North Island	Urb, Rural	Mm	MAT	100	y	n	n	n	y	n	y	2	67	3%	9	67	13%
(Brockie, 1977)	κ	North Island	Urb, Rural	Rn	MAT	100	y	n	n	n	y	n	y	7	79	9%	8	79	10%
(Brockie, 1977)	κ	North Island	Urb, Rural	Rr	MAT	100	y	n	n	n	y	n	y	1	16	6%	4	16	25%
(Buddle and Hodges, 1977)	κ	NZ – NS	Rural	"Rat"	MAT	100	y	y	y	y	y	n	n	0	15	0%	0	15	0%
(Buddle and Hodges, 1977)	κ	NZ – NS	Rural	Ee	MAT	100	y	y	y	y	y	n	n	0	1	0%	0	1	0%
(Hathaway et al., 1978)	κ	North Island	Farm	Tv	MAT	24	y	y	y	y	y	y	y	\	127	\	\	\	\
(Carter and Cordes, 1980)	κ	Waikato	Urb, Rural	Rn	MAT	20	y	y	y	y	y	n	n	26	134	19%	12	132	9%
(Carter and Cordes, 1980)	κ	Waikato	Urb, Rural	Rr	MAT	20	y	y	y	y	y	n	n				3	28	11%
(Hathaway, 1981a)	ε	\	\	Tv	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\

Reference	α	Place	Habitat†	Sp§	Test‡	Cut-off	B*	H*	P*	T*	C*	B'*	O*	Sero +ve	#S	Sero prev	Cult +ve	#C	Cult prev
(Hathaway, 1981b)	ε	\	\	Tv	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
(Hathaway and Blackmore, 1981b)	κ	North Island	Farm, Suburb	Fc	MAT	24	y	y	y	y	y	n	y	1	11	9%	0	11	0%
(Hathaway and Blackmore, 1981b)	κ	North Island	Farm	Me	MAT	24	y	y	y	y	y	n	y	0	9	0%	0	9	0%
(Hathaway and Blackmore, 1981b)	κ	North Island	Farm	Mf	MAT	24	y	y	y	y	y	n	y	0	9	0%	0	9	0%
(Hathaway and Blackmore, 1981b)	κ	North Island	Farm	Mn	MAT	24	y	y	y	y	y	n	y	0	4	0%	0	4	0%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	Ee	MAT	24	y	y	y	y	y	n	y	9	25	36%	5	27	19%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	Mm	MAT	24	y	y	y	y	y	n	y	3	39	8%	11	70	16%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	Rn	MAT	24	y	y	y	y	y	n	y	6	168	4%	63	245	26%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	Rr	MAT	24	y	y	y	y	y	n	y	8	29	28%	21	63	33%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	Tv	MAT	24	y	y	y	y	y	y	y	11	754	1%	0	27	0%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	Le	MAT	24	y	y	y	y	y	n	y	0	5	0%	0	5	0%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	Oc	MAT	24	y	y	y	y	y	n	y	0	9	0%	0	9	0%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	Ce	MAT	24	y	y	y	y	y	n	y	0	27	0%	0	3	0%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	Cn	MAT	24	y	y	y	y	y	n	y	0	4	0%	0	2	0%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	As	MAT	24	y	y	y	y	y	n	y	3	29	10%	0	29	0%

Reference	α	Place	Habitat†	Sp§	Test‡	Cut-off	B*	H*	P*	T*	C*	B'*	O*	Sero +ve	#S	Sero prev	Cult +ve	#C	Cult prev
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	An	MAT	24	y	y	y	y	y	n	y	0	29	0%	0	29	0%
(Hathaway et al., 1981)	κ	North Island	Farm, Forest, Urb	Pm	MAT	24	y	y	y	y	y	n	y	0	34	0%	\	0	\
(Hathaway and Blackmore, 1981a)	κ	Manawatū	Farm, Forest	Rn	MAT	12	y	y	y	y	y	n	y	6	168	4%	63	243	26%
(Hathaway and Blackmore, 1981a)	κ	Manawatū	Farm, Forest	Rr	MAT	12	y	y	y	y	y	n	y	8	30	27%	21	61	34%
(Schollum and Blackmore, 1981)	κ	Raglan county	Rural	Ch	MAT	24	y	y	y	y	y	y	y	4	116	3%	0	101	0%
(Anonymous, 1983)	π	Rotorua area	Forest	Me*	MAT	NS	y	y	.	.	.	.	.	4	39	10%	0	39	0%
(Anonymous, 1983)	π	North Island	NS	Ss	MAT	NS	.	.	.	.	.	.	.	0	60	0%	0	39	0%
(Inglis, 1984)	κ	Nelson area	Forest	Ce	NS	50	n	n	y	n	n	n	y	\	24	\	\	\	\
(Cowan et al., 1991)	κ	Orongorongo valley	Forest	Tv	MAT	24	y	y	y	y	y	y	n	1	261	0.4%	0	247	0%
(Horner et al., 1996)	κ	NZ	NS	Tv	MAT	50	n	n	n	n	n	y	n	\	\	\	\	\	\
(Day et al., 1997a)	ε	\	\	Tv	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
(Day et al., 1997b)	ε	\	\	Tv	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
(Day et al., 1998)	ε	\	\	Tv	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
(Kakrada, 1999)	κ	North Island	Farm, Urb	Rn	MAT	24	y	y	y	y	y	n	y	2	7	29%	0	7	0%
(Kakrada, 1999)	κ	North Island	Farm, Urb	Rr	MAT	24	y	y	y	y	y	n	y	4	17	23%	0	17	0%
(Caley and Ramsey, 2001)	μ	\	\	Tv	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
(Mackereth et al., 2005)	λ	Otago Peninsula	Seashore	Af	MAT	100	y	y	y	n	y	n	y	0	128	0%	\	\	\
(Roe et al., 2010)	κ	NZ islands	Seashore	Ph	MAT	24	n	y	y	n	n	n	n	\	147	\	\	\	\
(Fabri, 2016)	π	North Island	Rural	Tv	MAT	48	y	y	y	n	y	n	n	0	21	0%	0	1	\
(Fabri, 2016)	π	North Island	Rural	Ee	MAT	48	y	y	y	n	y	n	n	1	2	50%	0	2	0%

Reference	α	Place	Habitat†	Sp§	Test‡	Cut-off	B*	H*	P*	T*	C*	B'*	O*	Sero +ve	#S	Sero prev	Cult +ve	#C	Cult prev
(Fabri, 2016)	π	North Island	Rural	Rn	MAT	48	y	y	y	n	y	n	n	1	1	100%	\	\	\
(Fabri, 2016)	π	North Island	Rural	Mm	MAT	48	y	y	y	n	y	n	n	0	1	0%	0	1	0%
(Fabri, 2016)	π	North Island	Rural	Fc	MAT	48	y	y	y	n	y	n	n	0	14	0%	0	3	0%
(Fabri, 2016)	π	North Island	Rural	Mf	MAT	48	y	y	y	n	y	n	n	0	1	0%	\	\	\
(Fabri, 2016)	π	North Island	Rural	Me	MAT	48	y	y	y	n	y	n	n	0	1	0%	\	\	\
(Fabri, 2016)	π	North Island	Rural	Mn	MAT	48	y	y	y	n	y	n	n	\	\	\	\	\	\
(Fabri, 2016)	π	North Island	Rural	Oc	MAT	48	y	y	y	n	y	n	n	0	1	0%	0	1	0%
(van de Pol, 2016)	π	Palmerston North	Urb, Suburb	Tv	MAT	48	y	y	y	n	y	n	n	2	16	13%	0	26	0%
(van de Pol, 2016)	π	Palmerston North	Urb, Suburb	Ee	MAT	48	y	y	y	n	y	n	n	1	5	20%	1	5	20%
(van de Pol, 2016)	π	Palmerston North	Urb, Suburb	Me	MAT	48	y	y	y	n	y	n	n	0	2	0%	0	2	0%
(van de Pol, 2016)	π	Palmerston North	Urb, Suburb	"Rat"	MAT	48	y	y	y	n	y	n	n	\	0	\	\	\	\
(van de Pol, 2016)	π	Palmerston North	Urb, Suburb	Mm	MAT	48	y	y	y	n	y	n	n	\	0	\	\	\	\

NS: not specified: \ Not applicable; Periods indicate missing data;

α Type of study: ε = experimental, κ = cross-sectional survey, λ = longitudinal survey, μ = modelling, π = pilot study;

† Urb = Urban, Suburb = Suburban (defined according to its original meaning as areas in the periphery of urban centres, this category includes refuse dumps);

§ Sp= Species: Af = NZ fur seal Arctocephalus forsteri, An = Mallard duck Anas platyrynchos, As = Grey duck Anas superciliosa, Ce = Red deer Cervus elaphus, Ch = Feral goat Capra hircus, Cn = Sika deer Cervus nippon, Dd = Fallow deer Dama dama, Ee = European hedgehog Erinaceus europaeus, Fc = Feral cat Felis catus, Hj = Himalayan tahr Hemitragus jemlahicus, Le = European brown hare Lepus europaeus, Me = Stoat Mustela erminea, Me = not specified but likely Dama wallaby Macropus eugenii, Mf = Ferret Mustela putorius furo, Mm = House mouse Mus musculus, Mn = Weasel Mustela nivalis, Oc = European rabbit Oryctolagus cuniculus, Ov = White-tailed deer Odocoileus virginianus, Ph = NZ sea lion Phocarctos hookeri, Pm = Pukeko Porphyrio melanotus, Re = Kiore Rattus exulans, Rn = Brown rat Rattus norvegicus, Rr = Ship rat Rattus rattus, Ru = Chamois Rupicapra rupicapra, Ss = Feral pig Sus scrofa, Tv = Brushtail possum Trichosurus vulpecula;*

‡ MAT = Microscopic Agglutination Test, AT = Agglutination lysis test;

** Serovar included in MAT/AT panel (yes/no/pooled): B = Ballum, P = Pomona, H = Hardjo, C = Copenhageni, T = Tarassovi, B' = Balcanica, O = others. Except for Balcanica (same serogroup as Hardjo), the serovar was considered included when another serovar from the same serogroup was used (for instance serovar Icterohaemorrhagiae in the same serogroup as Copenhageni)*

(a) Maintenance host distribution

Brown rats, mice and ship rats arrived in NZ with the first European settlers in the 1770s – 1790s, 1790s – 1840s and 1860s – 1890s (King, 2016, King, 2017), and rapidly colonized both the North and South Islands. Brown rat populations that were thriving in the 1850s declined by the end of the 19th century and have since had a discontinuous distribution that has been attributed to competition with ship rats and/or predation from mustelids released as an attempt to control rodents and rabbits (King, 2017, King and Barrett, 2005).

Hedgehogs were first released around Christchurch and Dunedin in the 1870s – 1890s and had reportedly dispersed through most lowland areas of the South Island by 1910, when they were introduced and quickly spread in the North Island (Brockie, 1975a). They are now considered to be present throughout NZ except at high altitudes, and are found in gardens and urban areas, but also in grassland and shrubland. In their native range, they are known to avoid pastures because of the risk of predation by badgers (Young et al., 2006). Comparisons of road-kill counts along North Island highways indicate that the abundance of hedgehogs was similar in 1984 and 1994 but had dropped drastically (-82%) in a 2005 transect (Brockie et al., 2009).

The preferred habitat for species introduced to NZ can differ from their original habitat in their native range. Mice, known to be commensal and found only around human dwellings or farm buildings, benefit from the absence of other wild rodents and are also present in pastures and forests in NZ. The same is true for ship rats that benefit from the absence of other arboreal rodents (such as squirrels) and are also present in forests (King et al., 2011). Brown rats are the species that remained the most synanthropic. They are found more easily around dwellings, in suburbs and refuse dumps. They can be found in farm environments, but in lower densities than ship rats and preferentially around farm buildings (King and Barrett, 2005).

Almost all available density or abundance estimates published for rodents since 2004 were carried out in forests or islands in the frame of conservation projects (Table 2-3). Rodents density estimates in studies published before 2004 are given in detail in (Harper and Rutherford, 2016) and (Murphy and Nathan, in press). Populations are known to fluctuate greatly, with spikes associated with seed masting events (Walker et al., 2019, Veale et al., 2015). Although mice and rats are known to be present in pastures, there is a dearth of information on their abundance in farm habitats. A study in grazed or fenced fragments of native forest showed a higher density or ship rats in the fenced fragments, and the highest measured densities in mainland NZ (Innes et al., 2010). Mice

benefit from the removal of predators (Goldwater et al., 2012) and rats (Caut et al., 2007), with which they compete, and this effect is expected to be especially true in warmer forests of NZ (Walker et al., 2019).

One study in Tāwharanui Open Sanctuary (Northland) compared mice densities in forest, grazing pasture, coastal vegetation and rank grass before and after removal of other invasive species. While not detected in pastures before predator removal, there were up to 3.5 mice captured per 100 trap-night (C/100TN) after the removal of predators. This was significantly less than the density estimates in the three other habitats, that were between 120 and 190 C/100TN at the same period (Goldwater et al., 2012)). Another study described higher mice presence indices in former pastures being regenerated than in the neighbouring grazed pastures (Whitehead et al., 2014). In forests, abundance ranged between 0 – 73 mice C/100TN and up to 190 C/100TN in the absence of competitors (listed in (King and Barrett, 2005)).

Table 2-3 | Indices of abundance (in captures or corrected captures/100 trap.nights or *in sighting/100km) and density estimates (individuals per hectare) published in the literature for mice, rats and hedgehogs in New Zealand . *Table adapted and completed from (Murphy and Nathan, in press) with the kind permission of authors. Only literature published after 2004 was searched for rodents.*

Place	Is.	Habitat Type	Abundance	Density	Months	Years	Reference
Mus musculus							
Grebe Valley	SI	Beech forest	0.5–32.9	\	Feb, Dec	2000	(Purdey et al., 2004)
Borland Valley	SI	Beech forest	0–62	\	Nov, Feb, Dec	1999 – 2000	(Purdey et al., 2004)
Borland Valley	SI	Beech forest	\	0.02–1.8	Feb, May, Nov	2003 – 2004	(Wilson and Lee, 2010)
Waitutu Forest	SI	Beech—mixed forest	\	8–28	F, M, A, N[1]	2001 – 2003	(Ruscoe et al., 2004)
Orongorongo Valley	NI	Beech—mixed forest	0–13.8	\	F, M, A, N[1]	1973–1998	(Fitzgerald et al., 2004)
Maungatautari	NI	Podocarp—mixed forest†	\	9–46	F, M, A, N	2011 – 2016	(Wilson et al., 2018)
Moturekareka Is.	oiNI	Coastal forest/ scrub†	\	81	Apr	2014	(Reynolds, 2015)
Saddle Is.	oiNI	Coastal forest/ scrub†	\	8.8–19.2	Jan, Mar, May, Aug	2008	(MacKay et al., 2011)
Tāwharanui	NI	Coastal forest/ scrub†	1–190.16	14.6–156.7	F, A, J, A, O, D 2	2007	(Goldwater et al., 2012)
Maud Is.	oiSI	Coastal forest/ scrub†	\	138	Feb	2014	(Reynolds, 2015)
Moturekareka Is.	oiNI	Pine forest†	\	34	Apr	2014	(Reynolds, 2015)
Auckland Is.	OI	Rata forest (+ shrubland)	5.6–7.2	\	Jun, Jul	2007	(Harper, 2010)
Maud Is.	oiSI	Scrub (Manuka/Grass)†	\	102	Feb	2014	(Reynolds, 2015)
Antipodes Is.	OI	Tussock/Grassland†	\	55–104	Jan, Jul	2011 & 2013	(Russell, 2012, Elliott et al., 2015)
Borland Valley	SI	Tussock/Grassland	\	0.4–38.6	Feb/Mar, May, Nov	2003 – 2007	(Wilson and Lee, 2010)
Auckland Is.	OI	Tussock/Grassland	12.7	\	Jun, Jul	2007	(Harper, 2010)
Tāwharanui	NI	Grassland (grazed pasture)†	0–3.51	\	F, A, J, A, O, D	2007	(Goldwater et al., 2012)
Tāwharanui	NI	Grassland (rank grass)†	1.71–121.13	\	F, A, J, A, O, D	2007	(Goldwater et al., 2012)
Waikauri Bay	NI	Grassland (rank grass)	17.62–91.18	\	Apr, Aug, Dec	2007	(Goldwater et al., 2012)
Tāwharanui	NI	Supra-littoral vegetation†	40–130.44	\	F, A, J, A, O, D	2007	(Goldwater et al., 2012)

Place	Is.	Habitat Type	Abundance	Density	Months	Years	Reference
Rattus rattus							
Eglinton Valley	SI	Beech forest	\	0.38	Mar	2005	(Christie et al., 2015)
Orongorongo Valley	NI	Beech—mixed forest	2.3–7.5	\	F, M, A, N	1971 – 1998	(Efford et al., 2006)
Orongorongo Valley	NI	Beech—mixed forest	31	5–9	Apr, May	2003 – 2004	(Wilson et al., 2007)
Waikato	NI	Broadleaved forest fragment (fenced)	\	6.5	Jan, Feb	2008	(Innes et al., 2010)
Waikato	NI	Broadleaved forest fragment (grazed)	\	0.5	Jan, Feb	2008	(Innes et al., 2010)
Big South Cape Is.	oiStI	Supra-littoral vegetation	\	6.5–36.4	Dec, Jan	2003 – 2004	(Harper and Rutherford, 2016)
Erinaceus europaeus							
\	NI	Road-kill	0–58.3*	\	Jan, Feb	1987	(Morris and Morris, 1988)
\	SI	Road-kill	0–8*	\	Jan, Feb	1987	(Morris and Morris, 1988)
\	NI	Road-kill	6.7–6.9* (max 23–25*)	\	Feb	1984 – 1994	(Brockie et al., 2009)
\	NI	Road-kill	1.3*	\	Feb	2005	(Brockie et al., 2009)
\	NI	Road-kill	4–25*	\	\	2009 – 2014	(Sadleir and Linklater, 2016)
Macraes flat	SI	Tussock/Grassland	0.01	\	May	2013	(Glen et al., 2014)
Tasman Valley	SI	Tussock/Grassland (shrubs)	0	\	Jun, Jul	2013	(Glen et al., 2014)
Lake Wairarapa	NI	Grassland/Scrub	\	0.88	Oct to May	1995 – 1996	(Gorton, 1998)
Massey University	NI	Farmland	\	2.5	Nov to Jun	1970 – 1971	(Parkes, 1975)
Massey University	NI	Farmland	\	1.1	Jul to Oct	1970 – 1971	(Parkes, 1975)

Is. = Island: NI = North Island, SI = South Island, StI = Stewart Island, oiSI/NI/StI = outlying island of SI/NI/StI, OI = Offshore island

†Mice were the only non-native mammal species present

[1]. Quarterly trapping: February, May, August and November.

[2]. Bi-monthly trapping: February, April, June, August, October, December

(b) Infection dynamics in maintenance host

Ballum prevalence

The prevalence and seroprevalence described in the literature are listed in Table 2-2. In those studies, spanning 1964 – 1999, the overall seroprevalence for Ballum varied between 3 – 8% for mice, 6 – 28% for ship rats and 0 – 36% for hedgehogs, with a variable cut-off across studies (from 12 to 100) and the culture prevalence varied between 13 – 16%, 0 – 33% and 0 – 19% respectively. There is no recent estimate available.

Both Hathaway and Brockie found that among the rodents with a Ballum positive culture, a majority were seronegative and that serology was not appropriate to diagnose the infection status of these animals. Forty-six to 89% of brown rats, 67 to 75% of ship rats and 83 to 89% of mice with a Ballum isolate were seronegative (Hathaway et al., 1981, Brockie, 1977). Brockie also noted that one of five hedgehogs shedding Ballum had no detectable antibodies.

A difference was reported between hedgehogs in urban and farm environments. While 56% of 72 hedgehogs captured in dairy farms had evidence of infection, six urban hedgehogs showed no seropositive reactions and no isolation (Brockie and Till, 1977), in agreement with Smith's results in 98 urban hedgehogs with only 2 seropositive for Ballum (Smith, 1964). However, in a pilot study preliminary to this work, Ballum was isolated from one of five hedgehogs captured in an urban area (van de Pol, 2016).

The same difference in prevalence between animals in urban and rural areas was also described in rodents with a lower rate of infection in urban habitats (Brockie, 1977, Brockie and Till, 1977). However, contrasting results were also described with a higher prevalence of Ballum and Copenhageni in brown rats in urban habitats, while ship rats had a higher prevalence in rural areas (Carter and Cordes, 1980).

All the studies available in the literature investigating the seroprevalence and prevalence of Ballum are cross-sectional surveys, and therefore single time-point estimates. No work was conducted on the dynamics of infection in these populations. A study done in New Caledonia investigated the dynamics of rodents and *Leptospira* carriage over time and linked higher prevalence to hot and rainy seasons (Perez et al., 2011). Despite Ballum putatively identified in ship rats and mice, these results cannot be extrapolated to NZ where the climate is different.

Pathogenesis in animal hosts

The literature on the pathogenesis of *Leptospira* in laboratory animals is abundant, especially on mice that are widely used as models of sublethal infection (Richer et al., 2015) but descriptions of natural infections in wild populations are scarce (Adler, 2015). Infection is asymptomatic for mice and rats, and no experimental study on hedgehogs is available for Ballum. Hathaway determined a minimum infective dose of 10 bacteria for Ballum and Pomona in pathogen-free laboratory mice injected intraperitoneally (Hathaway, 1978, Hathaway et al., 1983). This dose was much higher (10^7 organisms) for Balcanica and Hardjo.

Experimental infections with a fatal outcome were described for hedgehogs infected with Pomona (route not specified, Webster, 1957) and a combination of Szwajizak and Canicola (instillation into the nostrils, Van der Hoeden, 1958). The natural route of infection for hedgehogs is thought to be by direct contact between nasal and/or buccal mucosa and contaminated water while foraging for food (Brockie and Till, 1977). Fennestad & Borg-Petersen (Fennestad and Borg-Petersen, 1972) described a positive correlation between interstitial nephritis and *Leptospira* infection in hedgehogs in Denmark, but the dominant serovar isolated from this population was Bratislava.

Specific attributes of invasive species

Mice, ship rats and hedgehogs are all invasive species in NZ. They were released, intentionally or not, in small numbers, and adapted successfully to a new environment. Several mechanisms have been proposed to explain the success of invasive species. They have been summarized by Morand for rats (2015): (1) the pathogen release hypothesis, (2) the immunocompetence advantage (spill-back), (3) the competition advantage (by spillover) and (4) the reallocation of energetic resources.

Briefly, (1) relates to the difference in the number of pathogens harboured by an invasive species in a new territory compared to the diversity available in their native range, thus providing a first advantage. Mechanism (2) relates to the correlation between pathogen biodiversity and immunocompetence, animals coming from a native territory with a high pathogen biodiversity will have a higher immunocompetence and be better armed to face new pathogens they could encounter in the invaded territory. (3) relates to the possibility for an invasive species to benefit from the transmission of a pathogen they harbour to a local species that is more susceptible to this new pathogen. Finally, (4) relates to the possibility for an invasive species cleared from parasites to reallocate energetic resources into reproduction and growth.

Another possible advantage that is linked to the invaded location is the 'vacant niches' hypothesis suggesting that "species-poor communities do not offer biological resistance to invasion" (Morand et al., 2015, p. 413).

(c) Pathogen release & survival in the environment

Pathogen release

The amount of *L. borgpetersenii* sv Ballum released in the urine of maintenance hosts has not been studied in natural environments. A recent meta-analysis (Barragan et al., 2017a) gives a quantitative estimate of *Leptospira* spp. shed by different hosts, including rats (5.7×10^6 *Leptospira*/mℓ of urine), mice (3.1×10^3 *Leptospira*/mℓ), cattle (3.7×10^4 *Leptospira*/mℓ) and humans (7.9×10^2 *Leptospira*/mℓ). However, the low number of subjects, the variation in the methods used to quantify the bacterial load between studies, and the fact that different species and serovars of *Leptospira* were considered, limit the possible comparisons between host species. It is often inferred in the literature that the presence and amount of *Leptospira* in the kidney of a host directly reflects the presence and amount of these bacteria in voided urine. Using qPCR (quantitative PCR) Costa et al. indeed found a significant positive correlation between the average load of *Leptospira* in kidneys and urine samples of brown rats (Costa et al., 2015b). Leptospiruria has however been described as being intermittent, of variable length both within and between species, and also depending on the infecting strain but this may be due to the use of insensitive methods like DFM (Hathaway, 1978). Experimental infections with Ballum in mice showed that after infection, they begin shedding rapidly and reach a plateau of 3×10^7 *Leptospira*/mℓ of urine after 117 days, and keep shedding virtually until the end of their life (Soupé-Gilbert et al., 2017). Desvars et al. described a positive association between renal *Leptospira* load and weight in male rats in Reunion island (2013). Brockie & Till (1977) hypothesized that since hedgehog urine was acidic, leptospires wouldn't survive long in their urine unless they were directly voided in water on pastures or soil.

Pathogen survival, development and dissemination outside the host

There appears to be no study published investigating the specific survival and development of Ballum *in situ* (*i.e.* in the environment). One study investigated Castellonis (strain Castellón 3), another serovar from the Ballum serogroup, and showed

that, *in vitro* with sterile conditions at pH 7.2, it survived nine days at 4°C, 32 days at ambient temperature and 155 days at 30°C (Addamiano, 1959). The presence of saprophytic leptospires (*L. biflexa* sv Patoc 1) in the culture medium did not affect the survival and virulence of this serovar on guinea pigs (Addamiano, 1959).

Leptospires of the species *L. borgpetersenii* have a smaller genome than *L. interrogans* and by comparing the two genomes (two strains of serovar Hardjo *vs*. serovars Lai and Copenhageni), Bulach et al. (2006) hypothesized that the former underwent a process of genome reduction, losing mainly genes important for its adaptation and survival in the environment. They linked this difference in genome size to a difference in the transmission process, with *L. borgpetersenii* sv Hardjo having a direct animal to animal transmission rather than an indirect transmission through the environmental compartment. By comparing *L. borgpetersenii* with both *L. interrogans* and *L. biflexa*, Picardeau et al. (2008) confirmed that the loss of transduction functions in *L. borgpetersenii* impacts its ability to survive outside its host. The absence of environmental transmission was confirmed for *L. borgpetersenii* serovar Balcanica where transmission is thought to occur during mating (Day et al., 1997b, 1998). The survival of *L. borgpetersenii* sv Ballum in the environment should thus be, at least theoretically, limited.

The importance of abiotic factors like pH, humidity, temperature, salinity and UV light for the survival of *Leptospira* spp. in the environment have long been recognized (Kirschner and Maguire, 1957, Ruys, 1946, Smith and Turner, 1961). The physicochemical properties of soil also play a role in the survival of leptospires, but very little is known on this topic. In Ontario, the distribution of pathogenic and saprophytic leptospires serovars was correlated to the type of bedrock, with titres to pathogenic leptospires (mainly Pomona) found only in animals from areas with Paleozoic bedrock while titres to saprophytes (*L. biflexa*) were more ubiquitous (Kingscote, 1970). Lall (2018) described a significant positive relationship between the presence of *Leptospira* and the soil concentrations in iron, manganese and copper.

The capacity to form biofilms and resist harsh environmental conditions has been more recently described (Trueba et al., 2004) in a variety of pathogenic and saprophytic strains of *Leptospira* spp. including *L. borgpetersenii* svs Castellonis, Hardjobovis, Sejroë and Tarassovi (Trueba et al., 2004, Ristow et al., 2008). Again, no information on this trait has been published on Ballum, but its capacity to aggregate in cultures suggests it can likely form biofilms as well.

Other bacteria present in the environment can interact with *Leptospira* spp. and decrease *Leptospira* survival: *Aerobacter cloacae*, *Pseudomonas* spp., … ; or on the contrary increase it: *Azospirillum* spp., *Escherichia coli, Mycobacterium rubra*,

Sphingomonas spp., *Micrococcus* spp., *Brevundimonas* spp., *Acinetobacter* spp., *Paracoccus* spp., … (Abdoelrachman (1947) in Smith and Turner, 1961, Barragan et al., 2011, Vinod Kumar et al., 2015). By forming biofilms with other bacteria, *Leptospira* are more resistant to ultraviolet light, temperature stress and antibiotics (Barragan et al., 2011, Vinod Kumar et al., 2015). More work is needed to understand factors affecting the environmental survival of *Leptospira* (Barragan et al., 2017b).

(d) Exposure and competency of cattle

Ballum was first isolated in NZ from two healthy calves, in the frame of an epidemiological study conducted on nine calf groups in the Hauraki Plains (Ris et al., 1973). For the same study in asymptomatic calves, the 1972 – 73 annual report of the Wallaceville Animal Research Centre mentions a seroprevalence of 44% (18/25 and 4/25 in two of the nine groups) for Ballum (Ris and Hodges, 1974). The fact that the cut-off for seropositivity is not specified and that the results were not detailed in the associated publication—only titres of leptospiruric calves were reported—call into question the reliability of this reported seroprevalence. Ballum was included in the strains used in an experimental infection of 8 to 10-week-old calves: two calves were inoculated intramuscularly with Ballum and blood-sampled at 7, 10, 12, 14 days post-infection (PI) and weekly thereafter. They had a peak of temperature three days PI that lasted no more than two days. Leptospiruria was observed by DFM between 24 and 68 days after inoculation, and maximum MAT titres of 10,000 were observed at 10 days PI and decreased afterwards. Complement fixation antibodies peaked at 10 days PI but declined more rapidly and were detectable, for all serovars, for only 8 to 23 weeks PI (Hodges and Ris, 1974). Hodges also found no haemolytic effect of Ballum antigens on cattle erythrocytes and suspected the presence of haemolysin inhibitors in convalescent sera from cattle infected with Ballum or Hardjobovis that only partially inhibited the haemolysis induced by Pomona antigens, and only at low dilutions (1974a). The inhibition of haemolysis induced by Pomona antigens was complete for convalescent sera from cattle infected with Pomona or Copenhageni (Hodges, 1974a).

Despite no evidence of pathogenicity of Ballum on cattle in peer-reviewed publications, there are clinical cases described in the grey literature: the Ruakura Animal Health Laboratory described severe clinical signs of photosensitisation attributed to Ballum in more than 17% of a mob of 3-month-old Friesian calves. Two calves died and showed severe subcutaneous oedema, skin necrosis and sloughing. Two other cases were sampled and showed leptospiruria and "seroconversion to Ballum only" (Anonymous, 1976, p. 12). Other serovars tested were not specified in the report. Gribbles Veterinary

Pathology diagnostic laboratory described the case of a calf "doing poorly" that had "red-discoloured urine" with analyses revealing a mild multifocal cortical interstitial nephritis, with a positive *Leptospira* PCR on urine, but no *Leptospira* visible by silver staining, and titres of 400 to Ballum but seronegative to Pomona and Hardjobovis (Varney K, 2009). Two of ten asymptomatic calves subsequently tested in the same mob also had titres to Ballum (400 and 1600). These results must be interpreted with caution, as the involvement of Ballum here is based on only indirect evidence. However, in another report for the Ruakura Animal Health Laboratory, Ballum was isolated from the liver and urine of a 4-week old calf that died of leptospirosis (Anonymous, 1977b). This calf had haematuria and pale mucous membranes and the report relates lesions of severe focal nephritis and "some haemoglobin casts" in the renal convoluted tubules (Anonymous, 1977b). The method used to type the Ballum isolate is not indicated.

A very small proportion (3/10680 in 1973, 3/6409 in 1974, 9/1020 in 1977, 0/257 in 1978) of abortive cows routinely tested by serology at the Ruakura Animal Health Laboratory showed antibodies to Ballum (titres ≥ 200, Anonymous, 1974a, 1974b, 1977a, 1978). The complement fixation test, not serovar-specific, was introduced in 1978 in this laboratory to replace MAT for the routine diagnosis of leptospirosis (Anonymous, 1978) and information on the serovar was subsequently not available.

Among the descriptive and analytical studies on *Leptospira* infection in cattle published in the last 40 years, only the most recent included Ballum in their MAT antigenic panel (Mannewald, 2016, Yupiana et al., 2017). The others only targeted Pomona and Hardjo (Subharat et al., 2012, Weston et al., 2012, Sanhueza et al., 2013, Fang et al., 2015, Fang et al., 2014b, Dreyfus et al., 2012b, Dreyfus et al., 2018).

The crude seroprevalence of Ballum (48 seropositivity threshold) was 13.7% [11.7, 16.0%] in beef cattle (Mannewald, 2016) and 3% [3, 4%] in dairy cattle (Yupiana et al., 2019a), with at least one positive animal in respectively 76% of beef herds and 38% of dairy herds. All titres described were low (< 384). The animals sampled in those two studies were all adults, but as dairy cattle are usually kept longer than beef cattle, the average age of animals tested likely differed. Although there is an apparent increase in the Ballum seroprevalence recorded between the 1970s and 2010s (Figure 2-2), age at sampling appears to be an important factor, with the highest prevalence observed in calves. This makes comparisons of studies and the identification of a real increase difficult.

There is no study available on Ballum seroprevalence dynamics in cattle naturally infected, or the duration of titres over time. However, the use of serology to assess exposure may be limited by the presence of seronegative carriers. Using a GyraseB PCR and subsequent sequencing of the amplicons, Yupiana et al. found in 94/4000 urine

samples of adult dairy cows PCR positive, with 13/81 successfully typed as Ballum, all from cows seronegative for Ballum (2019). This discrepancy between MAT titres and culture or PCR results has also been identified with other species of *Leptospira* (Libonati et al., 2017). Seroprevalence presented in the previous studies cited above is therefore likely to represent an underestimation of the true prevalence.

Impact of vaccination

The hypothesis of 'competitive exclusion' between serovars within a mammalian host has been proposed in the past (Hathaway, 1981c). Hathaway suggested that the widespread use of vaccines against Hardjobovis and Pomona in cattle would create an empty 'niche' that could benefit other serovars. Vaccination against Hardjobovis and Pomona has been in place since the early 1980s in NZ. Coverage varies according to the farming type, with 99.5% of dairy cattle vaccinated (Yupiana, 2019) compared with only around 5%-25% of beef and deer, and less than 1% of sheep (Sanhueza et al., 2017). Yupiana et al. showed that vaccination and antibiotic treatments are efficient in reducing the risk of shedding *Leptospira* spp. in dairy cows, but also reported evidence of some animals shedding Ballum after vaccination (Yupiana et al., 2019b). According to this 'competitive exclusion' hypothesis, the emergence of a new serovar in cattle populations would be more likely in dairy cattle than beef cattle, which contrasts with the seroprevalence observed in those two groups and described below (Figure 2-2).

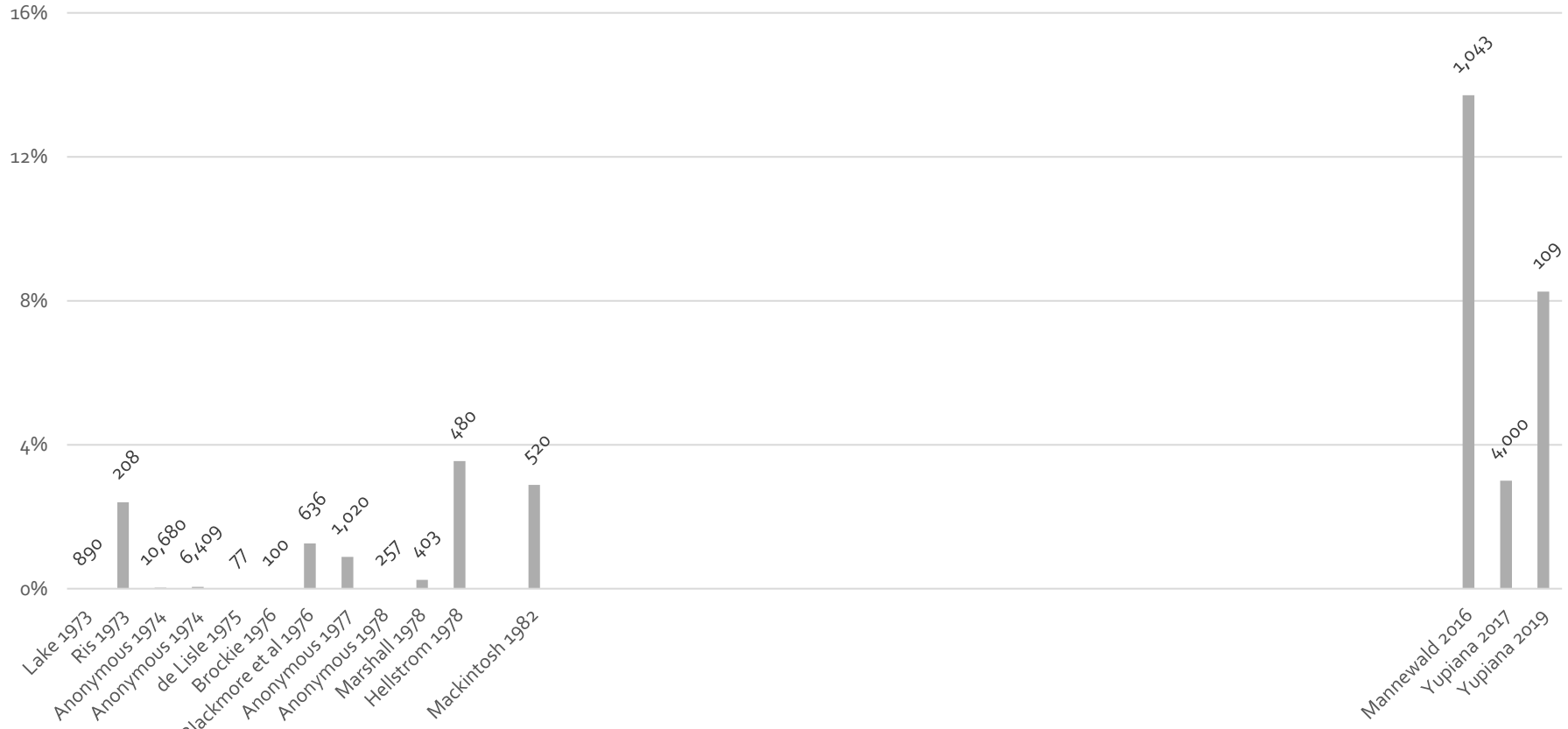

Figure 2-2 | Crude seroprevalence of *L. borgpetersenii* serovar Ballum and number of cattle tested reported in the New Zealand literature since the first description of this serovar in the country.

(e) Human exposure and risk factors

Leptospirosis is a notifiable disease in NZ and is also covered by the ACC (Accident Compensation Corporation) as an occupational disease for farmers and meat industry workers (Marshall and Manktelow, 2002). There is probably a bias in notification towards these occupations, but even so, the total number of severe cases is thought to be approximately three times higher than the notifications (Sanhueza et al., 2019). Sanhueza et al. (2019) estimated that both severe and mild human leptospirosis cases—that are more likely to be missed—were 22-fold under-ascertained compared with annual notifications.

While notified cases attributed to Hardjo and Pomona represented > 99% of the cases in the 1970s – 1980s (Mackintosh, 1981), cases attributed to Ballum now represent a third of all cases (ESR 2016, 2017). This apparent increase is relative and, at least partially, can be explained by the decreasing incidence of Hardjobovis and Pomona cases since the introduction of vaccination. The negligible incidence of cases attributed to Ballum in the 1970s increased from 0.2 in 1990-92 to 0.6/100,000 in 1996 – 1998 (Thornley et al., 2002). Nisa et al. (submitted) reported an incidence rate ratio of 1.56, 95% CI [1.18, 2.05] between 1999 – 2007 and 2008 – 2016 for this serovar. However, Ballum annual incidence varied between years and remained between 0.2 and 0.6/100,000 between 2006 and 2016 (Figure 1-2), the same order of magnitude as in the 1990s. Only in 2017, the last year with data available, was the incidence higher, 0.77/100,000 habitants (ESR 2019).

While Hardjobovis and Pomona cases are mostly related to farm and meat workers, infections with Ballum do not appear to share the same pattern (Nisa et al., submitted). Among the 1999-2016 notified cases, although farmers and meat workers showed a higher mean incidence for Ballum than the whole population, cases with Ballum were more likely to have an occupation other than farm or meat workers compared to other serovars (Nisa et al., submitted). People with Ballum were significantly older, more likely to be hospitalised and had a higher proportion of female cases compared with other serovars (Nisa et al., submitted). The incidence in people of European ethnicity was also significantly higher than in Māori, while the contrary was observed for Hardjobovis and Pomona (Nisa et al., submitted). Another study indicated that forestry-related workers were also an occupation at risk for Ballum, with 57.1% of cases due to this serovar in this occupation group (Thornley et al., 2002).

Among 302 veterinary students enrolled at Massey University, NZ, Fang et al. (2014a) found none reactive to *Leptospira* spp. using MAT and testing for Hardjobovis, Pomona and Ballum. Among 276 veterinarians, Sanhueza et al. (2015) found only one

with a titre of 48 for Ballum (0.4%, 95% CI = 0, 2.0%). Dreyfus et al. (2014) did not test for Ballum in their study on meat workers. A study was conducted in 1974 – 76 using the complement fixation test rather than the MAT on the NZ National Serum Bank. Sera from 879 donors in Wellington and Christchurch, with occupations others than farmer or meat workers had no detectable antibodies against *Leptospira* spp. (Metcalfe et al., 1979).

Ballum and Pomona were the most prevalent serovars (respectively 4/178 and 5/178) in a serosurvey on beef, sheep and deer farmers (Sanhueza et al., 2017). The risk factors for *Leptospira* seropositivity the authors identified (all serovars combined), were assisting in calving or fawning, having a high abundance of wild deer on farm, farming deer in combination with sheep and beef or alone, a proportion of flat terrain ≥ 25% and having a low abundance of possums. The latter was interpreted as the result of a confounding variable. The abundance of rodents and hedgehogs on farm (as estimated by the farmers in the questionnaire) was not identified as a risk factor.

The recreational risk of contracting leptospirosis, especially around water-related activities, has been described in numerous developed countries (Mwachui et al., 2015, Guillois et al., 2018, Pappas et al., 2008) but no specific study has been conducted on that aspect in NZ. New Zealand was listed as one of the top destinations (after Asia) where Australian travellers acquired leptospirosis (Lau et al., 2010).

Discussion

Leptospirosis due to *L borgpetersenii* sv Ballum is emerging as an important problem in humans in NZ (Nisa et al., submitted, El-Tras et al., 2018, Thornley et al., 2002), but has been little studied, and even overlooked in NZ in the past decades. There is a lack of current information on wildlife infection with Ballum in NZ, as most of the studies published were conducted almost 40 years ago. Mice are the main maintenance host of this serovar, and in NZ other species like hedgehogs, ship rats and brown rats are also able to maintain Ballum. This review also highlighted the possibility for Ballum to spillover to livestock.

Other species have been little studied and could also play a role. Feral pigs have been shown to harbour Ballum in other countries but only one unpublished study has been conducted in NZ and all 60 feral pigs sampled were negative (Anonymous, 1983). Very few lagomorphs have been tested (Hathaway et al., 1981, Blackmore et al., 1976) but Ballum was isolated on one occasion from a rabbit (Blackmore et al., 1976). Serosurveys on wild ruminants did not include Ballum in their MAT panels (Daniel, 1966, Daniel,

1967, Inglis, 1984), but recent serosurveys on farmed deer showed Ballum could infect those species too (Mannewald, 2016).

The densities of maintenance hosts, and more precisely the density of infected animals is an important parameter to understand the exposure to a pathogen. Mice, rats and hedgehogs are known to share the same habitat as humans and cattle, but there is limited information on their population dynamics in farm habitats.

There is an apparent increase in the seroprevalence for Ballum observed in cattle over time (Mannewald, 2016, Yupiana, 2019). Cattle are able to harbour and shed this serovar but the rate of transmission from wildlife to cattle is unknown. Vaccination (against Pomona and Hardjobovis) can lead to a shift in the predominant serovars found in cattle. Several questions then arise. Given the decrease in Hardjobovis and Pomona incidence as a result of vaccination, is the notion of nidality still valid for serovar Ballum? Recent information on what serovars are circulating in wildlife and vaccinated or unvaccinated livestock sharing the same environment would be needed to answer this question.

Once infected, mice can excrete leptospires for the remainder of their lives, but this is not certain for rats and hedgehogs. Again, questions arise as to how the dynamics of mice population impact shedding over space and time? The role the environmental reservoir plays in the survival and transmission of the pathogen to non-maintenance hosts also remains to be investigated. Leptospires of the species *L. borgpetersenii* are considered to be genetically disadvantaged for a long survival in the environment, but nothing is known on that aspect specifically for serovar Ballum.

Humans can be infected via different transmission pathways (Figure 2-1), but the relative importance of each transmission direction is currently unknown. Genetic, physiological and immunological attributes of humans as spillover hosts are also important determinants of transmission (Plowright et al., 2017) that were considered beyond the scope of this work and would also need to be taken into account. Do humans become contaminated mainly via contacts with the maintenance hosts, bridge hosts or the environment? Recent advances in molecular methods and genotyping to discriminate among those different sources could be a way to assess this. It emerges from descriptive studies of human notified cases that Ballum doesn't share the same transmission sources as Hardjobovis and Pomona, and understanding the diversity and relative importance of different sources of infection will be critical for an efficient control of leptospirosis.

In conclusion, most of the knowledge available for Ballum relies on studies performed in the 1970s in both domestic and wild animals. After a long gap with no available information on this serovar, recent investigations of livestock and human

epidemiological data indicate more information is needed about the role of wild hosts in the maintenance and transmission of Ballum. The possibility for this serovar to spillover to domestic hosts, that could thus act as bridge hosts, should be taken into account.

“I’m very conscious of the fact that you can’t do it alone. It’s teamwork. When you do it alone you run the risk that when you are no longer there nobody else will do it.”

— Wangari Maathai

Chapter 3

A cross-sectional investigation of *Leptospira* at the wildlife-livestock interface in a beef and sheep farm and a dairy farm in the Manawatū-Whanganui region, New Zealand

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Chapter 3.
Cross-sectional study

Abstract

There has been a recent upsurge in human cases of leptospirosis in New Zealand, with wild mammals a suspected emerging source, but up-to-date knowledge on this topic is lacking. We conducted a cross-sectional study in the Manawatū-Whanganui region to identify serovars present in wildlife and livestock.

Traps were set for 10 trap-nights in March-April 2017 on a dairy (A) and a beef and sheep (B) farm, targeting house mice (*Mus musculus*), ship rats (*Rattus rattus*), hedgehogs (*Erinaceus europaeus*) and brush-tailed possums (*Trichosurus vulpecula*). Trapped wild mammals and an age-stratified random sample of domestic animals, namely cattle, sheep and working dogs were blood sampled. Sera were tested by microagglutination test for 5 serogroups and titres compared using a Proportional Similarity Index (PSI). Wildlife kidneys were sampled for culture and qPCR targeting the *lipL32* gene. True prevalence in mice was assessed by collating different laboratory results using two different methods, latent class (LCM) and occupancy modelling.

Infection profiles varied by species, age-group and farm. At the MAT cut-point of ≥ 48, up to 78% of wildlife species were seropositive, and 16 to 99% of domestic animals. Five/9 hedgehogs, 23/105 mice and 1/14 ship rats reacted to Ballum. Four and 1/18 possums and 4 and 1/9 hedgehogs reacted to Hardjobovis and Tarassovi, respectively. In ruminants, seroprevalence for Hardjobovis and Pomona ranged 0 to 90% and 0 to 71% depending on the species and age group. Titres for Ballum, Tarassovi and Copenhageni were also observed in respectively 4 to 20%, 0 to 25% and 0 to 21% of domestic species. The PSI indicated rodents and livestock had the most dissimilar serological responses. Three/9 hedgehogs, 31/105 mice and 2/14 rats were carrying leptospires (PCR and/or culture positive). True prevalence in mice was similar with both models, respectively 36.8%, 95% Credible Interval [25.2, 49.6%] or 38% [26, 51%] on Farm A and 21.8% [9.8, 37.6%] or 22% [11, 40%] on Farm B by LCM and occupancy models.

Serovars found in wildlife species are commonly detected in livestock in the same environment. Transmission pathways between and within species should be assessed to mitigate efficiently exposure to *Leptospira*.

Introduction

Leptospirosis is a zoonosis caused by pathogenic bacteria of the genus *Leptospira* affecting a wide range of vertebrates worldwide. The advent of genomic methods has shed light on this complex genus (Picardeau, 2017). Currently 65 different species and more than 300 serovars are described (Thibeaux et al., 2018a, Vincent et al., 2019) and this number is likely to increase. In New Zealand, only two species and six serovars (sv) are known to be endemic in animals: *L. borgpetersenii* sv Hardjobovis, Ballum, Balcanica and Tarassovi and *Leptospira interrogans* sv Pomona and Copenhageni. *L. interrogans* svs Canicola and Australis have also been isolated but from human cases only (Marshall and Manktelow, 2002).

In New Zealand, this zoonosis has a clear occupational pattern, with more than two-thirds of the cases being notified in farm or abattoir workers (ESR 2017). Cattle, pigs, and subsequently sheep and farmed deer have been shown to maintain serovars Hardjobovis or Pomona independently of wildlife (Hellstrom, 1978, Bolt, 1990, Vallée, 2016, Subharat, 2010). These two serovars were responsible for 99% of human cases in the 1970s (Mackintosh, 1981). The uptake of vaccination in the 1980s in the pig and dairy industries combined with other hygienic measures was followed by a rapid and sustained reduction of cases due to these serovars (Marshall and Chereshsky, 1996).

Several studies in the 1950s – 1970s countries worldwide, rodents and wildlife were identified as a minor public health concern for leptospirosis in New Zealand (Hathaway et al., 1981, Smith, 1965, Hathaway, 1981a). Ship rats (*Rattus rattus*), brown rats (*R. norvegicus*), house mice (*Mus musculus*) and hedgehogs (*Erinaceus europaeus*) were described as maintenance hosts for Ballum and possums (*Trichosurus vulpecula*) for Balcanica (Hathaway, 1978). Both serovars were at the time extremely rare (< 1%) in the notified human cases. This, together with limited financial resources (Collins-Emerson, 2017), probably explains why there are no recent data available on *Leptospira* infection in wildlife (see Chapter 2 or (Moinet et al., 2017)).

Concurrently to the studies on wildlife mentioned above, studies conducted on livestock underlined the scarcity of reactions to serovars other than Hardjobovis and Pomona (Hellstrom, 1978). Titres for Tarassovi, Ballum or Copenhageni were scarce and at that time interpreted as cross-reactions (Hathaway, 1978, Hellstrom, 1978). Hathaway et al. (1981c) then described this epidemiological situation through the concept of nidality, where different serovars were considered to evolve in adjacent 'niches' with virtually no spillover between domestic and wild species. Subsequent studies focused on Hardjo and Pomona and other serovars were omitted from the test panel.

Although spillovers were formerly considered to be rare events in NZ, this needs to be questioned. Vaccinated dairy cattle have recently been shown to shed serovars not traditionally associated with cattle (Yupiana et al., 2017). Other strains are also more common among human cases, especially Ballum, responsible for 30% of the cases notified in 2015 and 2016 (ESR 2016, 2017). It is unknown if this change in livestock and human epidemiology is due to an increased exposure to maintenance hosts shedding those emerging serovars or a change in the role of livestock in maintaining them. Up-to date information on all serovars circulating in wildlife and livestock is needed.

The objectives of this study were to (1) describe the seroprevalence in wildlife and sympatric livestock in two farm environments; (2) assess PCR/culture prevalence in wildlife on those farms; (3) compare seroprevalence and prevalence between species, sex and age groups in the two farms, and (4) estimate the true prevalence of natural *Leptospira* infection in mice using Bayesian latent class models and site-occupancy models.

Materials & Methods

Study sites

We selected two farms where *Leptospira* infection had been detected in livestock during previous studies. Farm A was a dairy farm in coastal Manawatū, identified after an outbreak of leptospirosis due to sv Hardjobovis and Pomona among the farm workers (Benschop et al., 2017). Livestock from this farm had been investigated and an intervention study had been conducted to assess the effectiveness of livestock vaccination to decrease the shedding rates of sv Hardjobovis and Pomona. This study underlined the risk posed by two non-vaccine serovars, Ballum and Tarassovi (Yupiana et al., 2019b). Farm A comprised two dairy herds of 228 and 400 milking cows, 150 rising 1-year-old (R1) and 150 rising 2-year-old (R2). The farm spanned 130 ha of lowland pastures and was bounded by a pine forest on one side (average elevation: 20m). Farm B was a beef & sheep farm in the Tararua region, where sheep naturally exposed to Hardjobovis and Pomona had been studied previously (Vallée et al., 2017). It comprised *ca.* 2600 'hoggets' (1-year), 2300 '2-tooth' (2-year) and 4000 mixed-age ewes, 350 calves, 160 R1 and 100 R2 heifers and 300 mixed-age cattle. Around 20 working dogs were also present in the farm that spanned 2100 ha of hilly pastures bordered with native bush (average elevation: 444 m).

Fieldwork

Animal Ethics. The present research was done in accordance with the New Zealand Animal Welfare Act 1999 and the Massey University Code of Ethical Conduct. The Massey University Animal Ethics Committee approved the procedures done on animals under the protocol 16/93.

Wildlife trapping. In each site 72 Longworth small mammal live-traps (Penlon Ltd., Oxford, UK) targeting mice, 45 Tomahawk 202.5 collapsible live-traps (Tomahawk Live Trap, Hazelhurst, WI, USA) targeting rats, and 36 Havahart #1099 live-traps (Woodstream Corp., Lititz, PA, USA) targeting possums and hedgehogs were used. Those three types of traps were set on grids in locations favourable to rodents, possums and hedgehogs with approximately 10 m, 25 m and 50 m spacing, respectively (Russell, 2012, Harper and Rutherford, 2016, Cowan, 2001) and their GPS position was recorded. These traps were set in March-April 2017 for up to 10 days per site, baited with peanut butter, cat food and pieces of apple covered with a mix of sugar and cinnamon and checked daily.

Wildlife sampling. Wild animals were anaesthetised, a blood sample taken, and animals were then euthanized for organ sampling as described by Herbreteau et al. (2011). Mice and rats were anaesthetised using isoflurane (Attane®, Bayer) insufflated in a plastic bag and euthanized while sedated by cervical dislocation. Other species were anaesthetised by intramuscular injection of a mix of medetomidine (Domitor®, Zoetis NZ Ltd, 50 to 150 µg/kg) and ketamine (Phoenix pharm, 5 to 10 mg/kg) and euthanized while sedated with pentobarbital (Pentobarb 300®, Provet, 150 mg/kg, intracardial injection). In addition to blood, urine and a kidney were sampled aseptically for culture and PCR. The other kidney was stored in formalin for histology. Concomitantly other organs (spleen, liver, lung, heart, brain, gastro-intestinal tract) were collected and stored in cryotubes and formalin for other studies.

Trap number, species, sex, reproductive status, age (juvenile, sub-adult or adult) and body measurements (weight, head, body, tail and hind leg lengths and body-condition score) were recorded for each trapped animal.

Livestock sampling. We sampled domestic animals by mob according to the farmers' schedule (when animals were gathered for milking, drenching, shearing, pregnancy testing or annual vaccine booster). We expected a seroprevalence of 20% for dogs (which was the seroprevalence observed in working dog breeds in (Harland, 2015)), of 80% in sheep and beef cattle (which was the seroprevalence observed in beef & sheep farms in (Vallée et al., 2015) when *Leptospira* was present, and 50% in dairy cattle (which was the seroprevalence in the dairy farm at first sampling in (Benschop et al., 2017)). Assuming

the proportions of seropositive animals in each farm and group were as expected, and adjusting for a finite population, we used the formula for estimating the expected seroprevalence with 10% absolute precision and 95% confidence in (Dohoo et al., 2009) to calculate the sample size in each species or age-group (Table 3-1). Blood was collected by caudal or jugular venepuncture using a one-inch 20 G vacutainer needle and a CAT Plus Blood Collection Tube without anticoagulant (BD Vacutainer®). Blood samples were transported on ice in a cooling box to the Molecular Epidemiology and Public Health Laboratory (*m*EpiLab, Massey University, Palmerston North), where they were centrifuged at *2000 g* for ten minutes to obtain serum. A convenient sample of urine was collected for culture from cattle and sheep voiding urine during blood sampling. Urination was otherwise stimulated by tickling the vulva and mid-stream urine samples were collected in 60 mℓ sterile containers. A sample size of 30 urine samples per species and age-group was targeted.

Table 3-1 | Number of domestic animals sampled per age group and farm

	Group	Expected seroprevalence (%)	Approximate group size (#)	Sample size (#)	Sample date
Farm A	Milking cows	50	250	70	29/03/2017
	1-year-old dairy cattle (R1)		150	59	10/04/2017
	2-year-old dairy cattle (R2)		150	59	10/04/2017
Farm B*	Working dogs	20	25	18	23/05/2017
	1-year-old ewes (hoggets)	80	2600	61	23/05/2017
	2-year-old ewes (2-tooths)		2300	60	5/04/2017
	Mixed-age ewes		4000	61	10/03/2017
	2-year-old beef cattle (R2)		100	39	5/04/2017

**The 1-year-old and mixed-age beef cattle groups present on farm were not sampled*

Culture. To keep contamination to a minimum, cultures were processed on farm and the method adapted accordingly. A field lab was set up to process all wild animal samples, while livestock samples were processed next to the sampling area (yard, milking shed or paddock). Kidneys to be cultured were removed aseptically within half an hour of euthanasia. Kidneys were washed with 70% ethanol and flamed prior to 1 cm^3 (or the whole kidney if less) being placed with 1 mℓ (or an equivalent volume) of Phosphate Buffered Saline (PBS) in a sterile Petri dish. The piece of kidney was then dilacerated using a sterile scalpel blade. A 0.5 to 1 mℓ aliquot of this slurry was removed at this point into a cryotube for molecular work. The remaining kidney and PBS slurry was pipetted into a tube with 2 mℓ PBS and left to stand for approximatively 30 minutes. A culture vial containing 5 mℓ EMJH + 5'-fluorouracil was then inoculated with 0.5 mℓ of liquid and

two subsequent serial dilutions (1/10) were made. Similarly, livestock urine was first collected in a sterile container, and, within 1 h after collection, 0.5 mℓ was subsequently inoculated in 5 mℓ EMJH + 5'-fluorouracil with two subsequent serial dilutions (1/10). Culture vials were stored at ambient temperature and protected from the light in the field and placed at 28°C on a shaker in an incubator as soon as they reached the ^{m}EpiLab. They were checked under the dark field microscope at least every two weeks for 14 weeks.

Laboratory analyses

MAT. Microscopic agglutination test (MAT) was used to test all sera for antibodies to *Leptospira borgpetersenii* sv Hardjobovis, Ballum and Tarassovi and to *Leptospira interrogans* sv Pomona and Copenhageni (Table 3-2). The technique used was a modification of the technique described by Faine (1999) and done as described by Fang et al. (2014). Two-fold dilutions of the serum sample ranging from 1:24 to 1:3072 were made in 0.9% saline for each serovar. After being incubated for 1.5 – 4 h with a volume of live antigen suspension of each of the above-mentioned serovars, the presence of agglutination or lysis was checked under a dark-field microscope. A positive control (standard antiserum, World Health Organisation (WHO) Leptospirosis Reference Centre, Amsterdam) and negative control (0.9% saline) were included for each serovar tested, on each day of testing. The end-point of an agglutination reaction was deemed to be the dilution at which approximately 50% of the organisms have been agglutinated and expressed as a reciprocal titre (*e.g.* titre 24 for dilution 1:24). Since this serological test was used to assess previous exposure (seroprevalence) to leptospires at the population level, and not for clinical diagnosis, the positive threshold was set at a titre of 48 or higher (Blackmore et al., 1982).

Table 3-2 | Strains of *Leptospira* included in the Microscopic Agglutination Test

Genomospecies	Serogroup	Serovar	Strain
L. interrogans	Icterohaemorrhagiae	Copenhageni	M20
	Pomona	Pomona	68
L. borgpetersenii	Sejroë	Hardjobovis	180
	Ballum	Ballum	Mus 127
	Tarrassovi	Tarrassovi	Perepelitsin

***lipL32* real-time PCR on kidney**. DNA from 80 µℓ of the kidney PBS slurry was extracted with QIAamp DNA mini kit® (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand). A real-

time Polymerase Chain Reaction (PCR) assay was performed on each extract using a probe targeting the *LipL32* gene, only present in the pathogenic clade of the genus *Leptospira* (Stoddard, 2013). Reactions were performed in a total volume of 10 µℓ consisting of 0.4 µM each of forward and reverse primers, of sequences 5'-AAG CAT TAC CGC TTG TGG TG-3' (*lipL32*-45-F) and 5'-GAA CTC CCA TTT CAG CGA TT-3' (*lipL32*-286-R), 0.13 µM of probe, of sequence FAM-5'-AA AGC CAG GAC AAG CGC CG-3'-BHQ1 (*lipL32*-189P), 2 µℓ of ToughMix® (Quantabio), 2 µℓ PCR grade water and 2 µℓ of DNA template and analysed as described by (Stoddard, 2013, Galloway and Hoffmaster, 2015). We used a Qiagen Rotor-Gene Q machine (Bio-Strategy Ltd, Auckland, New Zealand), PCR grade water as a negative control and DNA extracted from approximately 3×10^8 cells/mℓ of pure culture of *L. borgpetersenii* serovar Hardjobovis as a positive control.

Data analysis

Except where otherwise stated, all analyses were conducted in ℝ version 3.4.2 (R core Team, 2017). We differentiated the seroprevalence (estimated by MAT), the prevalence (estimated by culture or PCR) and the true prevalence (estimated as a function of two or three of the laboratory tests). The probability of shedding amongst seropositive and seronegative animals was calculated by dividing the number of animals positive for PCR and/or culture by the total number of animals tested within each stratum.

Confidence Intervals for proportions. Exact confidence intervals (CI) of observed culture and PCR prevalence and seroprevalence were computed based on the binomial distribution (Dohoo et al., 2009).

Geometric Mean Titres (GMT). The geometric mean titre of positive sera (GMT) and of all sera (GMT0) was calculated using the formulas given in (Vallée et al., 2015). While sera for which no antibodies were detected (titre <24) were excluded from the calculation of the GMT, they were given a log-titre of 0 and included in the calculation of the GMT0.

True prevalence. Misclassification bias can arise from the use of imperfect tests giving false positive and false negative results. To limit this bias, two different methods were used to adjust the apparent prevalence in mice and their result compared. True prevalence for each farm was computed using (1) a Bayesian latent class modelling (LCM) for two independent tests and two populations and (2) occupancy modelling as in (Lachish et al., 2012).

(1) The parameters used in the LCM are presented in Table 3-3. Briefly, this method uses prior knowledge of the parameters and the observed data combined in a likelihood function to simulate a posterior distribution of these parameters via an

iterative Markov Chain Monte-Carlo (MCMC) technique. Priors were determined using the literature when available, otherwise one of the co-authors gave their opinion on their value prior to the modelling (Table 3-3). The MAT and PCR sensitivity and specificity evaluated by Hea (2014) were used as priors except the PCR specificity that was assumed to be 100% (Stoddard et al., 2009). Sensitivity and specificity were assumed to be the same in both farms. Prevalence in mice assessed by Hathaway (1981) was used as a prior for Farm A, and the prior for prevalence on Farm B suspected to be lower due to the topography of the farm was set to half the value on Farm A. We determined the beta distributions of the priors using the function epi.betabuster in package epiR version 0.9-97 (Stevenson et al., 2018). MAT and PCR measure different outcomes (*Leptospira* DNA in kidney *vs.* antibodies against *Leptospira* in blood), and titres are a poor predictor of shedding status (Fang et al., 2014b). We made therefore no adjustment for correlation. We ran 10,000 MCMC iterations and discarded the first 5000. We extracted from the posterior distributions the means and 2.5^{th} and 97.5^{th} percentiles to build the 95% credible intervals. To assess convergence, we used the last 5000 iterations in chain to plot a Gelman-Rubin-Brooks graph (Brooks and Gelman, 1998). A sensitivity analysis was conducted using weak and perturbed priors (weakly informative (Jeffrey's prior), high (80%) or low (1%) priors for prevalence and optimistic (99%) or pessimistic (55%) priors for test parameters, Table 3-3) for each parameter and magnitude and direction of changes in the posterior distributions were noted. LCM were run in ℝ with package R2OpenBUGS version 3.2-3.2 (Sturtz et al., 2005) and OpenBUGS version 3.2.3. Package coda version 0.19-2 (Plummer et al., 2006) was used for convergence diagnosis. Details on this model are presented in Appendix 4.

Table 3-3 | Parameters used in the Latent Class Model

A

Farm A	PCR –	PCR +
MAT –	48	8
MAT +	3	15
Farm B	PCR –	PCR +
MAT –	27	1
MAT +	0	5

B

	"best guess"	95% certain	α	β
π_A	0.16[a]	< 0.5[c]	2.04	6.47
π_B	0.08[c]	< 0.5[c]	1.36	5.12
Se_{PCR}	0.65[b]	> 0.6[c]	171.39	92.75
Se_{MAT}	0.84[b]	> 0.5[c]	6.47	2.04
Sp_{MAT}	0.73[b]	> 0.7[c]	466.48	173.16
Jeffrey's	\	\	0.50	0.50
High π	0.8	< 0.9	17.62	5.16
Low π	0.01	< 0.1	1.34	34.17
Opt Se/Sp	0.99	> 0.95	88.28	1.88
Pes Se/Sp	0.55	> 0.3	5.99	5.08

A- Contingency tables for the number of mice tested by Microscopic Agglutination Test (MAT) and lipL32 PCR on Farm A and B.

B-Determination of the beta prior distribution (α, β) for the 5 parameters to be estimated: the true prevalence on Farm A and B (πA, πB), the sensitivity of each diagnostic test (SePCR, SeMAT) and the MAT specificity (SpMAT), and perturbed values used in the sensitivity analysis: weakly informative (Jeffrey's prior), high (80%) or low (1%) priors for prevalence and optimistic (99%) or pessimistic (55%) priors for test parameters. PCR sensitivity was assumed to be 1. Estimations based on [a](Hathaway et al., 1981), [b](Hea, 2014), [c]expert opinion

(2) Occupancy models are widely used in ecology to estimate the proportion of sites occupied by an animal species while accounting for imperfect detection. Considering animals as sites occupied or not by a pathogen, these models can be adapted to infer the probability ψ an animal is 'occupied'—*i.e.* the true prevalence of infection—and the probability p of pathogen detection conditional on the pathogen presence—*i.e.* the sensitivity of the test(s) used (Lachish et al., 2012). Briefly, occupancy models can be formulated as Hidden Markov Models (HMM) where hidden states (exposed/not exposed) are linked to observable events (detected exposed/not detected exposed) in a Markov chain. The results of the three laboratory diagnostic methods used in this study (MAT, PCR and culture) were considered as detection occasions and for each mouse, a 'detection history' (*i.e.* an observed status being a combination of the three test results for a given mouse) was built to fit HMM to estimate the true prevalence ψ of *Leptospira* infection in mice. For instance, an animal with a positive MAT and PCR and negative culture had a 'detection history 110' (out of a total of eight possible observed status). HMM were implemented in software E-Surge version 2.1.2 as described by (Gimenez et al., 2014). While ψ was allowed to vary between farms, p was considered either constant across laboratory methods or method specific. As in (Lachish et al., 2012), model selection was based on QAICc (Akaike Information Criterion corrected for small sample size and adjusted for overdispersion). The model with the lowest QAICc was selected as the model that fitted the data best. An important assumption of this method was that all animals tested positive for any given test were considered as true positives (perfect specificity). Details on occupancy models and their parameterisation are presented in Appendix 5.

Kappa test for cross-reaction. Agreement beyond chance between MAT results for different serovars was tested using Kappa (κ) tests for all wild and all domestic species respectively. A Cohen's Kappa and a square-weighted Kappa were calculated for each pair of MAT serovar results (positive/negative) and each pair of MAT serovar log-titres. While Cohen's Kappa is adapted to binary data, the square-weighted Kappa gives more weight to bigger differences between titres (*e.g.* titres 24 – 3072 *vs.* 24 – 48) and is therefore more adapted for ordinal data. Values $\kappa \leq 0$ indicated no agreement while the strength of agreement was considered as poor for $0.01 \leq \kappa < 0.2$, fair for $0.2 \leq \kappa < 0.4$, moderate for $0.4 \leq \kappa < 0.6$, good for $0.6 \leq \kappa < 0.8$ and very good for $\kappa \geq 0.8$.

PSI-Czekanowski index. A proportional similarity index (PSI) in the serological responses between species was computed for all species. The PSI or Czekanowski index has first been used to measure the breadth of a population's niche in ecology, but also to measure the similarity between the frequency distributions of pathogens types among different animal species (Feinsinger et al., 1981, Rosef et al., 1985). It is calculated as $PSI = 1 -$

$0.5 \sum_i |p_i - q_i| = \sum_i \min(p_i, q_i)$ where p_i and q_i are the proportion of serovar i out of all serovars detected in animal species P and Q respectively. The same positivity threshold as MAT was used (titre ≥ 48). The closer the PSI is to 1, the more similar are the frequency distributions of *Leptospira* serovars between two species; the closer the PSI is to 0, the most dissimilar they are. We determined 95% credible intervals using a bootstrap simulation method with 2000 replications (Efron and Tibshirani, 1986). If the credible intervals included 0.5 the PSI was regarded as inconclusive.

Results

Species composition in the different study sites

There were respectively 720, 430 and 351 trap-nights for Longworth, Tomahawk and Havahart traps on Farm A. Because of an accident while setting the traps on Farm B, those figures were slightly lower on Farm B (648, 418, 332). Rats, hedgehogs and possums were trapped in both Tomahawk and Havahart traps. The number of animals captured per 100 trap-nights and sampled in each farm are detailed in Table 3-4. No possums were trapped on Farm A where possum control has been in place within the farm and the neighbouring forest for several years. Other wild mammals not targeted by the traps were also observed while on site: feral cats (*Felis catus*) and rabbits (*Oryctolagus cuniculus*) on both farms, Sambar deer (*Cervus unicolor*) on Farm A and Red deer (*C. elaphus*) on Farm B. Two Sambar deer hunted during the trapping session on Farm B boundaries and one by-catch feral cat from each farm were also sampled. All analyses were negative for those animals. We did not have the opportunity to sample the R1 and mixed-age beef cattle on Farm B during the study.

Seroprevalence and titres

Serological results are detailed in Table 3-4 for wildlife and Table 3-5 for livestock, along with culture and PCR results. All dairy cattle from Farm A and a majority of dogs from Farm B had been previously vaccinated against leptospirosis—albeit none recently—with a bivalent vaccine (Hardjobovis & Pomona) for the former and an unknown valence for the later.

Table 3-4 | Abundance (A) in captures per 100 trap-nights, total number of animals sampled (N) and with a positive result (bolded) for *Leptospira* infection by Culture, PCR or Microscopic Agglutination Test (MAT; cut-off 48). *The prevalence and 95% confidence interval are indicated in brackets when N>5, and when applicable, the serovar specific geometric mean titre of all sera (GMTo) or of sera with titres ≥ 24 (GMT) are indicated for each serovar.*

	Species	A (C/100TN)	N	Culture # positive (% [95% CI])	PCR # positive (% [95% CI])	MAT # positive (seroprevalence [95% CI]) and GMTo (GMT)					
						Ballum	Copenhageni	Hardjobovis	Pomona	Tarassovi	Overall
Farm A	*Erinaceus europaeus*	1.02*	8	**3 (38% [9, 76])**	**3 (38% [9, 76])**	**4 (50% [16, 84])** 62 (167)	0 (0% [0, 37]) 17 (24)	**4 (50% [16, 84])** 26 (42)	**2 (25% [3, 65])** 40 (305)	**1 (13% [0, 53])** 19 (29)	**6 (75% [35, 97])**
Farm A	*Mus musculus*	14.31	74†	**16 (26% [16, 39])**	**23 (31% [21, 43])**	**18 (25% [15, 36])** 30 (523)	**1 (1% [0, 7])** 12 (96)	0 (0% [0, 5]) 12 (24)	0 (0% [0, 5]) 0	0 (0% [0, 5]) 12 (24)	**18 (25% [15, 36])**
Farm A	*Rattus rattus*	1.02*	3	**1/3**	**1/3**	**1/3** 38 (384)	0/3 0	0/3 0	0/3 0	0/3 15 (24)	**1/3**
Farm B	*Erinaceus europaeus*	0.67*	1	0/1	0/1	**1/1**	0/1	0/1	0/1	0/1	**1/1**
Farm B	*Mus musculus*	5.71	33†	**6 (18% [7, 35])**	**6 (18% [7, 35])**	**5 (16% [5, 33])** 19 (253)	0 (0% [0, 11]) 12 (24)	0 (0% [0, 11]) 13 (24)	0 (0% [0, 11]) 0	0 (0% [0, 11]) 0	**5 (16% [5, 33])**
Farm B	*Rattus rattus*	2.40*	11	0 (0% [0, 28])	**1 (9% [0, 41])**	0 (0% [0, 28]) 0	0 (0% [0, 28]) 0	0 (0% [0, 28]) 0	0 (0% [0, 28]) 0	0 (0% [0, 28]) 13 (24)	0 (0% [0, 28])
Farm B	*Trichosurus vulpecula*	2.67*	18‡	0 (0% [0, 31])	0 (0% [0, 26])	0 (0% [0, 19]) 12 (24)	0 (0% [0, 19]) 12 (24)	**4 (22% [6, 48])** 34 (272)	0 (0% [0, 19]) 0	**1 (6% [0, 27])** 13 (34)	**5 (28% [10, 53])**

*† 1 animal lacked a blood sample and had only a PCR and culture done; ‡ 10 had all 3 analyses done, 2 had PCR and MAT only, and 6 had MAT only; * Calculated with Tomahawk and Havahart traps since animals were caught in both types*

Table 3-5 | Herd size, number of blood (S) and urine (U) sampled and with a positive result (bolded) for *Leptospira* infection by Microscopic Agglutination Test (MAT; cut-off 48). *The prevalence and 95% confidence interval are indicated in brackets, and when applicable the serovar specific geometric mean titre of all sera (GMTo) or of sera with titres ≥ 24 (GMT) are indicated for each serovar.*

	Group	Herd size	S	U	MAT # positive (seroprevalence [95% CI]) and GMTo (GMT)					
					Ballum	Copenhageni	Hardjobovis	Pomona	Tarassovi	Overall
Farm A	R1 Dairy cattle	150	60	30	**7 (12% [5, 23])** 19 (39)	**3 (5% [1, 14])** 14 (114)	**18 (30% [19, 43])** 23 (35)	**10 (17% [8, 29])** 17 (44)	0 (0% [0, 6]) 14 (24)	**26 (43% [31, 57])**
Farm A	R2 Dairy cattle	150	60	30	**12 (20% [11, 32])** 21 (34)	0 (0% [0, 6]) 13 (24)	0 (0% [0, 6]) 13 (24)	**2 (3% [0, 12])** 13 (68)	**7 (12% [5, 23])** 21 (28)	**19 (32% [20, 45])**
Farm A	Milking cows	228	83	30	**7 (8% [3, 17])** 16 (30)	**1 (1% [0, 7])** 13 (26)	**66 (80% [69, 88])** 66 (72)	**58 (70% [59, 79])** 98 (136)	**8 (10% [4, 18])** 18 (40)	**82 (99% [93, 100])**
Farm A	TOTAL Dairy cattle	528	203		**26 (13% [9, 18])**	**4 (2% [1, 5])**	**84 (41% [35, 48])**	**70 (34% [28, 41])**	**15 (7% [4, 12])**	**127 (63% [56, 69])**
Farm B	Working dogs	20	14	\	**2 (14% [2, 43])** 18 (36)	**3 (21% [5, 51])** 19 (34)	**9 (64% [35, 87])** 87 (101)	0 (0% [0, 23]) 18 (24)	0 (0% [0, 23]) 14 (24)	**9 (64% [35, 87])**
Farm B	Hoggets (1Y)	2600	58	\	**4 (7% [2, 17])** 15 (62)	**8 (14% [6, 25])** 17 (35)	**3 (5% [1, 14])** 15 (384)	**41 (71% [57, 82])** 185 (576)	0 (0% [0, 6]) 13 (24)	**42 (72% [59, 83])**
Farm B	2-tooth (2Y)	2300	61	\	**4 (7% [2, 16])** 16 (37)	**5 (8% [3, 18])** 15 (36)	**55 (90% [80, 96])** 267 (352)	**12 (20% [11, 32])** 28 (216)	**9 (15% [7, 26])** 20 (31)	**56 (92% [82, 97])**
Farm B	Mixed-age ewes	4000	61	11	**3 (5% [1, 14])** 15 (30)	0 (0% [0, 6]) 13 (24)	**40 (66% [52, 77])** 45 (59)	**25 (41% [29, 54])** 32 (64)	**15 (25% [14, 37])** 22 (34)	**49 (80% [68, 89])**
Farm B	TOTAL Sheep	8900	180		**11 (6% [3, 11])**	**13 (7% [4, 12])**	**98 (54% [47, 62])**	**78 (43% [36, 51])**	**24 (13% [9, 19])**	**147 (82% [75, 87])**
Farm B	R1 Beef cattle	160	\	\	\	\	\	\	\	\
Farm B	R2 Beef cattle	100	45	5	**2 (4% [1, 15])** 14 (34)	0 (0% [0, 8]) 0	0 (0% [0, 8]) 14 (24)	**1 (2% [0, 12])** 13 (28)	**4 (9% [2, 21])** 17 (28)	**7 (16% [6, 29])**
Farm B	Mixed-age Beef cattle	300	\	\	\	\	\	\	\	\

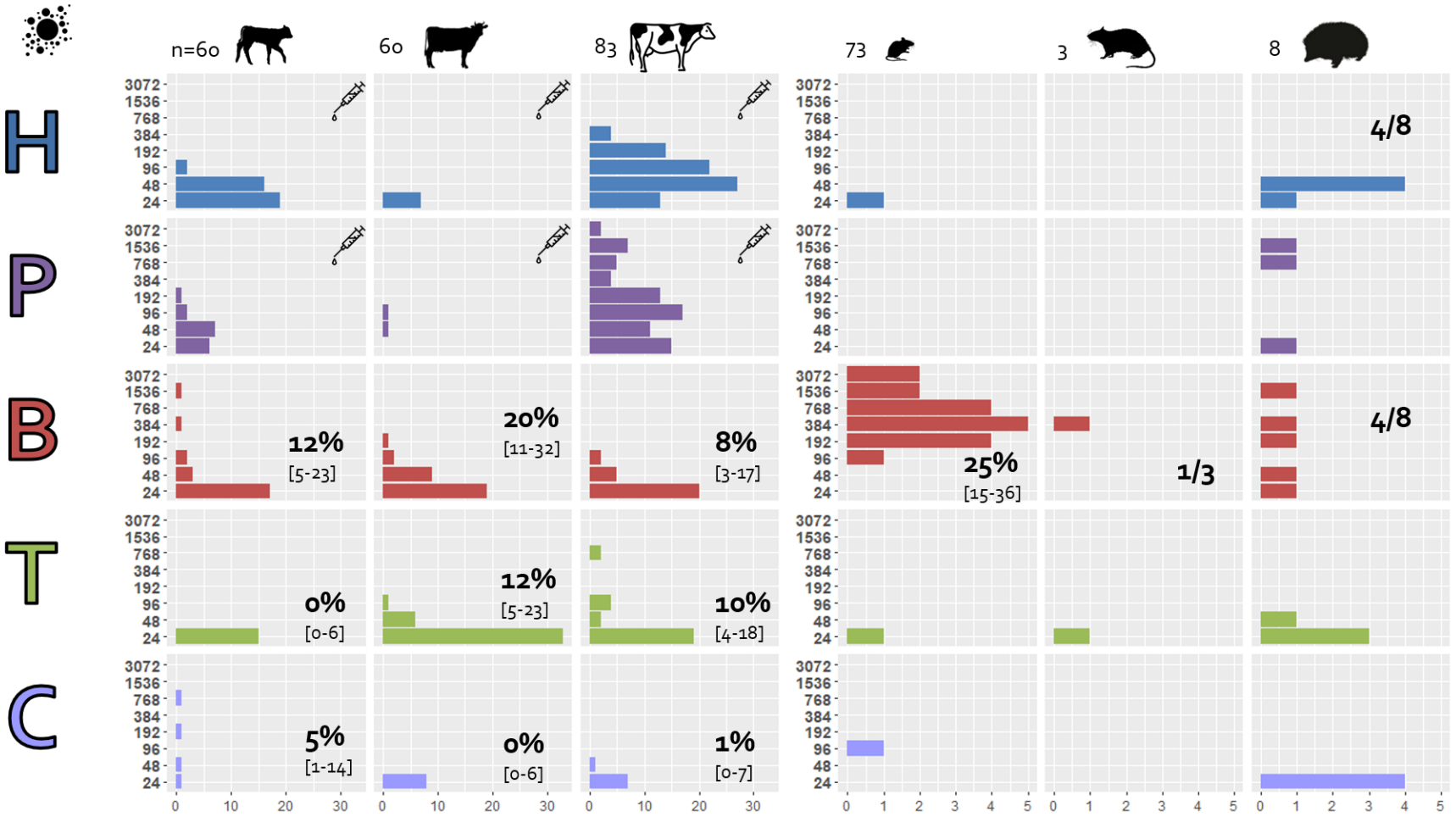

Figure 3-1 | Microscopic Agglutination Test titres observed in domestic and wild animals captured and sampled on Farm A. *Seroprevalence (cut-off 48) is also indicated. H = Hardjobovis (Sejroë serogroup), P = Pomona, B = Ballum, T = Tarassovi, C = Copenhageni*

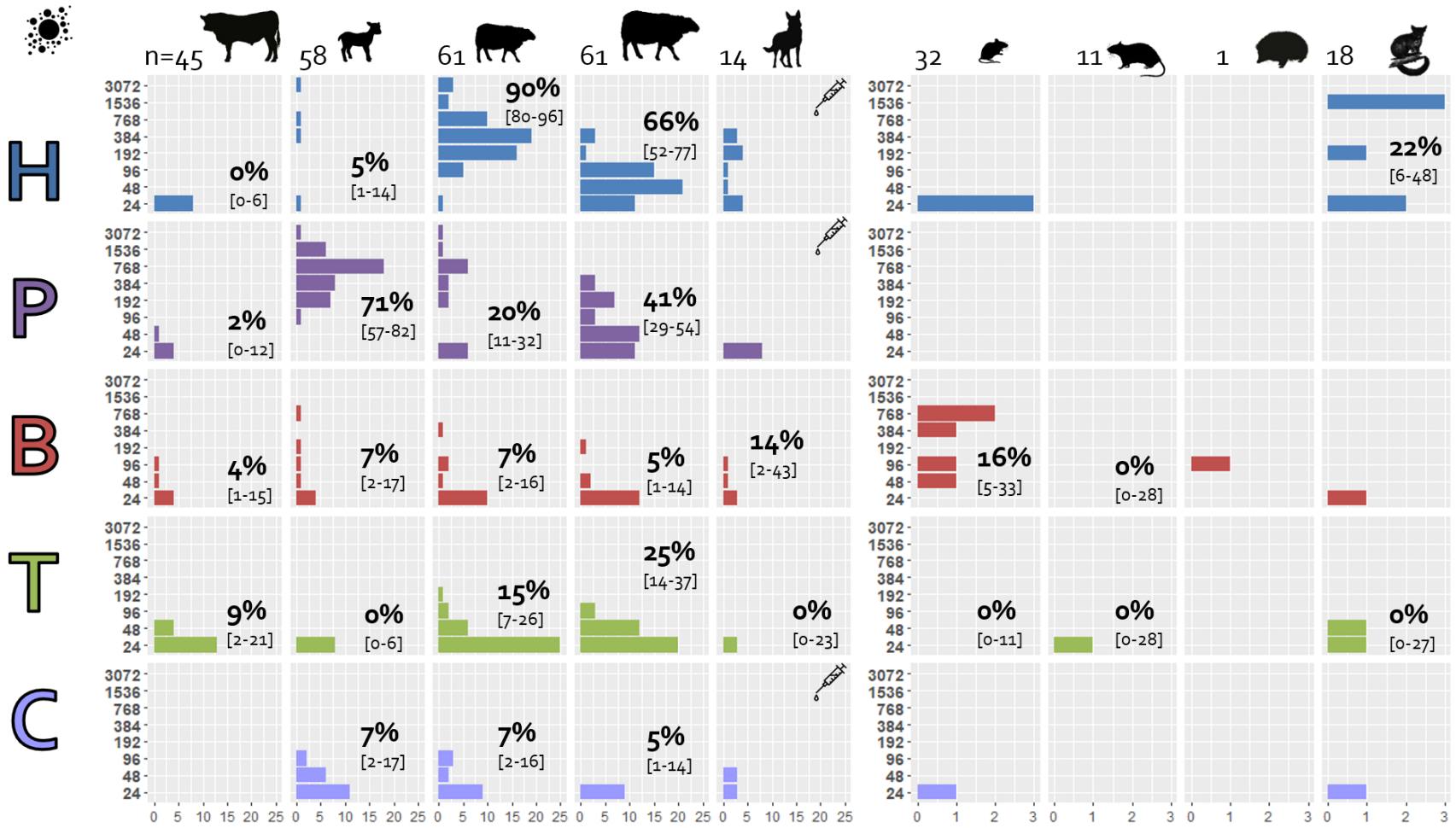

Figure 3-2 | Microscopic Agglutination Test titres observed in domestic and wild animals captured and sampled on Farm B. *Seroprevalence (cut-off 48) is also indicated. H = Hardjobovis (Sejroë serogroup), P = Pomona, B = Ballum, T = Tarassovi, C = Copenhageni*

The distribution of titres in different age groups and species is represented in Figure 3-1 and Figure 3-2. Among the unvaccinated livestock, respectively 43% [36-51%] and 54% [47-62%] of sheep had titres ≥ 48 for Pomona and Hardjobovis. Only 1/45 R2 beef cattle had a positive reaction for Pomona and none for Hardjobovis. In that group, four had titres of 24 for Pomona and eight for Hardjobovis (Figure 3-2). No ship rats or mice had positive reactions to those two serovars. Four mice had titres of 24 for Hardjo, three from farm B, one from Farm A. However, half of the hedgehogs (4/8) from Farm A had low titres (48) for Hardjo, two had high titres to Pomona (768 and 1536), and four possums from Farm B had titres to Hardjo (192 in a juvenile, and 1536 for three adults).

All groups and species had titres against Ballum except rats and possums on Farm B. Numerous livestock had a titre of 24 (Figure 3-1 and Figure 3-2). For instance, the seroprevalence for dairy cattle on Farm A increased from respectively 12%, 20% and 8% in R1, R2 and milking cows with a cut-off at 48 to 40%, 52% and 33% with a cut-off at 24.

Only livestock were positive for Tarassovi and Copenhageni, with the exception of one possum and one hedgehog for Tarassovi, and one mouse for Copenhageni. As with Ballum, numerous animals had a titre of 24 for Tarassovi (Figure 3-1 and Figure 3-2). For instance, the seroprevalence for sheep on Farm B increased from respectively 0, 15 and 25% in hoggets, two-tooth and adults with a cut-off at 48 to 14, 56 and 57% with a cut-off at 24. Except for the pair Copenhageni-Tarassovi that both had a very low seroprevalence, all Kappa calculated for wild species were < 0.2 (Table 3-6). There was a fair agreement between Ballum and Copenhageni results in domestic species.

The most similar serological responses between host species, as assessed with the PSI calculations, were observed between mice and rats, followed by cattle and sheep (Table 3-7). Rodents and possums had the most dissimilar serological responses, followed by rodents and sheep.

Table 3-6 | Unweighted Cohen's Kappa for agreement between MAT results (lower triangle) and weighted (squared weights) kappa for agreement between log-titres (upper-triangle) obtained with the MAT for the 5 different serovars tested. *Values ≥ 0.20 are bolded.*

ALL WILD SPECIES

	B	C	H	P	T
B	\	0.085	0.114	0.068	0.060
C	0.139	\	0.083	0.168	**0.466**
H	0.135	0.050	\	0.057	0.173
P	0.068	0.097	0.100	\	0.093
T	0.087	**0.380**	0.196	0.136	\

ALL DOMESTIC SPECIES

	B	C	H	P	T
B	\	**0.339**	0.012	-0.051	0.089
C	**0.215**	\	0.033	0.092	-0.029
H	-0.007	0.014	\	0.043	0.124
P	-0.047	0.055	0.157	\	-0.046
T	0.027	-0.032	0.126	0.019	\

B = Ballum, C = Copenhageni, H = Hardjobovis, P = Pomona, T = Tarassovi

Table 3-7 | Proportional Similarity Index values (lower triangle) and their associated bootstrapped 95% confidence intervals (upper triangle). *Values not bolded are values deemed inconclusive (i.e. for which the confidence interval includes 0.5)*

	Hedgehog	Mouse	Ship rat	Possum	Dog	Sheep	Cattle
Hedgehog	\	0.17-0.67	0.17-0.67	0.08-0.67	0.17-0.74	0.32-0.81	0.39-0.89
Mouse	0.42	\	**0.88-1**	**0-0**	**0-0.4**	**0.03-0.13**	**0.1-0.2**
Ship rat	0.42	**0.96**	\	**0-0**	**0-0.36**	**0.02-0.08**	**0.09-0.18**
Possum	0.42	**0**	**0**	\	0.36-0.86	0.38-0.6	0.36-0.56
Dog	0.48	**0.18**	**0.14**	0.64	\	0.44-0.6	0.4-0.61
Sheep	0.63	**0.09**	**0.05**	0.54	0.54	\	**0.81-0.94**
Cattle	0.72	**0.16**	**0.14**	0.5	0.56	**0.91**	\

Culture and PCR prevalence

Culture and PCR apparent prevalence for wildlife is synthesised in Table 3-4. All cultures from livestock urine were unsuccessful due to contamination. No leptospires were observed under the dark-field microscope in those cultures before they had to be discarded (up to six weeks following sampling), but a negative result cannot be ascertained.

The estimate of true prevalence in mice given by the LCM was 36.8% [95% Credible Interval 25.2-49.6%] on Farm A and 21.8% [9.8-37.6%] on Farm B. Although the current study did not aim at estimating the sensitivity and specificity of the tests used, the model estimates of MAT sensitivity and specificity were 62.6% [45.9-78.1%] and 75.4% [72.1-78.5%], and the estimate of PCR sensitivity was 66.9% [61.4-72.2%]. Results of the LCM sensitivity analysis can be visualized in the forest plot (Figure 3-3). Applying weakly informative priors for prevalence on Farm A or B did not substantially affect the posterior median values of prevalence (+2%), and extreme change (increase to 80% or decrease to 1%) in the priors only lead to a moderate change (same direction) in the posterior values (+ 16% and – 12% for Farm A, + 29% and – 10% for Farm B).

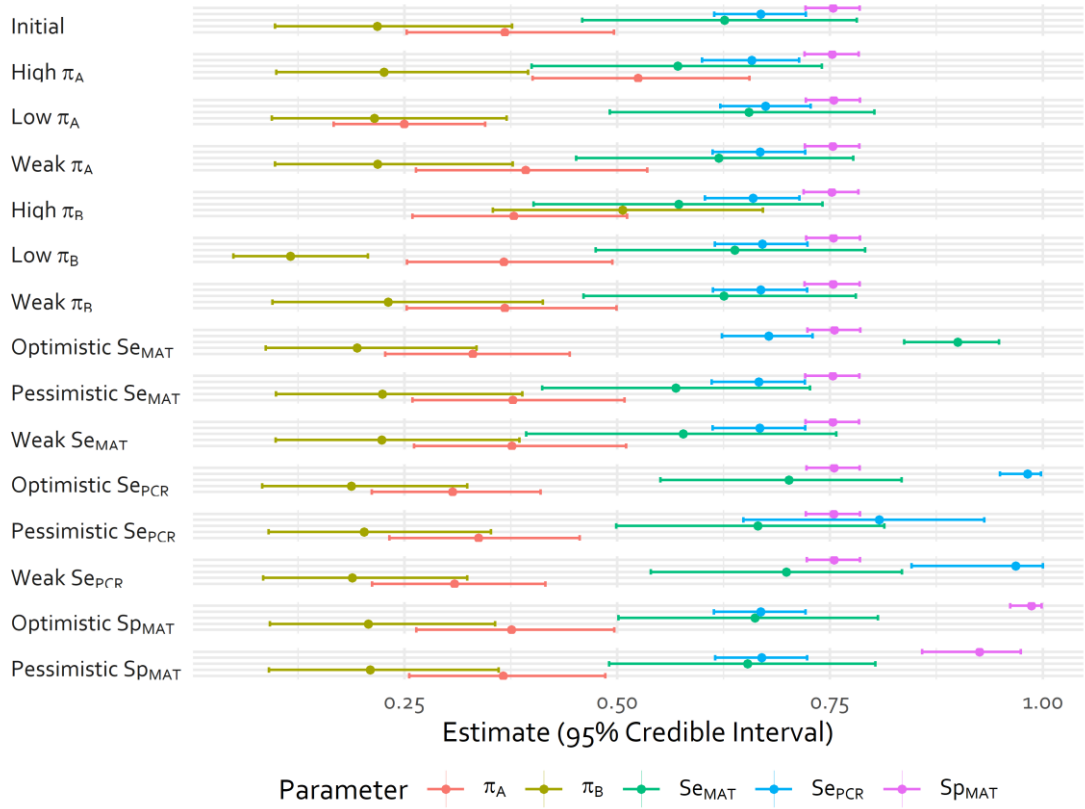

Figure 3-3 | Forest plot showing the parameters and their credible interval estimated by the initial latent class model and the other models run as a sensitivity analysis. *The five estimated parameters are the true prevalence on Farm A and B (π_A, π_B), the sensitivity of each diagnostic test (Se_{PCR}, Se_{MAT}) and the MAT specificity (Sp_{MAT})Weakly informative (Jeffrey's prior), high (80%) or low (1%) priors for prevalence and optimistic (99%) or pessimistic (55%) priors for test parameters were used in the sensitivity analysis.*

The occupancy model of best fit as determined by QAICc among those tested (listed in Table 3-8) was the model with a prevalence ψ varying by farm and a probability of detection (or sensitivity) p varying according to the laboratory method. This model estimated a true prevalence ψ of 38% [26-51%] on Farm A and 22% [11-40%] on Farm B.

Estimates of test sensitivity were 74% [55-87%] for culture, 88% [69-96%] for PCR and 64% [45-79%] for MAT.

Table 3-8 | E-Surge Outputs for four occupancy models tested in this chapter. *The model of best fit appears bolded*

Model	# IPa	Deviance	QAIC	QAICc
ψFarm.*p*Method	**5**	**194.20**	**204.20**	**205.23**
ψ.*p*Method	4	196.57	204.57	205.25
ψFarm.*p*	3	199.11	205.11	205.51
ψ.*p*	2	201.48	205.48	205.68

 # IPa = number of identifiable parameters

Probability of shedding

The probability of shedding was respectively 94% (17/18) and 9.4% (3/32) for seropositive and seronegative adult mice, and 60% (3/5) and 16% (8/50) for seropositive and seronegative young mice (juveniles + subadults). This probability was 43% (3/7) for seropositive hedgehogs (all serovars combined). No seronegative hedgehogs (0/2) were shedding leptospires. Of the nine hedgehogs sampled, seven were adults and two were subadults. Those two had only low titres detected (48 for Hardjobovis for one, 48 for Hardjobovis, Ballum and 24 for Tarassovi for the other) and no leptospires were detected or isolated from their kidneys. Two of the 14 rats sampled, both adults, were shedders (14%): one seropositive and one seronegative. The only non-adult rat captured was a subadult negative with all tests. No possums qualified as shedders.

Discussion

Our study demonstrates that livestock are exposed to serovars that circulate in wildlife in the same environment, especially Ballum. It adds weight to the growing body of evidence coming from surveys on domestic animal and humans that *Leptospira* infection in wildlife in NZ can be a source of infection and a concern for both livestock and public health. Prior to this current study, the most recent studies at the wildlife-livestock interface in NZ were conducted in the late 1970s, four decades ago. These concluded that "despite high prevalence of endemic infection of Hardjobovis and Pomona in cattle and pigs respectively and Ballum and Balcanica in wildlife, [there was] virtually no evidence of interspecies transmission" (Hathaway, 1981c, p. 111). Today, whether due to changes in vaccination practices, ecological factors, species distributions

or diagnostic techniques, we can see that this is no longer the case. Antibodies for serovars found in wildlife in our study were commonly found in livestock sharing the same environment, supporting the concept of inter-species disease transmission, or spillover.

Spillover has already been described between rodents and livestock. A European study described a higher seroprevalence for serovars associated with house mice and brown rats (Icterohaemorrhagiae, Ballum), in cattle kept indoors where those species were common, and a higher seroprevalence for Grippotyphosa, Australis & Sejroë, that are more associated with other wild rodents, in cattle kept outdoors (Trap, 1988). In New Zealand, those other rodents are absent, and mice and rats are presents not only in buildings but also in pastures, increasing the infection pressure.

Contrary to livestock, there is a dearth of information in wildlife about *Leptospira* prevalence or seroprevalence in NZ (Chapter 2). The sero- and culture prevalences we observed in mice were higher than the two previous estimates assessed in the 1970s. In the first study, seroprevalence was 3% (2/67, cut-off 100) and culture prevalence was 13% (9/67, Brockie, 1977). In the second, seroprevalence was 8% (3/39, cut-off 24), and culture prevalence was 16% (11/70, Hathaway et al., 1981). Differences observed could be due to a real difference in prevalence, or to the methods used. Both studies mentioned used a mix of snap-traps and cage-traps to catch mice and rats, and in (Brockie, 1977), blood for the MAT was extracted from hearts conserved in a saline solution and samples taken up to 24h after death. The second mentions the problem of contamination of kidneys from mice caught in snap-traps (Hathaway, 1978). This could reduce the sensitivity of both culture and MAT. Nevertheless, the higher farm prevalence of Ballum in mice by all tests (MAT, PCR, culture) in our study (16 – 31%) suggest a possible increase over time.

The interpretation of serological results is often challenging. Individuals infected with leptospires will first develop IgM antibodies, and IgG antibodies later, that have a longer half-life. MAT results are further complicated by the existence of cross-reactions between serovars and serogroups, especially in acute-phase samples (Levett, 2001). Some individuals can present paradoxical reactions where the highest titres are for a serogroup different than the infecting serovar, and anamnestic responses, where the early titres are predominantly against a serovar from a previous exposure (Levett, 2001). Even if the possibility of cross-reactions can hinder individual diagnosis, MAT results at the population level can give an overview of the serogroups circulating (Levett, 2001).

In the past, cattle titres to Tarassovi, Ballum and Copenhageni have been interpreted as being mainly due to cross-reactions (Hellstrom, 1978, Hathaway, 1981c). In our study, R2 (unvaccinated) beef cattle were negative for Hardjobovis or Pomona but

positive for Ballum or Tarassovi, making cross-reactivity less likely and true Ballum or Tarassovi infection more likely.

The poor strength of agreement between the different MAT serovar results indicated that cross-reactivity was of little consequence and titres more likely to be indicative of previous exposure to several serovars, except possibly for Ballum and Copenhageni in domestic species. This is consistent with an experimental study on calves infected with Pomona, Hardjo, Ballum or Copenhageni, where important cross-reactions were observed only between Ballum and Copenhageni (Hodges and Ris, 1974). The interpretation of titres in dogs was further hindered by the absence of information on the vaccine type used for working dogs. Although all vaccines licensed for dogs in NZ only cover the serogroup Icterohaemorrhagiae (*i.e.* sv Copenhageni), it is possible farm workers administered an off-label cattle trivalent vaccine (Hardjo, Pomona & Copenhageni) on dogs from Farm B as this practice is suspected to be common for working dogs (Harland et al., 2013, Cave et al., 2016).

Furthermore, the duration of detectable antibodies following infection by a serovar varies according to the species and serovar, and the rates of re-exposure to this serovar (Vallée et al., 2015). Vaccinal titres fade more rapidly than titres following natural infection (Kingscote and Proulx, 1986). There is also considerable individual variation in antibody decay (Blackmore et al., 1984). In a cross-sectional study like this one, the timing of infection is unknown, and animals previously infected with antibodies titres below the detection limit cannot be distinguished from animals never exposed (Gilbert et al., 2013).

On the other hand, culture and PCR methods add information about the true infection status. In this study, 35% of shedding mice (11/31) and one of two shedding rats had no detectable titres (hereinafter referred to as 'silent shedders'). This proportion was 67% to 89% in previous studies (Hathaway, 1978, Hathaway et al., 1981, Brockie, 1977). This difference might be due to a difference in the age ratios. Indeed, when stratified by age in our study, this proportion was 15% (3/20) of adult 'silent shedders' and 73% (8/11) of juvenile and subadult mice. Livestock could similarly be 'silent shedders' as well. The overall presence of low titres for this serovar in livestock reinforces this possibility. At the national scale, Yupiana et al. found 13/4000 (0.3%, 95% CI = 0.2, 0.6) urine samples of adult dairy cows, all seronegative for Ballum, PCR positive with sequences identified as Ballum (Yupiana, 2019). However, due to budgetary constraints and contamination of urine samples from livestock, it was not possible in this study to investigate the agreement between shedding and MAT titres in livestock species.

Serovars other than Hardjobovis and Pomona have been largely neglected in surveys conducted on cattle and sheep in NZ since 1983 (Chapter 2, p. 43). Only two

recent studies included other "atypical" serovars in their MAT panel and, as we did in this study, they found evidence of exposure in livestock. A nationwide study investigated sera from 1043 beef cattle and 1642 sheep sampled between 2009 and 2010. Seroprevalence in beef cattle was 13.7%, 95% CI [11.7, 16.0%] for Ballum and 18.0% [15.7, 20.5%] for Tarassovi. It was respectively 10.5% [9.0, 12.1%] and 14.0% [12.4, 15.8%] in sheep (Mannewald, 2016). In 4000 dairy cattle sampled throughout NZ in 2015, the seroprevalence was 3% [3, 4%] for Ballum and 17% [15, 20%] for Tarassovi (Yupiana et al., 2019a). MAT testing in both studies used the same cut-off and was performed in the same laboratory as the current study. In previous surveys conducted between 1967 and 1983, seroprevalence estimates for Ballum in adult cattle were 2.9% (15/520, cut-off 24; Mackintosh et al., 1982), 1.3% (8/636, cut-off 20; Blackmore et al., 1976), 3.5% (17/480, cut-off 17; Hellstrom, 1978), and did not exceed 0.9% at a 100 or 200 cut-off (listed in Chapter 2). Titres were reported for 5/208 calves, representing 2.4% (cut-off 200, Ris et al., 1973) but it is unclear whether serology was done only on those 5 leptospiruric calves or on all calves sampled.

Sheep and cattle from Farm B had been sampled previously but were tested only for sv Hardjobovis and Pomona (Dreyfus et al., 2018, Vallée et al., 2017, Vallée et al., 2015). Farm specific seroprevalence was reported only in (Vallée et al., 2015, Vallée et al., 2017) for a cohort of sheep sampled between 2011 and 2014 and showed important variations according to the age at sampling. Seroprevalence for Hardjobovis and Pomona that were between 0 and 5% in the first year of life reached 82 to 97% for Hardjobovis and 41 to 46% for Pomona in the second and subsequent years of life (Vallée et al., 2015). Although results they described for Hardjobovis are consistent with ours, seroprevalence and high titres for Pomona in hoggets sampled in our study (71%, GMT 576) contrast with the low seroprevalence they obtained in their cohort (0% in January to 2% in end of May to 42% in December 2012). These results suggest our sampling session took place shortly after the peak of exposure to Pomona, and suggest this peak happened earlier for hoggets born in 2016 compared to the 2011 cohort. It also confirms the variation between serovars and from years to years in exposure patterns already noted before (Vallée, 2016). The concomitant near total absence of titres to Pomona in the R2 beef group, in dogs, and in all wild species sampled is in favour of sheep independently maintaining this serovar on Farm B, contrary to what was previously thought (Hodges, 1974b). Pomona was however found in dairy cattle and hedgehogs on Farm A in the absence of sheep, an indication that the composition of the maintenance community of Pomona may vary geographically.

Cattle from Farm A had been sampled previously after three human cases were reported within three months (Yupiana et al., 2019b). That longitudinal study underlined

the likely efficiency of vaccination against Hardjobovis and Pomona and antibiotic treatments to reduce shedding in milking cows, previously unvaccinated. It also detected changing dynamics in seroprevalence for other serovars in the replacement stock. In the R1 group there was a low seroprevalence (0 to 2%) to all serovars before vaccination that increased to 73% for Ballum following vaccination, and in the R2 group before vaccination —group not resampled after vaccination—there was a high seroprevalence for Tarassovi only (55% *vs.* 0 to 5% for other serovars) that was not observed in the other groups (Yupiana et al., 2019b). In our study, all R1 and R2 had been vaccinated following recommendations (Heuer et al., 2012) and the herd of milking cows was composed of animals first vaccinated at eight months old or more. It is unknown if the lower seroprevalence for Ballum and Tarassovi observed by us in autumn 2017 compared to the seroprevalence measured by Yupiana et al. (2019b) in summer 2015 – 2016 are due to vaccination, a seasonal change in exposure to different serovars, a cohort effect linked to annual changes in weather, or another unidentified cause.

The true prevalence of mice exposed to leptospires, calculated by LCM and occupancy model, was higher than the apparent prevalence estimated by PCR, culture, or especially MAT, indicating a possible underestimation of the real portion of the population exposed to leptospires. The sensitivity of culture and PCR methods depends on the bacterial load. When high quantities of *Leptospira* are present in the kidneys, PCR and culture are more likely to give a positive result. The real-time PCR method we used was not quantitative. Although the cycle threshold gives an indication of the concentration of bacterial DNA in the sample, the sampling conducted in remote settings prevented the possibility to weight precisely the quantity of kidney used for extraction, and therefore the comparison between individuals. Had it been available, an estimate of the bacterial loads would have been helpful in refining the true prevalence occupancy model.

Similarly to prevalence, abundance indices of mice and ship rats estimated in our study (Table 3-4) were higher in both Farms A & B than those calculated by Brockie in farm and refuse dump environments (1977). Expressed in captures per 100 trap-nights (C/100TN), Brockie had respectively 2.97 and 0.76 C/100TN for mice and ship rats on farms, and 1.83 and 0.08 C/100TN on refuse dumps (1977). The absence of brown rats in our study suggests they were either absent or present at very low densities in the studied areas. As opposed to ship rats, brown rats prevalence was reported to be density-dependent (Hathaway and Blackmore, 1981a). The density, prevalence and seroprevalence of mice were higher in our study than in (Brockie, 1977). This was not observed for ship rats, for which numbers were insufficient to rule out a difference. Population dynamics of rodents are known to evolve cyclically, with rapid changes in

densities. To our knowledge, the relationship between *Leptospira* prevalence and species density has had little research attention. In Spain the prevalence of *Leptospira* in micromammals was not related to their relative abundance (Millán et al., 2018). To understand whether the density of infected rodents likely reflects the risk of spill over to other species, longitudinal studies would be useful.

Mat ha buan n'int ket unan

— breton proverb

Chapter 4

Population dynamics of house mice (*Mus musculus*) and the epidemiology of *Leptospira borgpetersenii* serovar Ballum in a dairy farm biotope

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Chapter 4.
Population dynamics

Abstract

Invasive house mice (*Mus musculus*) population dynamics have been widely studied in New Zealand (NZ)'s natural environment, but not in farm habitats. Moreover, mice are maintenance hosts of *Leptospira borgpetersenii* serovar Ballum, a serovar incriminated in an increasing proportion of notified human cases in NZ, and have rarely been studied on that aspect. Both prevalence and density of infected animals are important to consider for a better understanding of the risk they pose to other animals or humans. We estimated those parameters in a dairy farm biotope in a longitudinal survey conducted in Spring and Autumn over two years. In each session, mice live-trapped on grids were marked and released the first 5 days, and subsequently removed from the population for blood and kidney sampling. *Leptospira* exposure and shedding status were estimated using Microscopic Agglutination Test (MAT), and a combination of culture and PCR on kidneys. To estimate density and home range, we fitted a spatially-explicit capture-mark-recapture (SECR) model. We included MAT and shedding results to test for a null effect of infection on mice fitness, under the hypothesis that for a maintenance species, home range would be similar for positive and negative mice. Total densities varied from 3.6 to 55.9/ha, 95% CIs [0.9, 14.8] and [37.5, 83.1] depending on the grid and season. Prevalence was higher in spring (83% to 86%, 95% CIs [61, 95%] and [65, 97%]) than autumn (31% to 37%, 95CIs = 21, 43% and 26, 50%). Despite variations in prevalence and density, the density of infected—*i.e.* shedding—animals remained relatively constant over time (3 to 8/ha). Shedding or having antibodies against leptospires influenced the capture function. Shedding or seropositive mice had a wider home range, and seropositive mice were trapped on average one day earlier than seronegative mice. These results shed light on mice population dynamics in a farm biotope in NZ and contribute to the body of knowledge on *Leptospira* infection dynamics in a population of invasive maintenance hosts.

Introduction

Leptospirosis is a disease that affects humans and animals, caused by pathogenic bacteria of the genus *Leptospira* that are shed in the urine of infected hosts. It is most often acquired either by direct contact with infected urine, or by indirect contact with contaminated water environments. This zoonosis is present worldwide, with the highest rates in tropical areas. In New Zealand (NZ), the incidence is higher than in other temperate countries (Thornley et al., 2002). NZ's relative geographical isolation and the presence of only introduced terrestrial mammals—apart from native bats—make a unique ecological context. Of the multitude of existing serovars, only six have been isolated in animals in the country (Marshall and Manktelow, 2002). Those serovars are *Leptospira borgpetersenii* svs Hardjobovis, Ballum, Balcanica and Tarassovi and *L. interrogans* sv Pomona and Copenhageni. On the other hand, *L. interrogans* svs Australis and Canicola have been isolated from humans only and are thus not considered as endemic (Midwinter and Fairley, 1999). The disease mostly impacts the agricultural sector, with two-thirds of the notified cases in 2015 and 2016 being farm or meat workers (ESR 2016, 2017). Indeed, exposure to livestock urine is an important risk factor, and implementation of vaccination in dairy cattle and pig against Hardjobovis and Pomona, the serovars they maintain, along with increased awareness and use of personal protective equipment, has led to a decrease in the number of cases (Marshall and Chereshsky, 1996).

However, recent epidemiological findings question the role of livestock as the predominant source of leptospirosis. The incidence of human cases linked to Ballum, a serovar maintained by mice, rats and hedgehogs, increased in the 1990s (Thornley et al., 2002). It was the second serovar incriminated in human cases notified in NZ between 2010 and 2015, just after sv Hardjobovis (El-Tras et al., 2018). Recent studies on livestock highlighted the possibility for vaccinated livestock to shed other serovars, including Ballum, albeit at a low rate (Yupiana, 2019). The possibility for this serovar to spillover from wild maintenance hosts to livestock has been proposed (Chapter 2,Chapter 3),

Studies on serovar Ballum in its wild hosts in NZ are rare (Chapter 2). Studies conducted in the 1970s showed that mice (*Mus musculus*), ship and brown rats (*Rattus rattus* and *R. norvegicus*) and hedgehogs (*Erinaceus europaeus*) could harbour this serovar (Brockie, 1977, Brockie and Till, 1977, Hathaway et al., 1981). In Chapter 3, the most recent estimates of prevalence of *Leptospira* infection in mice caught in a dairy and a beef and sheep farm were higher than the previous estimates 22 to 38% *vs.* 13% to 16% in (Hathaway et al., 1981, Brockie, 1977). The presence of 'silent shedders', that had

leptospires detected in their kidney but no detectable antibodies, lowered the apparent seroprevalence.

Those studies were all cross-sectional studies, giving single time-point estimates of prevalence. As is often the case with wildlife, the population size, a fundamental parameter in epidemiological studies, was not available. Relative trapping abundance indices were given (captures per 100 trap-nights), but do not offer estimates of population size as reliable as capture-recapture methods (Wiewel et al., 2009). In terms of pathogen pressure and risk of transmission, the variations in densities of reservoir hosts is as important to consider as the prevalence, as both can affect the level of environmental contamination or human (or livestock) contact rate and exposure (Plowright et al., 2017).

Although mice populations have well described cyclical dynamics of population growth followed by crashes, the dynamics of *Leptospira* in populations of wild mice have rarely been studied. Moreover, studies investigating mice densities in New Zealand have been focused on forest and scrub habitats, in the frame of conservation projects, and density estimates on farm habitats are rare (Chapter 2).

In this chapter, we estimated the temporal dynamics of mice densities in an NZ farm biotope previously identified as a high-risk site for leptospirosis, using spatially explicit capture-recapture (SECR) methods, and we described the dynamics of *Leptospira* infection in the mice population. Using the estimates of home range and capture probability obtained from the SECR models, we also investigated the potential effect of *Leptospira* infection on the fitness of mice, under the hypothesis that the fitness should not be impacted for a maintenance host, and the home range and capture probability should be similar for infected and non-infected animals.

Materials & Methods

Study sites

The study site selected for a 2-year follow-up was a dairy farm in the coastal Manawatū-Whanganui region where investigations on *Leptospira* infection had already been conducted on humans (Benschop et al., 2017) and livestock (Yupiana et al., 2019b). The farm spans 130 ha of lowland exotic grass pastures and is bounded by a commercial pine forest plantation on one side. The terrain is flat (average elevation: 20 m) with patches of recently cut (1 – 5 years) pine trees around pastures (Figure 4-1). The dairy herd is moved to a new paddock after every milking, *i.e.* twice daily on a rotation length

of 17.5 days, and the grass kept low (<15 cm) by grazing. Around pastures, grass or vegetation is not cropped as frequently and can reach up to 50 cm. Rodent control is irregularly done around farm buildings and wasn't implemented just before or during this study. Poison baiting operations (cyanide) targeting possums had been conducted in the neighbouring forest before this study began (August 2016), and were repeated once in the months preceding the last sampling session.

Animal Ethics

The present research was done in accordance with the New Zealand Animal Welfare Act 1999 and the Massey University Code of Ethical Conduct. The procedures done on animals were approved by the Massey University Animal Ethics Committee under the protocol 16/93.

Capture and Sampling

Four sessions of capture (A1 – A4) were organised in Autumn and Spring between Spring 2016 and Autumn 2018. Session A2 (Autumn 2017) was described in detail in Chapter 3. At each session Longworth small mammal live-traps (Penlon Ltd., Oxford, UK) were set on two irregular grids of 36 traps with approximately 10 m spacing and their GPS position was recorded. Due to the risk of traps being trampled by livestock or flooded during heavy rain periods, the grids were set outside pastures around the palm kernel and milking sheds (Grid 1) and on a slightly elevated patch of cut pine trees (Grid 2). Grids were approximately 175 m apart and separated by pasture with a drain running in the middle (Figure 4-1). These traps were set for 10 to 12 nights per session, baited with peanut butter and pieces of apple covered with a mix of sugar and cinnamon and checked every morning.

Figure 4-1 | Aerial map of farm study site and trap layout. *Farm boundaries appear in white and pasture drain is highlighted in blue*

Table 4-1 | Numbers of mice trapped and sampled per session and grid.

Trapping session	Season	Grid	Session	Trap nights	Detections	Animals	Adults	Juveniles	Phase				MAT	Culture and/or PCR
									I only	II only	both	not sampled		
A1	Spring 2016	1	1	360	19	15	9	6	6	7	2	5	8	10
		2	2	360	22	17	14	3	2	12	3	3	14	13
A2	Autumn 2017	1	3	360	95	55	37	18	16	25	14	16	34	39
		2	4	360	82	48	17	31	8	24	16	8	39	40
A3	Spring 2017	1	5	432	12	11	9	2	1	9	1	1	10	10
		2	6	432	27	14	11	3	3	5	6	2	12	12
A4	Autumn 2018	1	7	432	45	37	26	11	3	30	4	1	35	36
		2	8	432	43	34	19	15	5	22	7	3	31	31
Total				3168	345	231	142	89	44	134	53	39	183	191

For each session, during the first 5 nights of trapping (Phase I – CMR), mice were anaesthetised using isoflurane (Attane, Bayer) insufflated in a plastic bag, ear-tagged (Mouse eartag Style 1005-1, National Band & Tag Company, Newport, KY, USA), weighed and released. The subsequent 5 to 7 nights of trapping (Phase II – Removal) captured or recaptured mice were anaesthetized, blood-sampled and euthanized to retrieve serum, and kidneys for *Leptospira* serology, PCR, histology, culture and subsequent genomic analysis of isolates (see Chapter 3 and Chapter 5 for more details). The first two sessions, urine samples were also collected opportunistically when animals urinated during handling. In both phases, weight, sex, age and an estimation of the reproductive status (immature/active) were recorded. In phase II, other body measurements (tail length, total body length and hind leg length) were also noted. We used a 50 g Pesola scale and weighed mice to the nearest 0.5 g, a 40 cm ruler to measure the tail and total length to the nearest mm, and a calliper to measure the hind leg to the nearest 0.1 mm.

Leptospira infection status determination

Laboratory analyses are described in detail in Chapter 3. The presence of antibodies against *Leptospira* spp. was tested using the Microscopic Agglutination Test (MAT) as described in Chapter 3, with a panel representing all serogroups known to circulate in animals in NZ: *Leptospira borgpetersenii* svs Hardjobovis, Ballum and Tarassovi and *Leptospira interrogans* svs Pomona and Copenhageni. The direct presence of *Leptospira* spp. in the kidneys was tested by culture in 3 serial dilutions of EMJH + 5-fluorouracil (first two sessions), later replaced by a unique mix of EMJH+STAFF (Sulfamethoxazole, Trimethoprim, Amphotericin B, Fosfomycin, and 5-Fluorouracil; Chakraborty et al., 2011) to avoid contamination (two last sessions). Cultures were checked weekly to fortnightly under the dark field microscope for 14 weeks. In some instances, leptospires were visible but could not be isolated. Cultures were considered positive for those animals. The presence of pathogenic *Leptospira* spp. DNA was tested by real-time PCR targeting the *lipL32* gene as described by Stoddard, Galloway et al. (2013, 2015).

Mice with a titre of 48 or higher for Ballum were considered **seropositive**. As continuous excretion of leptospires in the urine has been described in mice experimentally infected by Ballum (Soupé-Gilbert et al., 2017), all mice with a positive culture and/or PCR were considered to be **shedding** leptospires. Finally, all mice positive with at least one method were considered to have been **exposed** to *Leptospira*. Exact confidence intervals of observed prevalence and seroprevalence were computed based on the binomial distribution (Dohoo et al., 2009).

Sympatric wild mammals

Other species of animals were concurrently trapped and sampled on the farm. The same samples and laboratory analyses were conducted. Traps used were 45 Tomahawk 202.5 collapsible live-traps (Tomahawk Live Trap, Hazelhurst, WI, USA) targeting ship rats (spacing = 20 – 25 m), and 36 Havahart #1099 live-traps (Woodstream Corp., Lititz, PA, USA) targeting possums and hedgehogs (spacing = 50 m). The layout of those traps (Figure 4-1) covered Grid 1 and Grid 2 and extended over the farm buildings, the edge of the pastures and into the neighbouring forest. Except for rats that had a gaseous anaesthesia like mice, animals were anaesthetised by intramuscular injection of a mix of medetomidine (Domitor, Zoetis NZ Ltd, 50 to 150 µg/kg) and ketamine (Phoenix pharm, 5 to 10 mg/kg) and euthanized while sedated with pentobarbital (Pentobarb 300, Provet, 150 mg/kg, intracardial injection).

Sampling bias

Since some animals caught in phase I and released were not caught in phase II and therefore not tested, we also checked for the possibility of a selection bias in the time of sampling for *Leptospira* infection status. We used χ^2 test (or Fischer's exact test when expected frequencies were insufficient) to test for an association in age or sex of animals captured in phase I only, phase II only or both, and a t-test to check if serological or shedding status influenced the first day of capture.

SECR modelling

Densities of mice were estimated using spatially explicit capture-recapture (SECR) models using R version 3.4.2 and package secr version 3.2.0 (Efford, 2019b 2235). Borchers & Efford (2008) gave a detailed description of the statistical methods on which these models rely. Briefly, the population of mice living in the trapping area was described as a Poisson spatial distribution of points that represent the individual home range centres, and were assumed to be fixed. Each mouse had a declining probability of capture in a given trap when the distance between the trap and its home range centre increased. To account for the removal of mice from the population, mice sampled in phase II (as well as seven accidental deaths or euthanasias during phase I) were assigned known capture histories of 0 with probability equals 1 following removal.

The capture function was described as a half normal curve with two parameters, g_0 (probability to detect a mouse when home range centre and trap coincide), and σ (sigma, spatial scale over which this probability declines). The density D was derived

from the capture and distribution functions (Borchers and Efford, 2008). As in Russell et al. (2012), the parameter σ was interpreted as a proxy of the distance an animal moves from its home range centre, and, derived from it, the 95% circular probability density area of capture A (where $A = \pi [2.45 \sigma]^2$) gave an indication of the home range.

We treated each grid (Grid 1, Grid 2) within trapping session (A1 – A4) as a distinct 'session' (*i.e.* sessions 1 – 8) and used as session covariates the season, year, grid, and 'trapping session' number (*i.e.* A1 – A4). Conditional likelihood methods allow inclusion of individual covariates, so we also included the age (adult/juvenile) and sex of all individuals. Furthermore, because the information on serological and/or shedding status of mice was missing for some individuals, hybrid mixture models were used to estimate *Leptospira* status differences in mice detection. These mixture models combine known classes (*e.g.* session or age) and a latent class, hence the name hybrid. They include a mixing proportion parameter, 'pmix', that was allowed to vary between trapping sessions, and that represented the proportion of either seropositive or shedding mice in the population (Efford, 2019a). To build the models, we first checked for between session effects of year, season, grid and trapping session. Except for trapping session and grid, and season and year, these session covariates were included in the models exclusively of other session covariates. We retained only the session and year covariates for the subsequent models. We then investigated individual covariates and included the best models' parameters in the hybrid mixture models. Models were compared (when comparable) using an AIC framework (Borchers and Efford, 2008).

Results

Across all sessions, 231 different mice were caught for a total of 345 captures (Table 4-1). Forty-four mice were caught in phase I only. Of them, 12 were sampled: four died during anaesthesia and had all laboratory tests done, one had serology only, two were found dead in traps and had culture and PCR done but no serology, and four only had a urine sample submitted to culture. One mouse was preyed upon in the trap by a weasel in Spring 2017, and was counted in the capture history, but sex and *Leptospira* status could not be investigated. Analyses were run without this individual. Morphological summaries are given for adult mice in Table 4-2.

Female and male mice and juvenile and adult mice were as likely to be caught in phase I only, in phase II only, or in both (respectively $\chi^2 = 0.62$, df = 2, p-value = 0.73 for sex and $\chi^2 = 3.05$, df = 2, p-value = 0.22 for age). The number of mice tested for serology, PCR and culture is displayed in Table 4-1 and their results in Table 4-3. All PCR assays with positive amplification had a cycle threshold value < 37. All seropositive mice were

positive for Ballum, except for one mouse that had a titre of 96 for Hardjobovis, but no detectable antibodies against Ballum. This mouse, which also had a negative PCR and culture, was considered as seronegative in the subsequent analyses. Several mice also had titres against other serovars (Table 4-3).

Table 4-2 | Average weight and body measurements of trapped mice (± SD)

	Sex	#	Weight (g)		Body length (mm)		Tail length (mm)		Hindleg length (mm)	
Adult	F	52	15.2	±2.6	81.8	±5.2	80.6	±4.8	17.0	±1.7
	M	89	14.7	±2.2	80.3	±5.2	81.2	±5.0	17.4	±0.5
Juvenile	F	31	9.6	±1.3	70.0	±7.0	72.3	±5.1	16.9	±0.5
	M	58	9.3	±1.4	69.7	±4.9	70.6	±5.8	16.6	±0.8

Across all sessions, the proportions of seropositive and shedding mice were significantly different in juvenile and adult mice. Adults were more likely to be seropositive (52/112 adults *vs.* 16/71 juveniles, $\chi^2 = 9.63$, df = 1, p-value = 0.0019) and to be shedders (69/118 adults *vs.* 26/73 juveniles, $\chi^2 = 8.53$, df = 1, p-value = 0.0035). No significant difference between sex was found among seropositive (23/62 females *vs.* 45/121 males, $\chi^2 = 7.71\ e^{-31}$, df = 1, p-value = 1) or shedding mice (32/66 females *vs.* 63/125 males, $\chi^2 = 0.0099$, df = 1, p-value = 0.92). Seroprevalence in animals captured in both phases (50% [24/48]) was significantly different from seroprevalence in animals captured in phase II only (32% [42/130], $\chi^2 = 4.70$, df = 1, p-value = 0.030) is the number of re-captures.

No significant difference was found in time of first capture—*i.e.* the number of days since the beginning of the session—for adult *vs.* juvenile mice, or male *vs.* female, or *Leptospira* shedders *vs.* non-shedders (respectively t = – 0.76 , df = 201.05, p-value = 0.45 for age; t = – 0.14, df = 174.43, p-value = 0.89 for sex; t = 0.23, df = 188.77, p-value = 0.82 for shedding). There was no significant difference in time of first capture for mice captured only in phase I, or in both phases (two-sample t = -0.43, df = 94.08, p-value = 0.67). There was a small but significant difference in the mean time of first capture for seropositive ($\overline{m}$ = 5.4 nights, SD = 2.7) and seronegative ($\overline{m}$ = 6.6 nights, SD = 2.7) mice (two-sample t = – 2.91, df = 140.16, p-value = 0.0042), with seronegative mice being first captured on average a day later than seropositive mice.

Most of the non-shedding mice were captured during autumn sessions, and when both shedding and serological status were known, about a third (33/92 or 36%) of the shedding animals were 'silent shedders' (*i.e.* had no detectable antibodies) (Figure 4-2 and Appendix 6, Table S 6-1). This proportion was as high as 70% for juvenile mice in both autumn sessions (respectively 7/10 in 2017 and 5/7 in 2018).

Table 4-3 | Session prevalence and seroprevalence assessed by Culture, PCR and MAT in mice and other species sampled. *When numbers sampled were ≥ 5 the % and [95% CI] are indicated. Data from session A2 was detailed in Chapter 3*

		#	Shedding status		Microscopic Agglutination Test (MAT)					
	Species		**Culture**	**PCR**	**Hardjobovis**	**Pomona**	**Ballum**	**Tarassovi**	**Copenhageni**	**Overall**
A1 – Spring 2016	Feral cat	2	0/1	**1/1**	0/2	0/2	0/2	0/2	0/2	0/2
	European hedgehog	4	0/4	**¼**	**2/4**	0/4	**3/4**	**2/4**	**1/4**	**4/4**
	House mouse	24	**10/24 42% [22, 63]**	**19/23 83% [61, 95]**	**7/22 32% [14, 55]**	**1/22 5% [0, 23]**	**12/22 55% [32, 76]**	**3/22 14% [3, 35]**	**2/22 9% [1, 29]**	**13/22 59% [36, 79]**
	Ship rat	1	0/1	0/1	**1/1**	0/1	0/1	0/1	0/1	**1/1**
A2 – Autumn 2017	Feral cat	1	0/0	0/0	0/1	0/1	0/1	0/1	0/1	0/1
	European hedgehog	8	**3/8 37% [9, 76]**	**3/8 37% [9, 76]**	**4/8 50% [16, 84]**	**2/8 25% [3, 65]**	**4/8 50% [16, 84]**	**1/8 13% [0, 53]**	0/8 0% [0, 37]	**6/8 75% [35, 97]**
	House mouse	74	**22/61 36% [24, 49]**	**23/74 31% [21, 43]**	0/73 0% [0, 5]	0/73 0% [0, 5]	**18/73 25% [15, 36]**	0/73 0% [0, 5]	**1/73 1% [0, 7]**	**18/73 25% [15, 36]**
	Ship rat	3	**1/3**	**1/3**	0/3	0/3	**1/3**	0/3	0/3	**1/3**
A3 – Spring 2017	European hedgehog	5	**3/5 60% [15, 95]**	**4/5 80% [28, 99]**	**1/5 20% [1, 72]**	**1/5 20% [1, 72]**	**2/5 40% [5, 85]**	**1/5 20% [1, 72]**	**2/5 40% [5, 85]**	**4/5 80% [28, 99]**
	House mouse	22	**16/22 73% [50, 89]**	**19/22 86% [65, 97]**	**4/22 18% [5, 40]**	**1/22 5% [0, 23]**	**17/22 77% [55, 92]**	0/22 0% [0, 15]	**2/22 9% [1, 29]**	**17/22 77% [55, 92]**
	Weasel	2	0/2	0/2	0/2	0/2	**1/2**	0/2	0/2	**1/2**
	Ship rat	2	0/2	**2/2**	0/2	0/2	0/2	0/2	0/2	0/2
	Possum	1	**1/1**	**1/1**	**1/1**	0/1	0/1	0/1	0/1	**1/1**
A4 – Autumn 2018	Feral cat	4	0/3	0/3	0/4	0/4	0/4	0/4	0/4	0/4
	European hedgehog	14	**4/14 29% [8, 58]**	**5/14 36% [13, 65]**	**2/14 14% [2, 43]**	0/14 0% [0, 23]	**11/14 79% [49, 95]**	0/14 0% [0, 23]	**1/14 7% [0, 34]**	**11/14 79% [49, 95]**
	House mouse	67	**21/67 31% [21, 44]**	**25/67 37% [26, 50]**	**1/66 2% [0, 8]**	0/66 0% [0, 5]	**20/66 30% [20, 43]**	0/66 0% [0, 5]	**3/66 5% [1, 13]**	**21/66 32% [21, 44]**
	Ship rat	5	**1/5**	**1/5**	0/5	0/5	**2/5**	0/5	0/5	**2/5**
	Ferret	2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	**0/2**

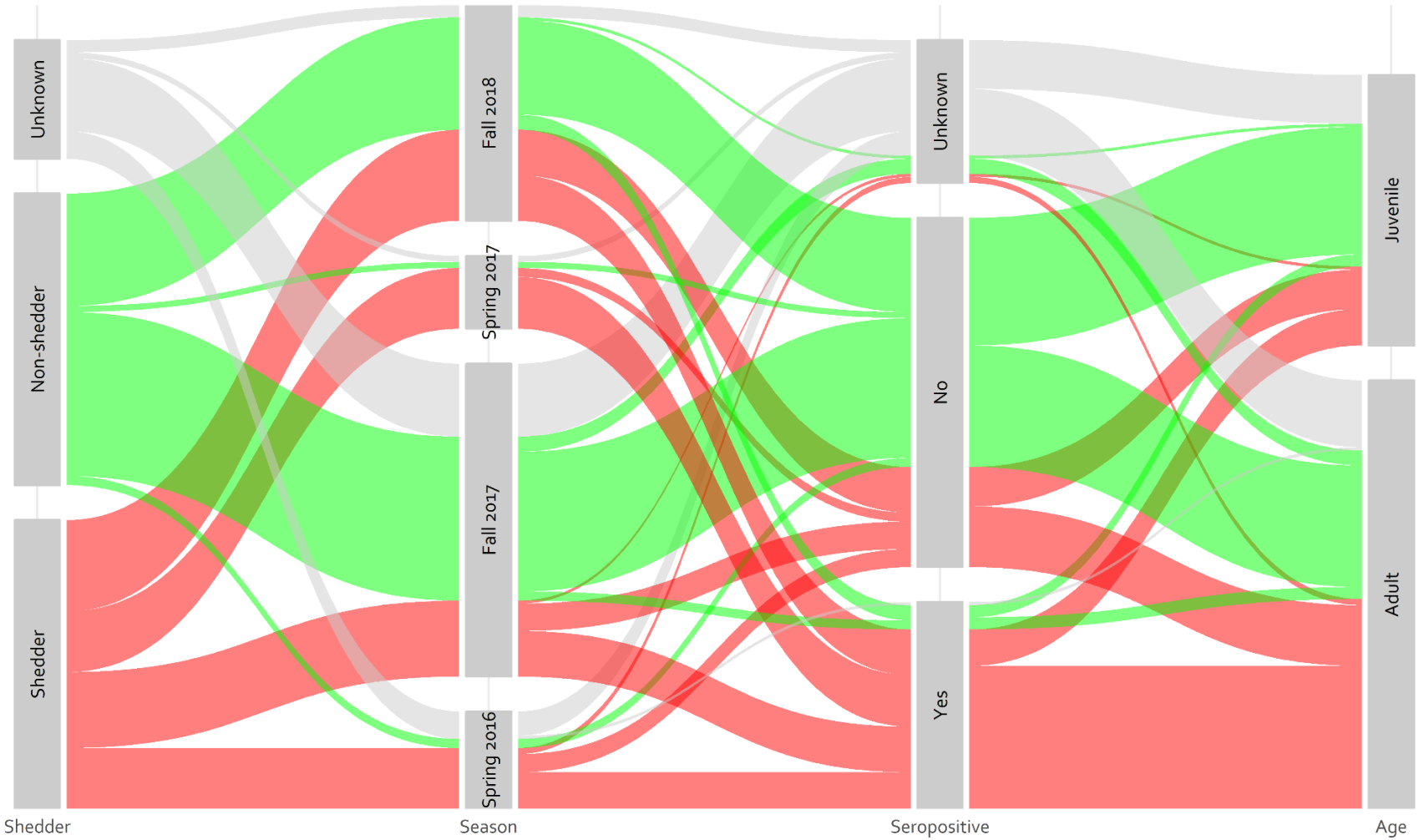

Figure 4-2 | Breakdown of mice captured by shedding status (PCR and/or Culture positive), season, serological status and age, shown as a parallel sets plot. *The band colouring highlights the proportion of shedders and non-shedders in the population studied.*

The spatially explicit capture-recapture model that had the greatest AICc weight was the model where the probability g_0 to detect a mouse when home range centre and trap coincide varied with the session and age and σ, the spatial scale over which this probability declines varied with the session (AICc weight = 29%), followed by the model where both g_0 and σ varied with session and age (AICc weight = 16%). A list of models ranking first is included in Appendix 6.The AICc weight of a model can be interpreted '*approximately*' as the probability, given the data, that this model is the best within the set of models compared (Burnham and Anderson, 2001). When hybrid mixture models were used to model detection including *Leptospira* seropositivity, the best model was where g_0 varied with the session and age, σ varied with the session and seropositivity status, and the mixing proportion varied by trapping session (AICc weight = 50%). It was followed by a similar model with g_0 also influenced by the seropositivity status (AICc weight = 24%). The best hybrid mixture model incorporating *Leptospira* shedding was also the one where g_0 varied with the session, age and shedding status, σ varied with the session and shedding status, and the mixing proportion varied by trapping session (AICc weight = 42%). Estimates of g_0, σ and the resulting densities and home ranges are presented for the latter (Table 4-4, Table 4-5, and Figure 4-3). Only one individual parameter with two levels can be included in hybrid mixture models in secr v3.2.0, so no models with both shedding and seropositivity status were built. Juvenile mice had a lower g_0 than adult mice. Mice shedding *Leptospira* had a lower g_0 but a higher σ, and similarly, mice with antibodies against *Leptospira* had a higher σ.

Table 4-4 | Estimates of g_0 (probability to detect a mouse when home range centre and trap coincide) by age, *Leptospira* shedding status, trapping grid and season.

Season	Grid	Age	*Leptospira*	g_0	lcl	ucl
Spring 2016	Grid 1	Adult	Non-shedder	0.036	0.009	0.127
			Shedder	0.016	0.005	0.051
		Juvenile	Non-shedder	0.015	0.004	0.056
			Shedder	0.007	0.002	0.022
	Grid 2	Adult	Non-shedder	0.012	0.003	0.051
			Shedder	0.005	0.001	0.02
		Juvenile	Non-shedder	0.005	0.001	0.022
			Shedder	0.002	0.001	0.009
Fall 2017	Grid 1	Adult	Non-shedder	0.128	0.072	0.216
			Shedder	0.061	0.034	0.108
		Juvenile	Non-shedder	0.056	0.029	0.105
			Shedder	0.026	0.013	0.051
	Grid 2	Adult	Non-shedder	0.146	0.086	0.236
			Shedder	0.07	0.035	0.138
		Juvenile	Non-shedder	0.064	0.037	0.109
			Shedder	0.03	0.014	0.062
Spring 2017	Grid 1	Adult	Non-shedder	0.014	0.002	0.106
			Shedder	0.006	0.001	0.052
		Juvenile	Non-shedder	0.006	0.001	0.048
			Shedder	0.002	0	0.023
	Grid 2	Adult	Non-shedder	0.241	0.096	0.487
			Shedder	0.124	0.058	0.245
		Juvenile	Non-shedder	0.114	0.042	0.273
			Shedder	0.054	0.024	0.117
Fall 2018	Grid 1	Adult	Non-shedder	0.044	0.018	0.104
			Shedder	0.02	0.007	0.052
		Juvenile	Non-shedder	0.018	0.007	0.047
			Shedder	0.008	0.003	0.024
	Grid 2	Adult	Non-shedder	0.038	0.015	0.089
			Shedder	0.017	0.006	0.044
		Juvenile	Non-shedder	0.015	0.006	0.039
			Shedder	0.007	0.002	0.02

lcl and ucl lower and upper 95% confidence limits, respectively,

Table 4-5 | Estimates of σ and resulting home range for mice according to their *Leptospira* shedding status, trapping grid and season.

Season	Grid	*Leptospira*	σ	lcl	ucl	A
Spring 2016	Grid 1	Non-shedder	15.56	6.16	39.31	0.46
		Shedder	36.93	16.81	81.13	2.57
	Grid 2	Non-shedder	32.31	7.76	134.56	1.97
		Shedder	76.68	19.05	308.62	11.09
Fall 2017	Grid 1	Non-shedder	9.02	6.12	13.30	0.15
		Shedder	21.41	16.50	27.78	0.86
	Grid 2	Non-shedder	8.33	6.68	10.39	0.13
		Shedder	19.77	11.54	33.88	0.74
Spring 2017	Grid 1	Non-shedder	23.25	6.86	78.80	1.02
		Shedder	55.18	14.79	205.89	5.74
	Grid 2	Non-shedder	9.37	4.74	18.50	0.17
		Shedder	22.24	13.59	36.39	0.93
Fall 2018	Grid 1	Non-shedder	12.11	7.32	20.03	0.28
		Shedder	28.74	14.33	57.64	1.56
	Grid 2	Non-shedder	14.69	8.72	24.75	0.41
		Shedder	34.87	17.16	70.87	2.29

lcl and ucl lower and upper 95% confidence limits, respectively, A 95% home-range probability density (hectares)

Although the variation of prevalence (Table 4-3) and densities (Figure 4-3) over time showed important changes, with prevalence varying between 31 and 86% and total densities varying between 3.6 and 56 mice/ha, the densities of shedding animals were more stable over time and varied between 3 and 8 shedding mice/ha (Figure 4-3).

Other species captured include feral cats (*Felis catus*), European hedgehogs (*Erinaceus europaeus*), ship rats (*Rattus rattus*), weasels (*Mustela nivalis*), ferrets (*M. putorius furo*) and a possum (*Trichosurus vulpecula*). Number sampled and results of the laboratory investigations are summarised in Table 4-3. Most of them were captured at the entrance of the neighbouring forest, although a weasel, a ferret and eight hedgehogs were captured within Grid 1 or just around, and several cats were captured on the side of the road in the vicinity of Grid 1. No other animals were captured on traps present in and around Grid 2, and Tomahawk and Havahart traps placed on this grid during trapping sessions A1 to A3 were moved to the forest for A4. The impact of weasels on captures in Grid 1 during A3 was conspicuous, with only two mice captured during phase I on that grid (including the one preyed on), and eight other individuals captured following the capture of the weasels on day 4 and 5.

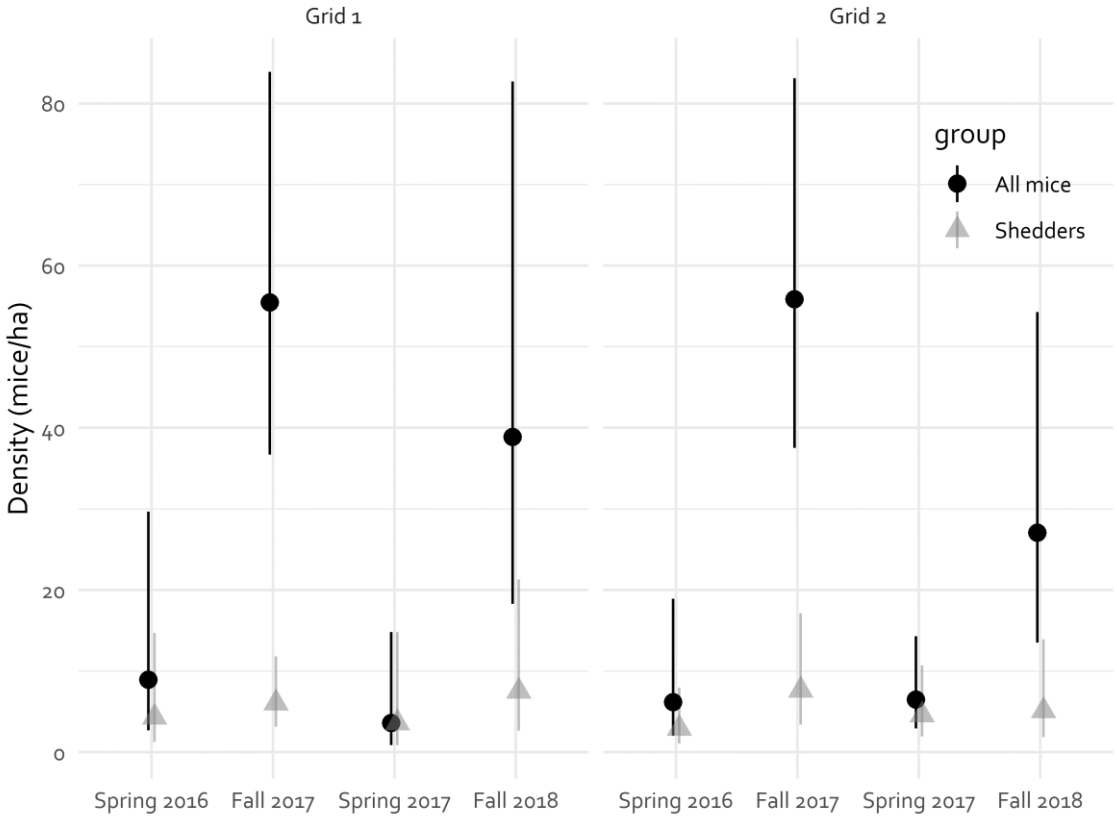

Figure 4-3 | Densities of all mice, and mice shedding *Leptospira* derived from the hybrid mixture SECR model. *Figures presented in this graph are available in Appendix 6, Table S 6-5.*

Discussion

There are few studies describing the prevalence of *Leptospira borgpetersenii* serovar Ballum in wild mice, their maintenance host, and even fewer describing its temporal dynamics, and the relationships between pathogen and population dynamics. Using a SECR framework, we found evidence of temporal heterogeneity in *Leptospira* prevalence within a single population of house mice in a dairy farm biotope. Moreover, we found that shedding or having antibodies against leptospires influenced the capture function.

As for many other rodent species, mice densities fluctuate cyclically and the shedding of leptospires into the environment is also expected to fluctuate according to the prevalence in a given population (Hathaway and Blackmore, 1981a). We demonstrated that on the contrary, despite variations in the demographic structure of the population, with a higher prevalence during spring sessions when the population is mostly composed of adults that survived after winter, the density of infected (shedding) animals remained relatively constant over time. This has several implications.

Firstly, if the number of shedders in the population represents a constant source of contamination for the environment, only the variations in the level of shedding, and subsequent survival of leptospires in the environment will affect the level of exposure and therefore the probability of spillover for other non-maintenance species (including humans). Experimental infections of mice showed that the quantity of Ballum shed increased the first two months after infection to reach a plateau, and then remained constant throughout their lifetime (Soupé-Gilbert et al., 2017). The heterogeneity in shedding levels of *Leptospira* in naturally infected populations of house mice in NZ remains to be studied. As described in Chapter 2, knowledge on the survival of Ballum in the environment is also fragmented and mostly inferred from in vitro studies on other serovars or theoretical inference from genomic analyses that give contradictory data. Leptospires of the species *L. borgpetersenii* have lost genes important in the survival outside of their host that is thus theoretically limited compared to other pathogenic leptospires like *L. interrogans* or saprophytic strains from which they diverged (Bulach et al., 2006, Thibeaux et al., 2018b). In contrast, in vitro studies of *L. borgpetersenii* isolates showed a long survival (up to 144 days) in controlled conditions (Addamiano, 1959). Acquiring more knowledge on the subject will be important to understand *Leptospira* epidemiology and ways to control this zoonosis. Indeed, if survival of Ballum in the environment is sustained, a precise knowledge of infection dynamics in mice will not be necessary to assess environmental exposure to *Leptospira* (Plowright et al., 2017). But if a variation in the levels of environmental shedding is more important to explain the quantity of leptospires present in the environment, knowledge of factors affecting the density of shedders and the individual level of shedding will be important to devise ways to limit this shedding.

The second implication that can be drawn from our results is that different control strategies of mice populations can also impact differently Ballum epidemiology. In terms of control of leptospirosis in the population, trapping or poisoning animals during winter or early spring, before densities are high and prevalence diluted by the recruitment of young uninfected individuals, will be more effective to decrease the prevalence and the density of infected animals. It will also be where the risk of direct contamination for people involved in trapping management is the highest, and measures like appropriate PPE should be applied to protect people in contact with mice, or with water potentially contaminated by mice. Hedgehogs and ship rats that were not the target species of this study were also shown to harbour Ballum and these precautions should be extended to work involving these species.

This study has shown that densities of mice in a farm environment were comparable with densities observed in other habitats, like forests and shrub (King and

Barrett, 2005). The *Leptospira* infection prevalence in mice was extremely high after the winter season, and this species therefore represents a risk for people and other animal species in direct or more commonly indirect contact with this ubiquitous species. Studies on *Leptospira* carriage in other habitats would be needed. In 2016, the NZ government put forth Predator Free 2050, a nation-wide plan to eradicate stoats, rats and possums from the country (Department of Conservation, 2016, Russell et al., 2015). Given the advantage gained by mice after removal of their predators and competitors (Caut et al., 2007, Goldwater et al., 2012), the risk that the density of *Leptospira* infected mice increases has to be taken into account in the future. This is all the more important as modelling of rodent population dynamics in forest habitats points out that mice will benefit more from the eradication of rats in warmer forests (Walker et al., 2019), where the survival of Ballum in the environment is also likely to be higher.

Mice being maintenance hosts for Ballum, we expected little impact on their fitness, and therefore no difference in their home range, but we found that on the contrary, seropositive and shedding mice had a larger home range than seronegative or non-shedding mice. These findings could be due to a true biological effect of *Leptospira*, to confounding by another un-measured factor, or to sampling and measurement biases.

Concerning the latter, one of the assumptions of SECR models is that the detection is homogeneous across animals and time. The determination of *Leptospira* status in this study required euthanasia of mice captured, and we had to choose a trade-off between CMR (phase I) and sampling (phase II). As a result, the status of most of the mice captured only in phase I remained unknown, and mice captured only in phase II were removed from the population at the first capture. However, we captured seropositive mice on average one day earlier than seronegative ones. There is therefore a possible bias in the assessment of seroprevalence and density modelling. Mice not sampled in phase I could have a higher proportion of seropositive than the rest of the animals sampled, and mice sampled during phase II when the movement of animals between traps was not being assessed could have a higher proportion of seronegative, with a negative impact on σ in that group. Despite this, we found no difference in sex, age, and first day of capture between animals captured in phase I only and animals captured in both. We would expect no difference in their serological status either. In other words, mice captured in both phases—and therefore sampled—are expected to be a representative sample of mice captured in the first phase (phase I only + both phases).

The main difference between animals captured in both phases (seroprevalence = 50%) or in phase II only (seroprevalence = 32%) is the number of re-captures. It is possible that the handling stress during the first phase elicited an enhanced immune response, and a better detection of antibodies in samples taken the following days,

especially for animals with a titre initially just below the detection threshold (48). The field of 'wild immunology' is a recent discipline (Pedersen and Babayan, 2011) and there is a dearth of information on the impact of captures on immune parameters, but it is likely this impact exists, and remains to be quantified (Pedersen and Babayan, 2011, Abolins et al., 2018). It is interesting to note that, like Byers et al. (unpublished, cited in Minter et al., 2019) in brown rats infected with *L. interrogans*, we found no significant association between infection status (*i.e.* shedding) and first day of capture.

On another hand, confounding factors could explain the association we found between *Leptospira* infection and variation in capture probability and home range. For instance, animals with a larger home range are more likely to be in contact with a contaminated environment and to encounter traps. Similarly, behavioural components like 'boldness', 'wariness' or a more aggressive nature could interact with both the probability to get infected and be captured, as illustrated by the association between the presence of wounds and *Leptospira* status described in brown rats (Minter et al., 2019, Himsworth et al., 2013). Lee et al. (2018) found that culling rats increased the odds that surviving rats carried *L. interrogans*, and considered that culling could destabilize family groups and lead to enhanced opportunities of disease transmission by an increased number of fights and resulting wounds. We did not collect information on the presence of wounds on the mice we trapped, nor test for an effect of removal on prevalence. Our results show that, on the other hand, a difference in trappability could also bias early estimates of prevalence, especially if trapping sessions are too short to encompass the heterogeneity in capture probabilities in different groups (note that the two notions are not incompatible: both a difference in trappability and culling can influence the assessment of prevalence in a population). The long timespan of capture in our study (10 to 12 nights) allowed encompassing this heterogeneity, but studies investigating prevalence in wild mice are usually shorter. In Europe, the APHAEA project (harmonised Approaches in monitoring wildlife Population Health, And Ecology and Abundance, https://aphaea.org/cards/species/voles) recommends 4 to 5 days of capture to estimate population densities of wild mice. Studies assessing prevalence and seroprevalence in wild rodents should take into account the possibility of a sampling bias.

Mouse capture probability and ranging behaviour varied widely in our study with no clear spatio-temporal (*i.e.* grid or season) patterns. External factors like the presence of predators, the change over time of vegetation cover or weather conditions were not included in the modelling but could also impact the detection of mice. The presence of a weasel on Grid 1 during the Spring 2017 capture session impacted the number of captures and recaptures, but not the number of mice present, as almost as many mice were captured in Grid 1 as in Grid 2 after capture and removal of the weasel. The concept of

'landscape of fear' has been used to describe this change in behaviour in the presence of predators or competitors (Mahlaba et al., 2017). The span of the study was limited and the vegetation cover remained relatively similar over sessions, but weather conditions changed drastically. While the last session in March 2018 was conducted at the end of a particularly dry summer, with water restrictions in place as early as December 2017, the Autumn 2017 session took place during cyclone Debbie, with a heavy rain every day during the captures and the lower parts of the pastures flooded in several places. Those variations could influence our results.

The laboratory techniques used to determine the serological or shedding status of animals have an imperfect sensitivity, and misclassification biases could impact the model results. Sensitivity of the laboratory tests conducted on mice during Autumn 2017 were estimated to be respectively 64%, 95% CI [45, 79%] for MAT, 88% [69, 96%] for PCR and 74% [55, 87%] for culture (Chapter 3), and uncertainty on those parameters was not included in the models in the present study. Although some CMR models like multi-state and multi-event capture recapture models can deal with imperfect observations and uncertainty in the disease status (Conn and Cooch, 2009, Buzdugan et al., 2017, Robardet et al., 2017), it is not the case with SECR models to date, and the nature and extent of the effect of misclassification on the model outputs remain to be assessed. However, those estimates are based on the assumption that tests sensitivities are fixed for all animals, and do not take into account individual heterogeneity. The timing of infection and the level of individual immune response or the burden of infection and quantity of *Leptospira* present in the kidneys indeed impact the tests ability to detect the bacteria, its DNA or antibodies against it. There were several seropositive non-shedders and 'silent shedders' detected during the study, and it is possible some individuals wrongly classified as negative were actually exposed to *Leptospira*, with prevalence thus being underestimated. Investigations are underway to model the sensitivity of laboratory tests as a function of shedding levels. But if mice with low levels of bacteria shedding are less likely to be detected, they are also less likely to play an important role in the transmission of *Leptospira*, so the non-detection of low-shedding mice is relatively unimportant in the context of this chapter.

The population of invasive mice sampled in this study is not a sample representative of mice in other parts of their wide range worldwide. They are descendants of mice introduced from various locations since the end of the 18th century, in majority Europe and Asia (Veale et al., 2018). The genetic diversity of this population is therefore limited compared to other populations in their original range, and the diversity of pathogens they harbour is also likely to be limited. This has several implications in terms of host-pathogen interactions and disease transmission. Within-

host pathogens interactions that impact—negatively or positively—the fitness of the host have been described in other rodent species (Telfer et al., 2010, Bordes and Morand, 2011). One of the hypotheses explaining the success of rodents as invasive species is the parasite release hypothesis (Morand et al., 2015). The apparent lack of impact of Ballum on mice in this study therefore does not necessarily apply elsewhere. The absence of a pathogen in the population can make individuals more resistant to others, and it could be the case in NZ.

“Give a small boy a hammer, and he will find that everything he encounters needs pounding”

— Abraham Kaplan, *The law of the instrument*

Chapter 5

Can sequencing methods help us decipher transmission pathways of *Leptospira* spp. within a community of hosts?

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Chapter 5.
Sequencing methods

Abstract

Methods like amplicon sequencing, Multi-Locus Sequence Typing (MLST) or whole genome sequencing (WGS) allow a deeper understanding of *Leptospira* diversity and evolution than serotyping. We applied all of these methods to investigate reservoir dynamics in farm environments, progressively incrementing the theoretical resolution of isolate classification by increasing the total proportion of the organism's genome included in the analysis.

Kidneys from wild mammals captured in two farms, and urine from sympatric livestock were sampled for culture and PCR. Samples were initially screened using a *lipL32* PCR. Those positive without an isolate were screened with a second PCR, targeting either the *glmU* (differentiates all known endemic strains) or *gyrB* gene (all but two), and amplicons sequenced. *GlmU* and *gyrB* sequences were aligned in Geneious and assigned to a species and associated serovar.

Genomic DNA was extracted and sequenced from 81 isolates using Illumina HiSeq™. Raw read data were assembled *de novo*, and assembled draft genomes were used to classify each isolate by different genetic typing methodologies. These included i) three recognised MLST schemes, ii) a core-genome MLST (cgMLST) scheme, and iii) single nucleotide polymorphism (SNP) analysis from core gene components. Genomes were compared with similar genomes available in public repositories and local unpublished isolates.

Four genotypes were detected by *glmU*/*gyrB*, MLST and cgMLST with total concordance between methods: *Leptospira borgpetersenii* genotype Ballum in mice, hedgehogs, rats and cattle, genotype Hardjobovis in cattle and genotype Balcanica (possum) in brush-tailed possum and red deer; and *L. interrogans* genotype Pomona in cattle and hedgehogs. The intra-genotype diversity was too low for source attribution. For instance, among the 73 Ballum isolates, the SNP analysis showed 0 to 6 SNPs despite covering > 95% of the genome. Ballum strains were most similar to an isolate from the Netherlands, in agreement with the European origin of invasive mice in New Zealand.

Sequencing methods are a valuable addition to serotyping for eco-epidemiological studies but are limited by within-genotype low variation and a low

sensitivity. They are currently dependent on successful isolation, and therefore better isolation and enrichment methods would improve how genomics could be applied to leptospirosis research.

Introduction

Contrary to many countries in the world, leptospirosis in NZ is traditionally associated with livestock rather than rodents. It was first described as "dairy farm fever" in the 1950s and is indeed the most common occupational zoonosis in the country, with more than two-thirds of the notified cases still being farm or meat workers (ESR 2019). The situation is evolving, and invasive wildlife have been identified as a source of infection for people and livestock (Chapter 2 and Chapter 3).

Diagnosis and epidemiosurveillance of this pathogen in human and animal populations has historically relied on serological typing. Until the end of the 1980s, the classification of leptospires was based on (i) their pathogenicity, with two species described (pathogenic *L. interrogans vs.* saprophytic *L. biflexa*), and (ii) their antigenic serological properties, with more than 300 serovars (sv) and 30 serogroups described (Levett, 2001). The microscopic agglutination test (MAT) has been, and still remains a reference test for serology (World Organisation for Animal Health (OIE), 2014). However, this test has some limitations that hinder its use as a diagnosis as well as a typing method. As all serological methods, this test relies on the host serological response. While leptospiraemia occurs early in the incubation phase, the presence of detectable antibodies in the blood only occurs after several days of infection. Chronically infected individuals—animals or humans—can also shed leptospires with low or undetectable concentrations of antibodies to the infecting serovar (Velasco-Castrejón et al., 2009, Libonati et al., 2017), and even at an acute stage of infection, an early course of antibiotics can reduce the production of antibodies (World Health Organization (WHO), 2003, Gerritsen et al., 1993), hindering both *Leptospira* detection and typing. When interpreting a positive MAT, cross-reactions within and even between serogroups are the main issue but not the only one. Paradoxical reactions (serological response to a completely different organism, or to a previously encountered serovar rather than the infecting one) are also described (Levett, 2001). Vaccinal or maternal antibodies can also further complicate the interpretation of results. For these reasons, and the complexities of maintaining live bacterial cultures for MAT testing, molecular techniques are becoming favoured for leptospirosis diagnosis. The isolation of leptospires from the blood, urine or tissues and subsequent typing is the ultimate way to identify and classify the infecting serovar

Alternatively, molecular classification relies on genetic heterogeneity between strains. DNA-DNA hybridization was until recently the recommended method for the determination of a species and is increasingly being replaced by *in silico* comparison methods—*e.g.* average nucleotide identity, genome-to-genome distance—based on genetic or genomic data (International Leptospirosis Society (ILS) – Taxonomic Subcommittee, 2018). Under this genotypic classification, there are—at the time of writing—65 described genomospecies in the genus *Leptospira* and this number is expected to rapidly increase in the future (Thibeaux et al., 2018a, Vincent et al., 2019). There are two distinct clades, saprophytes and pathogens, with two sub-clades in each clade, the former pathogenic (P1) and intermediate (P2) groups being the two subclades in the pathogen clade (Vincent et al., 2019). Of this wide variety of species and serovars described worldwide, only two species and eight serovars, from seven serogroups, have been isolated in NZ: *Leptospira borgpetersenii* svs Hardjobovis, Ballum, Balcanica and Tarassovi and *L. interrogans* svs Pomona, Copenhageni, Australis and Canicola. Australis and Canicola have been isolated exclusively from humans (Midwinter and Fairley, 1999).

Typing, by MAT (at the serogroup level) or cross-agglutination absorption test (CAAT, at the serovar level), has increasingly been replaced by direct methods targeting the bacterial DNA rather than the host antigenic response. One of the first methods, developed in NZ at the beginning of the 1980s, was BRENDA (Bacterial Restriction Endo-Nuclease DNA Analysis, also known as REA) and it allowed for the differentiation of *L. interrogans* sv Hardjoprajitno (exotic to NZ), *L. borgpetersenii* svs Hardjobovis and Balcanica, otherwise undistinguishable by MAT (Robinson et al., 1982). Other methods like RFLP (restriction fragment length polymorphism) or PFGE (Pulse-Field Gel Electrophoresis) were subsequently used but depend on the successful isolation of leptospires (Herrmann et al., 1992, Corney and Colley, 1996, Zuerner et al., 1993). With the uptake of PCR methods, culture independent techniques like DNA barcoding, VNTR (variable number tandem repeat), multispacer sequence typing or MLST have been used to type *Leptospira* strains isolated or detected in biological or environmental samples (Ayral et al., 2015, Li et al., 2013, Guernier et al., 2018, Cosson et al., 2014). With the development of WGS, methods covering a larger portion of the genome, such as core genome MLST will probably replace other methods relying on a single or small number of genes (Guglielmini et al., 2019).

So far, genomic methods have been applied to typing strains and comparing genomes from different species (Fouts et al., 2016, Xu et al., 2016) or serovars (Santos et al., 2018). There is, to our knowledge, only one published study investigating closely related strains using WGS. In that study, Llanes et al. (2018) investigated genetic variation between field and laboratory-adapted isolates of *L. borgpetersenii* sv

Hardjobovis with a focus on gene mutations and their potential impact on associated proteins. However, having access to whole genome data should, in theory, allow for an in-depth comparison and study of the bacterial population structure and diversity over space and time. In such analyses, the advantage of genomics in a public health setting is that minute variations in genomic content can lead to a more complete reconstruction of the evolutionary history of the organism (Grad and Lipsitch, 2014), mutations then allow infection transfer chains to be decrypted and sources of infection to be better defined.

The aim of this chapter was to type *Leptospira* strains detected previously in different species of sympatric animals (Chapter 3, Chapter 4) over two years, and to use comparative genetic and genomic techniques to explore associations, and likely evolutionary histories of similar *Leptospira* isolates both from this study, and from data available in public repositories. One simple hypothesis that we tested was that the population of strains isolated from different locations would show more genetic divergence than strains isolated from the same place. We also hypothesized that strains found in different host species would be more variable than strains from the same host species.

Materials & Methods

Origin of samples

The strains of *Leptospira* characterised in this chapter originate from kidneys and urine sampled from wild animals and sympatric livestock in two different farms in the Manawatū-Whanganui region, North Island of New Zealand (Chapter 3, Chapter 4). For wildlife, sampling sessions took place biannually in spring and autumn in a dairy farm (Farm A) between October 2016 and March 2018, (*i.e.* four sampling sessions) and a unique sampling session was organised in March 2017 in a beef & sheep farm (Farm B). The linear distance between the farms is 76 km, and Massey University Turitea campus is roughly midway between the two farms. Traps targeting mice (*Mus musculus*), ship rats (*Rattus rattus*), hedgehogs (*Erinaceus europaeus*) and possums (*Trichosurus vulpecula*) were set near pastures where livestock could not trample them. Wild animals were euthanized and urine and/or a kidney sampled for PCR and culture. Detailed procedures are presented elsewhere (Chapter 3, Chapter 4). Livestock (sheep and cattle) urine samples were collected between March and May 2017 on both farms and again on Farm A in March 2018 (Moinet et al., 2019). Ten kidneys from 2-year old beef cattle from Farm B sent to the slaughterhouse were sampled in May 2017. In October 2017, the veterinarian

from Farm A advised us that an abortion storm due to leptospirosis was happening in a neighbouring beef farm (Farm A'). We also sampled 20 beef kidneys from that farm at the slaughterhouse but no captures of wild animals were conducted there. The species sampled are detailed in Table 5-1. In Chapter 3 and Chapter 4, the presence of *Leptospira* spp. in urine or kidneys had been assessed using a real-time PCR targeting the *lipL32* gene and culture. Only animals with a positive culture and/or *lipL32* PCR were included in the present chapter.

Table 5-1 | Sampling sessions and numbers of animals sampled and analysed

Session	Dates	Farm	Medium	Species	Sampled	*lipL32* PCR+	*glmU* PCR+	With isolate(s)
WA1	Oct-Nov-16	A	Kidney	Hedgehog	4	1	0/1	0
			±Urine	Mouse	23	19	5/11	9*
				Rat	1	0	\	0
				Feral cat	1	1	0/1	0
WB	Mar-17	B	Kidney	Hedgehog	1	0	\	0
			±Urine	Mouse	33	6	1/3	5*
				Rat	11	1	0/1	0
				Possum	12 (12P/10C)‡	0	\	0
WA2	Mar-Apr-17	A	Kidney	Hedgehog	8	3	\	3
			±Urine	Rat	3	1	\	1
				Mouse	79 (74P/61C)‡	23	7/11	13*
LB	Mar-May-17	B	Urine	Sheep	11	\	\	#
				Beef cattle	5	\	\	#
	May-17	B	Kidney	Beef cattle	10[&]	4[&]	4[&]	0
LA1	Mar-Apr-17	A	Urine	Dairy cattle	90	\	\	#
WA3	Sep-17	A	Kidney	Hedgehog	5	4	0/1	3
				Mouse	22	19	2/4	16
				Rat	2	2	0/2	0
				Possum	1	1	\	1
LA2	Sep-Oct-17	A+A'	Kidney	Dairy + Beef cattle	23	11	0/10	3*
WA4	Mar-18	A	Kidney	Hedgehog	14	5	1/4	3*
				Mouse	67	25	3/12	19**
				Rat	5	1	0/1	1
LA3	Mar-18	A	Urine	Dairy cattle	92[$]	2 + 4[$]	0/6	0
				TOTAL	523	129 + 4[&]	18/67 + 4[&]	77

** leptospire-shaped organisms observed by dark-field microscopy in 1 additional animal culture, but not isolated*

*** leptospire-shaped organisms observed by dark-field microscopy in 2 additional animal cultures, but not isolated*

‡ Numbers in brackets refer to the samples processed by PCR (P) and Culture (C) when different

[#] all cultures (medium EMJH + 5'F) were contaminated 5 weeks post-sampling, no leptospires observed by dark-field microscopy before contamination

[$] due to an extraction kit contaminated by Ballum DNA, only 24 urine samples had a PCR as per protocol. A PCR on boiled preparation on the 68 leftovers from urine pellets gave 4 late amplifications (37 < Ct < 42), those 4 samples were also submitted to the glmU PCR.

[&]samples processed with a gyrB PCR

\ Not applicable

Cultures and isolates

All cultures were processed on farm, except for livestock kidneys that were received and processed directly at the Molecular Epidemiology and Public Health Laboratory (^{m}EpiLab). Prior to September 2017, 0.5 mℓ of urine or kidney slurry (kidney aseptically mashed with an equivalent volume of PBS) was pipetted into a tube with 5 mℓ of EMJH + 5'-fluorouracil, and two subsequent serial dilutions (1:10) were made. Because of contamination issues, samples taken from September 2017 were inoculated in a single dilution of EMJH supplemented with 'STAFF' (Sulfamethoxazole, Trimethoprim, Amphotericin B, Fosfomycin, and 5-Fluorouracil; Chakraborty et al., 2011). Culture tubes were kept at ambient temperature and protected from sunlight in the field and subsequently placed at 28°C on a shaker as soon as they reached the ^{m}EpiLab and checked under the dark field microscope at least every two weeks for 14 weeks. Detailed culture procedures are presented in Chapter 3. When positive, 3 mℓ sub-cultures of *Leptospira* were grown to the densest possible culture into EMJH or EMJH + STAFF medium and harvested by centrifugation. The centrifugation pellet was stored at -20°C and subsequently resuspended in 80 µℓ of PBS and submitted to DNA purification.

DNA purification

We purified cultures following the Qiagen Spin-Column protocol (DNA mini kit). A volume of 100 µℓ ATL buffer and 20 µℓ Proteinase K were added to the sample and incubated at 56°C on a shaking heat block for 1-2h. The sample was then incubated with 4 µℓ of RNase at 37°C for 30-60 minutes, and at 70°C for 10 minutes with 200 µℓ of Buffer AL. Finally, 200 µℓ of high-grade ethanol was added and the sample vortexed before being transferred onto a spin-column. The spin column was centrifuged at 6000 g for one minute and the flow-through discarded. The column was washed with 500 µℓ of Buffer AW1 and centrifuged at 6000 g for one minute. It was washed again with 500 µℓ of Buffer AW2 and centrifuged at 21000 g for 3 minutes, the flow-through discarded, and dry-spun for another minute to dry the membrane. The column was then placed on a new collection tube, eluted with 50 µℓ of molecular-grade water (Milli-Q®), and incubated at 37°C for three minutes before being centrifuged for one minute at 21000 g. DNA was stored at -20°C before library preparation was done.

Library preparation and WGS

DNA concentration was checked before and after library preparation using Qubit Fluorometric Quantification (ThermoFisher). DNA samples were diluted to the

concentration of 0.3 µg/mℓ. Library preparation was performed using the Illumina® Nextera® XT Library Prep Kit following the manufacturer's instructions. The genomes were then sequenced at Novogene (Hong Kong, China) by Illumina® HiSeq™. The target average read depth was ~ 50-fold coverage with read length 150 bp. Initial draft sequences for all strains were assembled *de novo* using the Nullarbor pipeline version 2.0.20180910 (Seemann et al., 2018), using the assembler skesa version 2.2.1 (Souvorov et al., 2018).

glmU and *gyrB* genes sequence typing

For animals with a negative culture but a positive *lipL32* PCR from kidneys, a conventional PCR assay was repeated on the DNA extracted from the kidney targeting the *glmU* gene (Nisa et al., in prep). In addition, kidneys from seven PCR-negative animals with leptospires seen by dark-field microscopy but not isolated, as well as two animals with signs of contaminated sequences by WGS were also submitted to this PCR. Ten early samples were analysed before the *glmU* PCR was developed using a conventional PCR targeting the *gyrB* gene as described by Slack et al. (2006b). The primers used for the different PCR assays conducted are presented in Table 5-2. Amplicons were sequenced by Massey Genome Services (Palmerston North, NZ) using an ABI3730 DNA Analyzer.

Table 5-2 | Primers used for *Leptospira* species PCR assays

Target	Amplicon size	Primers	Sequence*	Reference
lipL32	242 bp	*lipL32*-45-F	5'-AAG CAT TAC CGC TTG TGG TG-3'	(Stoddard et al., 2009)
		lipL32-286-R	5'-GAA CTC CCA TTT CAG CGA TT-3'	
		Probe:		
		lipL32-189P	FAM-5'-AA AGC CAG GAC AAG CGC CG-3'-BHQ1	
glmU	551 bp	*glmU*_DW_F	5'-CCC GTA TGA AAA CGG ATC AGC C-3'	(Nisa et al., in prep)
		*glmU*_DW_R	5'-ATT CTC CCT GAG CGT TTT GAT TTC-3'	
gyrB	502 bp	2For	5′- TGA GCC AAG AAG AAA CAA GCT ACA-3′	(Slack et al., 2006b)
		504Rev	5′- MAT GGT TCC RCT TTC CGA AGA-3′	

**Mixed base definition: M = A or C, R = A or G*

All *glmU* and *gyrB* sequences were aligned using Geneious® version 10.2.3 (Biomatters Ltd.) and compared to sequences from the laboratory reference cultures or to sequences available in GenBank. The sequences of the *glmU* gene differ for all six endemic serovars described in NZ and those of the *gyrB* gene differ for all but Balcanica

and Tarassovi (Edwards, 2019). According to this comparison, the positive samples were assigned to a species and serovar of *Leptospira*.

MLST and cgMLST

The three existing MLST schemes (Ahmed et al., 2006, Boonsilp et al., 2013, Varni et al., 2014) were used to type our isolates and the other genomes using the ℝ package MLSTar version 0.1 (Ferrés and Iraola, 2018) in ℝ version 3.5.1. The number of genes for *L. interrogans* and *L. borgpetersenii* is estimated to be respectively 3762 – 4990 and 4053 – 4443 (Fouts et al., 2016, Xu et al., 2016). As MLST schemes only use 6-7 genes, they represent only a small portion of the genome which limits the possible resolution of discrimination between strains. To increase the portion of genome being compared, we additionally used the cgMLST scheme developed by the Pasteur Institute (Guglielmini et al., 2019). This scheme targets 545 core genome genes (excluding the genes already present in the pre-existing MLST schemes) and present in all clades of *Leptospira*, and, in addition to the cgMLST type (cgST), provides a clustering type (CT) that groups cgSTs that have less than 40 allelic differences. When some of the 545 genes or portion of them are lacking and prevent the determination of a cgST, a group of compatible cgSTs is assigned instead of a specific cgST. We used GrapeTree to visualise the results (Zhou et al., 2018).

Comparison with other genomes

To determine the relationship between the genomes sequenced from our isolates and other *Leptospira* strains described internationally, we retrieved a comprehensive set of draft and closed genomes of *Leptospira* spp. strains published in NCBI (hereafter called NCBI genomes) and the associated metadata from the PATRIC database (Wattam et al., 2017). The available contigs and metadata associated with all the isolates present in the *Leptospira* PubMLST database (Jolley and Maiden, 2010) were also retrieved (hereafter called PubMLST genomes). All downloads (NCBI, PATRIC and PubMLST) were performed on 15 February 2019. Finally, a set of unpublished contigs from local or reference isolates available at the ^{m}EpiLab were also included (hereafter called ^{m}EpiLab genomes). Details on the ^{m}EpiLab genomes are presented in Table 5-3. All genomes retrieved were submitted to MLST. Genomes sharing at least one MLST type (ST) with our samples where then extracted to be included in the subsequent SNP analysis.

Core SNP alignment and phylogenomic tree

We aimed at including only closely related genomes to maximize the size of the core-genome being compared (*i.e.* the portion of genome that is conserved between all isolates). SNP (Single-Nucleotide Polymorphism) analyses were performed for the set of genomes that possessed the "Ballum profile" (n_{this_study} = 72, $n_{mEpiLab\_genomes}$ = 5, and n_{NCBI} = 8), the Hardjobovis profile (n_{this_study} = 2, $n_{mEpiLab\_genomes}$ = 12, and n_{NCBI} = 12) and the "Pomona profile" (n_{this_study} = 6 and n_{NCBI} = 14) using snippy version 4.4.5 (Seemann, 2017). The core SNP alignment was used to build a phylogenomic tree using the ℝ packages ape version 5.3 (Paradis and Schliep, 2019) and ggtree version 1.10.5 (Yu et al., 2017) in ℝ version 3.5.1.

Table 5-3 | Summary of information on *m*EpiLab genomes included in the genomic comparison.

Strain name	Sp	Serogroup	Serovar	Year	Location	Host	ST1	ST2	ST3	Sequencing Depth	Genome Length	Reference/Origin
MU_Rr_LW046	*Lb*	Ballum	Ballum	2017	Palmerston Nth, NZ	Ship rat, *Rattus rattus*	149	99	146	x113	3889386	local isolate
MU_Rr_LW001	*Lb*	Ballum	Ballum	2016	Palmerston Nth, NZ	Ship rat, *Rattus rattus*	149	99	146	x108	3955956	local isolate
H5	*Lb*	Ballum	Ballum	2015	Palmerston Nth, NZ	Hedgehog, *E. europaeus*	149	99	146	x116	3909893	(van de Pol, 2016)
Ballum (Mus127)	*Lb*	Ballum	Ballum	1944	Denmark	Mouse *M. musculus*	149	99	146	x110	3889845	LRC, Brisbane, Au
Arborea	*Lb*	Ballum	Arborea	1955	Arborea, Sardegna, Italy	Wood mouse, *Apodemus sylvaticus*	149	99	146	x173	3904112	LRC, Amsterdam, NL
SN_Ce_RL4	*Lb*	Sejroë	Balcanica	2017	Manawatū region, NZ	Red deer, *Cervus elaphus*	290	272	147	x179	3806021	local isolate
SN_Ce_RL16	*Lb*	Sejroë	Balcanica	2018	Manawatū region, NZ	Red deer, *Cervus elaphus*	290	272	147	x90	3795082	local isolate
P44	*Lb*	Sejroë	Balcanica	2015	Palmerston Nth, NZ	Possum, *T. vulpecula*	290	272	147	x106	3812314	(van de Pol, 2016)
Balcanica_Possum	*Lb*	Sejroë	Balcanica	.	NZ	Possum, *T. vulpecula*	290	272	147	x174	3820643	local isolate
Balcanica 1627 Burgas	*Lb*	Sejroë	Balcanica	1958	Bulgaria	Human	150	60	127	x169	3796252	LRC, Brisbane, Au
Tarassovi	*Lb*	Tarassovi	Tarassovi	1939	.	Human	153	212	147	x108	3728684	LRC, Brisbane, Au
D12	*Lb*	Sejroë	Hardjobovis	2010	Waikato region, NZ	Sheep	152	175	145	x109	3693980	(Fang, 2014)
D25	*Lb*	Sejroë	Hardjobovis	2010	Waikato region, NZ	Sheep	152	175	145	x104	3694206	(Fang, 2014)
E44	*Lb*	Sejroë	Hardjobovis	2010	Waikato region, NZ	Sheep	152	175	145	x130	3705261	(Fang, 2014)
EVK11	*Lb*	Sejroë	Hardjobovis	2011	NZ	Farmed deer	152	175	145	x200	3692148	(Vallée, 2016)
EVK2	*Lb*	Sejroë	Hardjobovis	2011	NZ	Farmed deer	152	175	145	x160	3696467	(Vallée, 2016)
EVK25	*Lb*	Sejroë	Hardjobovis	2011	NZ	Farmed deer	152	175	145	x164	3690250	(Vallée, 2016)
EVK5	*Lb*	Sejroë	Hardjobovis	2011	NZ	Farmed deer	152	175	145	x136	3687947	(Vallée, 2016)
EVK8	*Lb*	Sejroë	Hardjobovis	2011	NZ	Farmed deer	152	175	145	x128	3690771	(Vallée, 2016)
G18	*Lb*	Sejroë	Hardjobovis	2010	Waikato region, NZ	Cattle	152	175	145	x169	3707674	(Fang, 2014)
I53	*Lb*	Sejroë	Hardjobovis	2010	Waikato region, NZ	Cattle	152	175	145	x179	3708831	(Fang, 2014)
I89	*Lb*	Sejroë	Hardjobovis	2010	Waikato region, NZ	Cattle	152	175	145	x101	3693904	(Fang, 2014)
K4_12	*Lb*	Sejroë	Hardjobovis	2008	NZ	Farmed deer	152	175	145	x168	3708698	(Subharat, 2010)
AO25ii	*Li*	Pomona	Pomona	.	NZ	Sheep	140	52	58	x98	4519439	(Dorjee et al., 2011)

Strain name	Sp	Serogroup	Serovar	Year	Location	Host	ST1	ST2	ST3	Sequencing Depth	Genome Length	Reference/Origin
B33	*Li*	Pomona	Pomona	2010	Waikato region, NZ	Sheep	140	52	58	x115	4500617	(Fang, 2014)
EMY7780	*Li*	Pomona	Pomona	2013	Waikato region, NZ	Human	140	52	58	x121	4522174	local isolate
ESR8	*Li*	Pomona	Pomona	.	Waikato region, NZ	Human	140	52	58	x124	4523717	local isolate
P5661	*Li*	Pomona	Pomona	.	.	Farmed deer	140	52	58	x122	4526353	local isolate (NZVP)
Pomona_str68	*Li*	Pomona	Pomona	<1977	.	"American Skunk"	140	52	58	x152	4500542	Wallaceville ARC, NZ

Sp = Species; Lb = Leptospira borgpetersenii; Li = L. interrogans; Year is the year of isolation; ARC = Animal Research Centre; LRC = Leptospirosis Reference Centre; NZVP = New Zealand Veterinary Pathology; Periods indicate missing data

Results

Number of isolates and amplicons sequenced

We isolated 73 strains from wildlife on Farm A (from 69 different animals), five from wildlife on Farm B and three strains from beef cattle from Farm A. Those isolates are listed in Appendix 7. Although 21 cows had positive *lipL32* or *gyrB* PCR results, we obtained no isolates from the livestock urine samples taken on both Farm A and B (Table 5-1). For six animals (four mice, a rat and a beef cattle beast), isolation and WGS was successful but the *lipL32* PCR was negative. For seven animals (five mice, a beef cattle beast and a hedgehog), leptospire-shaped bacteria were seen under the dark-field microscope (DFM) but could not be isolated. The *lipL32* PCR were negative for all seven, but these samples were nevertheless submitted to *glmU* PCR. Two genomes submitted to WGS showed signs of contamination with exotic DNA (one mouse, one hedgehog), and the associated kidneys were also submitted to *glmU* PCR for confirmation. In total, 77 animals had a *glmU* or *gyrB* PCR conducted. Out of the 67 *glmU* PCR conducted, 18 gave positive results, and out of the 10 *gyrB* PCR conducted, four were positive (Table 5-1). The number of samples analysed for each step is synthetized in Figure 5-1 opposite.

Classification of isolates and amplicons sequenced

In total, samples of 146 animals were submitted to sequencing, and the infecting strain could be determined for 86. Four allelic single-locus profiles, MLST profiles or cgMLST cluster types were detected by *glmU*, MLST and cgMLST with direct correlation of the groups assigned to each genotype between all typing methodologies: *Leptospira borgpetersenii* sv Ballum—MLST profile ST1.149 ST2.99 ST3.146, or cgMLST cluster type CT15, hereafter called 'Ballum profile'—was detected in mice, hedgehogs, rats and cattle. *L. borgpetersenii* sv Hardjobovis—MLST profile ST1.152 ST2.175 ST3.145, or cgMLST CT72, hereafter called 'Hardjobovis profile'—was detected in cattle only. *L. interrogans* sv Pomona—MLST profile ST1.140 ST2.52 ST3.58, cgMLST CT5, hereafter called 'Pomona profile'—was found in hedgehogs and cattle, and *L. borgpetersenii* sv Balcanica (possum)—a novel MLST profile ST1.290 ST2.272 ST3.147, cgMLST CT312, hereafter called 'Balcanica (possum) profile'—was detected in a possum. Details of typing methods are given in Table 5-4.

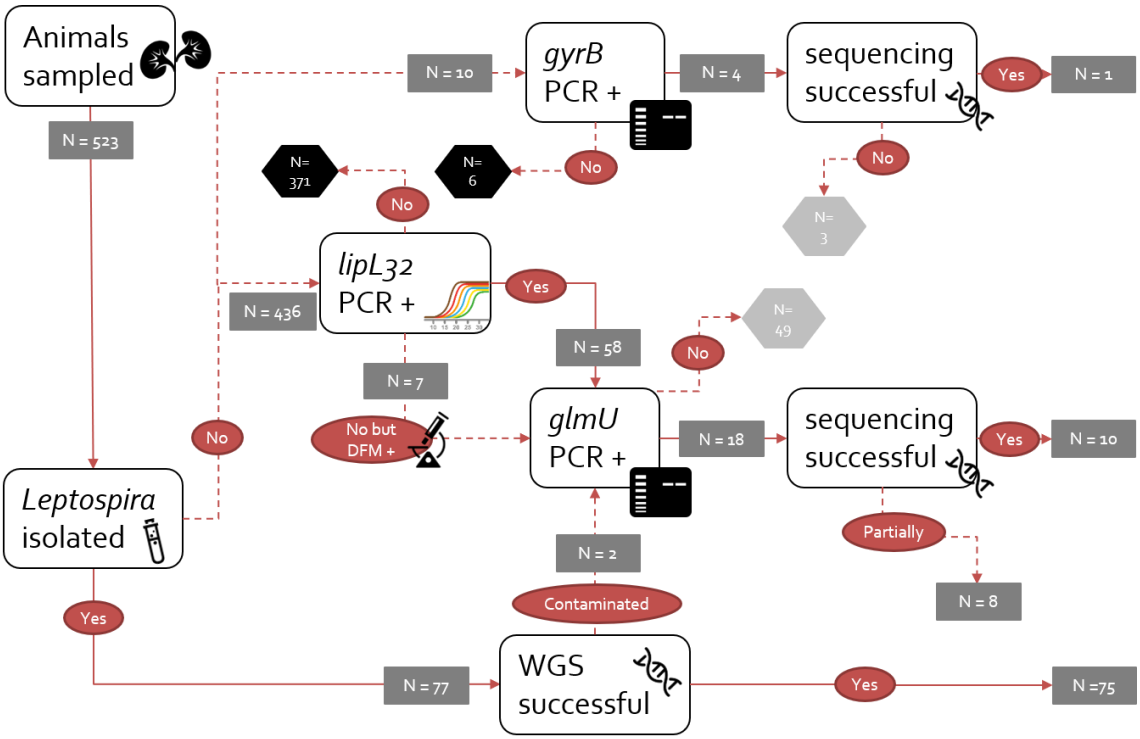

Figure 5-1 | Flow diagramme showing numbers of samples processed and tested. *Four mice had two isolates submitted to WGS (urine and kidney), but are represented only once in this diagramme*

Sequencing of amplicons failed for 52 animals, mainly cattle and mice (Table 5-4), and although the sequences were too low quality to assign a serovar, the species *L. borgpetersenii* could be assigned for eight additional mice. All seven samples that were *lipL32* PCR negative but had visible leptospire-shaped bacteria in DFM were negative by *glmU* PCR and remained untyped.

One hedgehog had signs of a mixed infection. The kidney PCR product sequenced as *L. borgpetersenii* sv Balcanica, while WGS data from positive cultures from this animal were suggestive of a mixed culture (possibly a mix of two strains of *L. borgpetersenii*, which resulted in incoherent genomic assembly) with a MLST result indicative of Ballum. A subsequent agglutination test using the culture obtained and standard antisera against Ballum (Mus 127) and Balcanica (1627 Burgas) showed a very good reaction to Ballum (titre ≥ 3072), but no reaction at all to Balcanica (titre < 24). However, it is important to note that the culture tested by MAT had been sub-cultured several times between the initial culture (13/03/2018) and the agglutination test (30/11/2018), while the sample submitted to WGS originated from the first subculture (3/05/2018). Growth rates between different *Leptospira* species vary greatly, and sequential passages of mixed cultures are likely to result in the selection of the better-growing isolate.

Table 5-4 | Results of *gyrB* typing, *glmU/gyrB* typing and MLST/cgMLST

Profile†	Species	Typing method			Total
		GyrB typing	*GlmU* typing	MLST/cgMLST	
L. borgpetersenii sv Balcanica ST1.290 ST2.272 ST3.147 CT 312	Hedgehog	\	*1**	.	1
	Possum	\	.	1	1
L. borgpetersenii sv Ballum ST1.149 ST2.99 ST3.146 CT 15	Mouse	\	*9*	*62*	70
	Hedgehog	\	.	*6**	5+1*
	Rat	\	.	2	2
	Cattle	1	.	.	1
L. borgpetersenii sv Hardjobovis ST1.152 ST2.175 ST3.145 CT 72	Cattle	.	.	2	2
L. interrogans sv Pomona ST1.140 ST2.52 ST3.58 CT 5	Hedgehog	\	.	3	3
	Cattle	.	.	1	1
L.borgpetersenii	Mouse	\	8	.	8
Untyped *Leptospira* spp.	Mouse	\	23	1‡	23
	Hedgehog	\	5	.	5
	Rat	\	4	.	4
	Feral cat	\	1	.	1
	Cattle	3	16	.	19

†STx. represents the profile for scheme x (x = 1 to 3), sv: serovar, CT is the cgMSLT clustering type

‡Contaminated genome sequence whose MLST results were congruent with the confirmatory glmU typing results

**Hedgehog MM305 showed signs of a mixed infection Ballum (MLST) / Balcanica (glmU typing)*

Comparison with other genomes

The searches in NCBI, PATRIC and PubMLST databases allowed retrieval respectively of 498 genomes, 550 sets of genome metadata, and 1293 sets of isolate metadata (for which 646 assemblies were available). Not including data from this study, we obtained a list of 1352 isolates, with at least one ST available for 1226 of them (Table

5-5). The isolates published internationally and sharing at least one common ST with one of the four profiles we obtained in this study are listed in Table 5-6.

Table 5-5 | Origin of genomes in public repositories compared with our isolates

# of genomes	Present in
465	all 3 databases
6	not in PATRIC
27	not in PubMLST
26	not in NCBI
32	only in PATRIC
796	only in PubMLST
1352	**Total**

Among the *m*EpiLab genomes, cultures from two rats captured within the Massey University campus and an urban hedgehog captured in the same town were assigned to the Ballum profile. Isolates with this Ballum profile were assigned a new ST profile ST3.146. Other isolates with the same profile have been isolates of svs Ballum and Castellonis originating from Europe, Asia, South America and French islands (Table 5-6) as well as the laboratory reference strain for sv Arborea. Other strains from the serogroup Ballum (as listed on the Amsterdam LRC website) present in the dataset of published isolates that had a different MLST typing result were *L. borgpetersenii* sv Kenya (ST1.154 ST2.178 ST3.132) and *L. mayottensis* sv Kenya (strain 200901122, ST1.new ST2.250 ST3.new).

Other isolates with the Pomona profile originated from the Americas, Australia and Egypt (Table 5-6), while isolates from China only had the same ST1 profile (ST1.140 ST2.3 ST3.34). A single strain of *L. interrogans* sv Lai (Col-P036) shared the same profile, whereas all other strains of sv Lai had profile ST1.1 ST2.7 ST3.47, suggesting that the serovar designation of this isolate may have been erroneous. Other strains from the serogroup Pomona present in the dataset of published isolates that had a different MLST typing result were: *L. interrogans* serovar Pomona strain UT364 (ST1.38 ST2.124 ST3.new); *L. kirschneri* serovar Mozdok strains Bren 166, Vehlefans 2 and 3, 61H and M36/05 (ST1.117 ST2.101 ST3.98); *L. kirschneri* serovar Mozdok strain 'B 81/7 type 3/Tsaratsovo' (ST1.100 ST2.146 ST3.new); and *L. kirschneri* sv Pomona strain M110/06 (ST1.232 ST2.102 ST3.new).

Other isolates with the Hardjobovis profile came from Australia, the USA, Brazil and the Netherlands. Two reference strains (strains JB197 and NVSL S 818) had an allele difference on gene *icdA* (in both schemes 2 and 3) and therefore another profile (ST1.152

ST2.176 ST3.new). Among the mEpiLab genomes, an isolate from a wild deer hunted in the Manawatū region was assigned to the Balcanica (possum) profile. There were two isolates with the same ST3 profile as the Balcanica profile published in China. Because no genome of sv Balcanica was available, we sequenced the reference strain (Balcanica strain 1627 Burgas) and the in-house Balcanica-possum reference strain, and while the Balcanica-possum strain had the same profile as our isolate, Balcanica-1627 Burgas was assigned to a different profile (ST1.150 ST2.new ST3.127). Other strains from the serogroup Sejroë present in the dataset of published isolates that had an MLST typing result different from both Hardjobovis and Balcanica (possum) were *L. interrogans* sv Medanensis (ST1.46 ST2.111 ST3.new), *L. interrogans* sv Hardjoprajitno (ST1.20 ST2.6 ST3.74) and *L. santarosai* sv Guaricura (ST1.225 ST2.103 ST3.140).

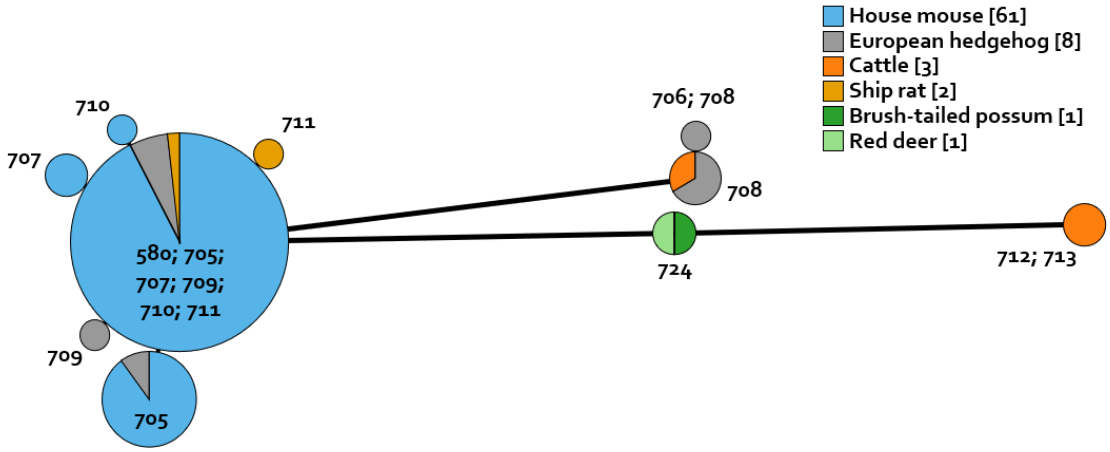

Figure 5-2 | Minimum spanning tree of 76 New Zealand isolates of *Leptospira* spp. submitted to cgMLST, 75 from the present study and one from a deer isolate. *Numbers indicated represent the cgST (see main text). The cluster on the left corresponds to Ballum isolates (Cluster Type CT15), the cluster on the far right to Hardjobovis isolates (CT72), the cluster in the centre (top) to Pomona isolates (CT5) and the cluster in the centre (bottom) to Balcanica isolates (CT312).*

Table 5-6 | Genomes and associated metadata extracted from NCBI, PATRIC and PubMLST sharing at least one MLST type with our isolates clustered in a 'Ballum profile' (A), 'Pomona profile' (B), 'Hardjobovis profile' (C) and 'Balcanica (possum) profile' (D). *Metadata is reproduced as presented in online repositories and may contain errors (e.g. strain Mus 127 was isolated in 1944, not 2000)*

PubMLST ID	Assembly Accession	Sp	Serogroup	Serovar	Strain name	Year	Country	Host	ST1	ST2	ST3	cgCT	Seq. Platform	Seq. Depth	Assembly Method	Genome Length
A -MLST Profiles similar to ST1.149 ST2.99 ST3.146 ('Ballum profile')																
819; 863	SRR513125; GCF_000244495.1	*Lb*	.	Castellonis	str. 200801910	.	Guadeloupe	Human, *Homo sapiens*	149	99	146	15	454; Illumina	62x	Celera[1]	3,970,478
875	GCF_000244535.1	*Lb*	.	.	str. Noumea25	.	New Caledonia	.	149	99	146	15	454 G	13.3x	Celera[1]	3,957,863
1079	GCF_000758045.1	*Lb*	.	Ballum	str. Muis5 (NAL2638)	2008	Netherlands	Mouse, *Mus musculus*	149	99	146	15	Illumina	53.0x	Velvet[2]	3,887,643
1204	GCF_001444465.1	*Lb*	.	Ballum	str. 56604-Rat	1978	China	Rat, *Rattus sp.*	149	99	146	15	454	29.5	Newbler[3]	4,037,579
1104	GCF_001569465.1	*Lb*	.	Ballum	str. 56604-Mouse	1964	China	Mouse, *Mus musculus*	149	99	146	15	IHiSeq	350.0x	Velvet[4]	3,900,819
1105	GCF_001568495.1	*Lb*	.	.	str. 56607	1959	China	Horse, *Equus caballus*	149	99	146	.	IHiSeq	333.0x	Velvet[4]	3,900,903
1107	GCF_001569155.1	*Lb*	.	.	str. 56648	1971	China	Lesser ricefield rat, *R. losea*	149	99	146	.	IHiSeq	356.0x	Velvet[4]	3,892,822
1244	GCF_001652525.2	*Lb*	.	.	str. 4E	2005	Brazil	Mouse, *Mus musculus*	149	99	146	.	SOLiD	400.0	Bowtie[5]	3,916,189
506; 1221	*SAMN01048185;* GCF_002018035.1	*Lb*	Ballum	Ballum	str. LO-24	2011	Brazil	Brown Rat, *R. norvegicus*	149	99	146	.	IMiSeq	60.0x	CLC[6]; Geneious[7]	3,963,883
423		*Lb*	Ballum	Ballum	str. Mus_127	2000	Denmark	Mouse, *Mus musculus*	*149*	.	.	.	.	.	.	.
424		*Lb*	Ballum	.	str. MC-OW08-2	2008	Japan	"mouse"	*149*	.	.	.	.	.	.	.
425		*Lb*	Ballum	Castellonis	str. Castellon_3	2002	Spain	"mouse"	*149*	.	.	.	.	.	.	.
B- MLST Profiles similar to ST1.140 ST2.52 ST3.58 ('Pomona profile')																
1007	GCF_000347155.1	*Li*	.	Pomona	str. CSL4002	.	USA	Otariidae	140	52	58	5	IGAIIx	52.6x	Celera[1]	4,589,471
1006	GCF_000346695.1	*Li*	.	Pomona	str. CSL10083	.	USA	CA sea lion, *Z. californianus*	140	52	58	5	IGAIIx	60.2x	Celera[1]	4,569,425
997	GCF_000343025.1	*Li*	.	Pomona	str. 2006006962	.	Egypt	Human, *Homo sapiens*	140	52	58	5	IHiSeq	58.86x	Celera[1]	4,562,852
1120	GCF_001568935.1	*Li*	.	.	str. 56133	1936	Australia	Human, *Homo sapiens*	140	52	58	.	IHiSeq	231.0x	Velvet[4]	4,518,153
18;1202	GCF_001857845.1	*Li*	Pomona	Pomona	str. AK-RFB	2007	Argentina	Cattle, *Bos taurus*	140	52	58	.	IMiSeq	100.0x	SPAdes[8]	4,627,117

PubMLST ID	Assembly Accession	Sp	Serogroup	Serovar	Strain name	Year	Country	Host	ST1	ST2	ST3	cgCT	Seq. Platform	Seq. Depth	Assembly Method	Genome Length
1223	GCF_001969075.1	*Li*	Pomona	Pomona	str. GR5	2004	Brazil	Pig, *Sus scrofa*	140	52	58	.	IMiSeq	60.0x	CLC[6]; Geneious[7]	4,594,696
814;957	GCF_000216355.3	*Li*	.	Pomona	str. Pomona	.	Australia	Human, *Homo sapiens*	140	52	58	.	454 G; IGAIIx	62.2x	Celera[1]	4,581,018
811;956	GCF_000243635.3	*Li*	.	Pomona	str. Kennewicki_LC82-25	.	USA	Human, *Homo sapiens*	140	52	58	.	454 G; IGAIIx	58.5x	Celera[1]	4,608,327
1238	GCF_002076815.1	*Li*	.	Lai	str. Col-P036	1994	Colombia	Pig, *Sus scrofa*	140	52	58	.	Illumina	12.0x	Newbler[9]	4,403,805
1061	GCF_000342365.1	*Li*	.	Pomona	str. Fox_32256	.	USA	Fox, *Urocyon littoralis*	140	52	-	5	IGAIIx	55.63x	Celera[1]	4,583,716
998	GCF_000343345.1	*Li*	.	Pomona	str. 2006006968	.	Egypt	Human, *Homo sapiens*	new	52	58	5	IHiSeq	58.53x	Celera[1]	4,551,186
1142	GCF_001567945.1	*Li*	.	.	str. 56630	1960	China	Rat, *Rattus sp.*	140	3	34	.	IHiSeq	359.0x	Velvet[4]	4,564,593
1129	GCF_001569065.1	*Li*	.	.	str. 56608	1958	China	Human, *Homo sapiens*	140	3	34	.	IHiSeq	616.0x	Velvet[4]	4,563,098
1163	GCF_001569045.1	*Li*	.	.	str. 56608-V	1958	China	Human, *Homo sapiens*	140	241	new	.	IHiSeq	345.0x	Velvet[4]	4,397,635
57		*Li*	Pomona	Pomona	str. Pomona	1936	Australia	"human"	*140*	*4*	*58*	.	.	.	.	.
79		*Li*	Pomona	Kennewicki	str. LT1026	.	USA	"bovine"	.	*3*	*58*	.	.	.	.	.
407		*Li*	Grippotyphosa	.	str. R235	1957	Sri_Lanka	"human"	*140*	.	.	.	.	.	.	.
408		*Li*	Pyrogenes	Guaratuba	str. An_7705	2006	Brazil	"opossum"	*140*	.	.	.	.	.	.	.
15		*Li*	Pomona	.	str. Bovino_bibi	.	Argentina	"bovine"	.	*52*	.	.	.	.	.	.
25		*Li*	Pomona	.	str. CañuelasI	1982	Argentina	"porcine"	.	*52*	.	.	.	.	.	.
26		*Li*	Pomona	.	str. CañuelasII	1986	Argentina	"porcine"	.	*52*	.	.	.	.	.	.
70		*Li*	Sejroë	Hardjoprajitno	str. V3P	.	Argentina	"rat"	.	*52*	.	.	.	.	.	.
74		*Li*	Pomona	.	str. 417	1999	Argentina	"human"	.	*52*	.	.	.	.	.	.
75		*Li*	Pomona	.	str. Macedo_Balcarce	1981	Argentina	"bovine"	.	*52*	.	.	.	.	.	.
77		*Li*	Pomona	.	str. Bayur_P	1989	Argentina	"bovine"	.	*52*	.	.	.	.	.	.
C- MLST Profiles similar to ST1.152 ST2.175 ST3.145 ('Hardjobovis profile')																
428; 630; 638	GCF_000013945.1	*Lb*	Sejroë	Hardjobovis	str. L550	.	Australia	Human, *Homo sapiens*	152	175	145	72	.	.	.	3,931,782
426; 634	GCF_000013965.1	*Lb*	Sejroë	Hardjobovis	str. JB197	.	USA	Cattle, *Bos taurus*	152	176	new	98	.	.	.	3,876,235
986	GCF_000342885.1	*Lb*	.	Hardjobovis	str. Lely_607	.	Netherlands	Cattle, *Bos taurus*	152	175	145	72	IHiSeq	71x	Celera[1]	3,774,172
987	GCF_000346975.1	*Lb*	.	Hardjobovis	str. Sponselee_CDC	.	Netherlands	Cattle, *Bos taurus*	152	175	145	72	IGAIIx	60.8x	Celera[1]	3,776,198
427; 1111	GCF_000355135.1	*Lb*	Sejroë	Hardjobovis	str. Sponselee	1924	Netherlands	Cattle, *Bos taurus*	152	175	145	.	IHiSeq	71.34x	Celera[1]	3,755,643
1205	GCF_001618445.1	*Lb*	.	Hardjo	str. BK-30	2014	USA	Cattle, *Bos taurus*	152	175	145	.	IMiSeq	300x	SPAdes[10]	3,947,069

PubMLST ID	Assembly Accession	Sp	Serogroup	Serovar	Strain name	Year	Country	Host	ST1	ST2	ST3	cgCT	Seq. Platform	Seq. Depth	Assembly Method	Genome Length
1209	GCF_001618485.1	*Lb*	.	Hardjo	str. NVSL_S_818	1986	USA	Cattle, *Bos taurus*	152	176	new	.	IMiSeq	300x	SPAdes[10]	3,884,697
1206	GCF_001618525.1	*Lb*	.	Hardjo	str. BK-6	2014	USA	Cattle, *Bos taurus*	152	175	NA	.	IMiSeq	300x	SPAdes[10]	3,967,801
1207	GCF_001618565.1	*Lb*	.	Hardjo	str. BK-9	2014	USA	Cattle, *Bos taurus*	152	175	NA	.	IMiSeq	300x	SPAdes[10]	3,949,086
1208	GCF_001618585.1	*Lb*	.	Hardjo	str. NVSL_S_1343	1986	USA	Cattle, *Bos taurus*	152	175	NA	.	IMiSeq	300x	SPAdes[10]	3,932,487
	GCF_003254845.1	*Lb*	.	Hardjobovis	str. 203	1986	USA	Cattle, *Bos taurus*	152	175	NA	.	PacBio; Illumina	165x	SMRT[11]	3,907,328
	GCF_003716785.1	*Lb*	.	Hardjobovis	str. L49	.	Brazil	Cattle, *Bos taurus*	152	175	NA	.	Illumina	173.0x	Velvet[12]	3,935,911
D- MLST Profiles similar to ST1.290 ST2.272 ST3.147 ('Balcanica (possum) profile')																
1110	GCF_001568305.1	*Lb*			str. 56676	1979	China	Human, *Homo sapiens*	153	212	147	.	IHiSeq	407.0x	Velvet[4]	3,750,210
1106	GCF_001569485.1	*Lb*			str. 56613	1965	China	Human, *Homo sapiens*	153	212	147	.	IHiSeq	344.0x	Velvet[4]	3,758,628

Lb = Leptospira borgpetersenii: Li = L. interrogans. ST indicated in italics are ST reported in the PubMLST database for which no sequences were available. IGAIIx/IHiSeq/IMiSeq = Illumina GIIx/HiSeq/MiSeq; 454 G = 454 GS-FLX Titanium. [1]v.7.0; [2]v.1.1.05; [3]v.April 2nd 2015; [4]v.1.2.03; [5]v.1.0; [6]NGS Cell v. 7.5.1; [7]v. R10; [8]v. 3.6.2; [9]v. 2.0.01.14; [10]PAGIT package v. SEP-2015; [11]SMRT analysis v. 2.3.0; [12] v. JULY-2018

Comparison of typing methods

The same four profiles found by *gyrB* or *glmU* typing and MLST were obtained by cgMLST, with all isolates of each MLST profile belonging to a same clustering type: Ballum to CT15, Pomona to CT5, Hardjobovis to CT72 and Balcanica to CT312. The variation within each CT was minimal, with both Balcanica isolates (possum isolate –this study and deer isolate –^{m}EpiLab genome) belonging to the same cgST (724), all Pomona isolates in cgST 708 or cgST group 706; 708 (*i.e.* some genes or portions of them among the 545 genes included in the cgMLST scheme were lacking and the corresponding cgST may either be cgST 706 or cgST 708) and Hardjobovis isolates in cgST group 712; 713. Ballum isolates were classified into five cgSTs (705, 707, 709-711), or, for most of them, in cgST group 705; 707; 708; 709; 710; 711; 580 (therefore encompassing those five cgSTs and cgST 580). All isolates from Farm B were in that cgST group. There was no clear pattern emerging from the typing (Figure 5-2).

Profile-specific core-genome SNP analyses

We used SNP analyses within each profile to quantify the level of variability of isolates of similar profiles, with the aim of using differences to query population structure. We aimed at including only closely related genomes to maximize the size of the core-genome being compared (*i.e.* the portion of genome that is conserved between all isolates). For this reason, the strain of Ballum Noumea 25 (New Caledonia) was left aside, as it was relatively different from the other strains in that profile.

There were between zero and six SNP differences among the 72 isolates of Ballum from this study (65 from mice, five from hedgehogs, two from rats), three to eight SNPs with two rat isolates from Massey campus (MU_Rr_LW001 and 046), and two to six SNPs with a hedgehog isolate from Palmerston North (H5). Isolates obtained from urine and kidney of the same animal were among the genomes with no SNPs differences between them. The portion of genome covered in the SNP analysis (or coverage) was 94.59% to 95.58% for our genomes and ranged 92.11% – 95.58% for other genomes compared (detail available in Appendix 7). No clear pattern emerged between species, geography (although isolates from Farm B were more similar with each other, they were among isolates from Farm A), or time. The sub-group identified with the cgMLST (cgST 705) was also identified and contained a hedgehog and mice from sessions A2 to A4 (Figure 5-3). In total, there were 18 variant sites among all 72 Ballum isolates from this study. Five were in non-coding regions, four were synonymous variants, eight missense

variants, and one resulted in a start lost. Affected sites and proteins are detailed in Appendix 7.

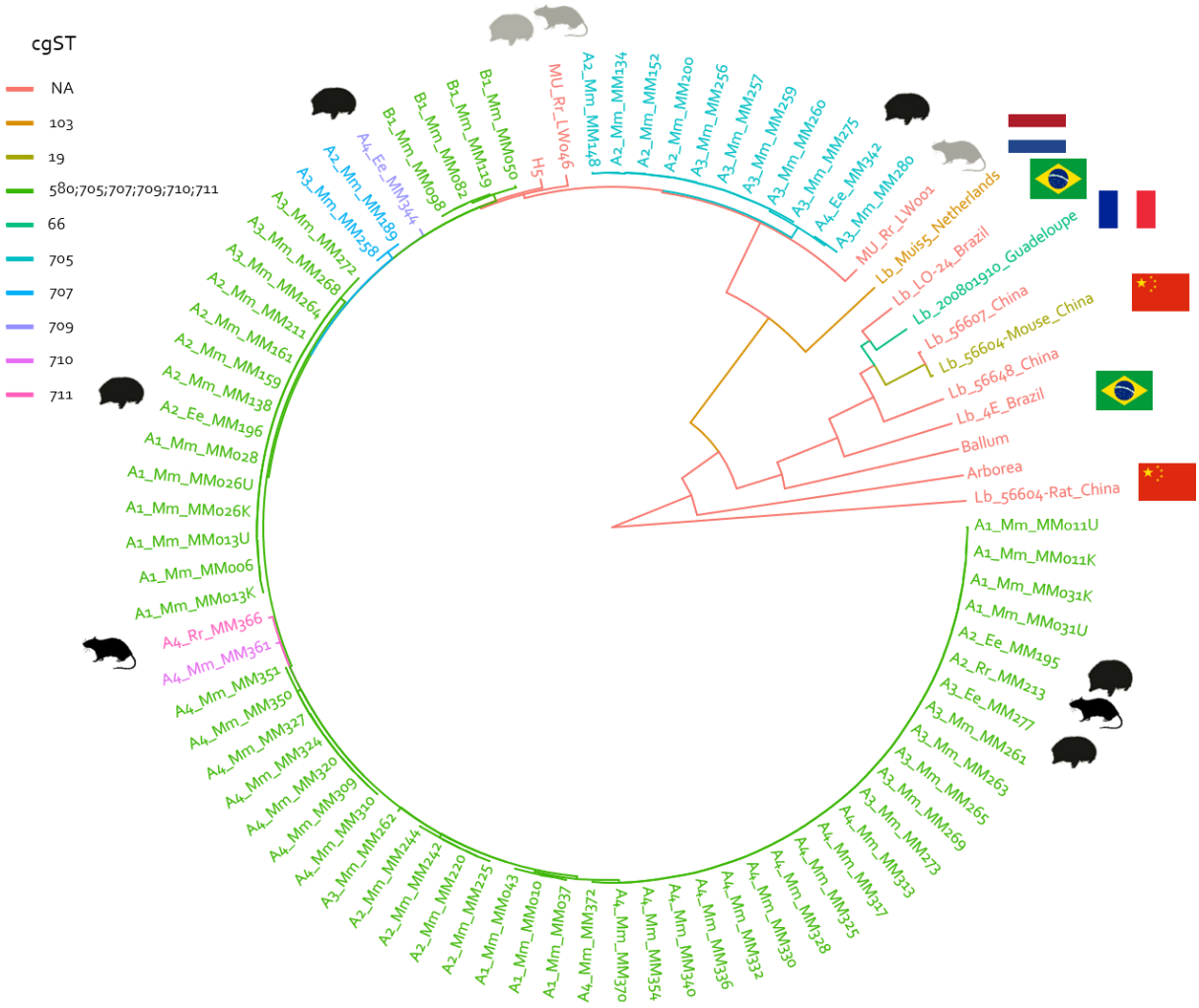

Figure 5-3 | Phylogenomic tree of Ballum isolates (MLST profile ST1.149 ST2.99 ST3.146, cgCT15) from this study compared with isolates publicly available sharing the same profile and reference strains Ballum (Mus 127) and Arborea (Arborea). *For a better visualization the tree is represented circularly. Colours represent the core-genome ST (NA = not available). Icons represent species other than mice isolated in this study (black) or elsewhere in New Zealand (grey)*

For isolates from the Pomona profile, the three isolates from hedgehogs had no SNP differences on the portion of genome included in the SNP analysis, and only one with the cattle isolate (in a non-coding region). This cattle isolate had no SNP differences with a human local isolate, ESR8. Other local isolates had 1 to 3 SNP differences with our isolates. Coverage was 91.29% to 91.65% for the four genomes from this study and ranged 89.04 – 93.68% for other genomes compared (detail available in Appendix 7). The international strain closest to isolates from this study was the in-house reference strain of Pomona, strain 68 (originally from an American skunk), followed by the strain Col-Po36 from Colombia, and strains from Australia and Egypt (Figure 5-4).

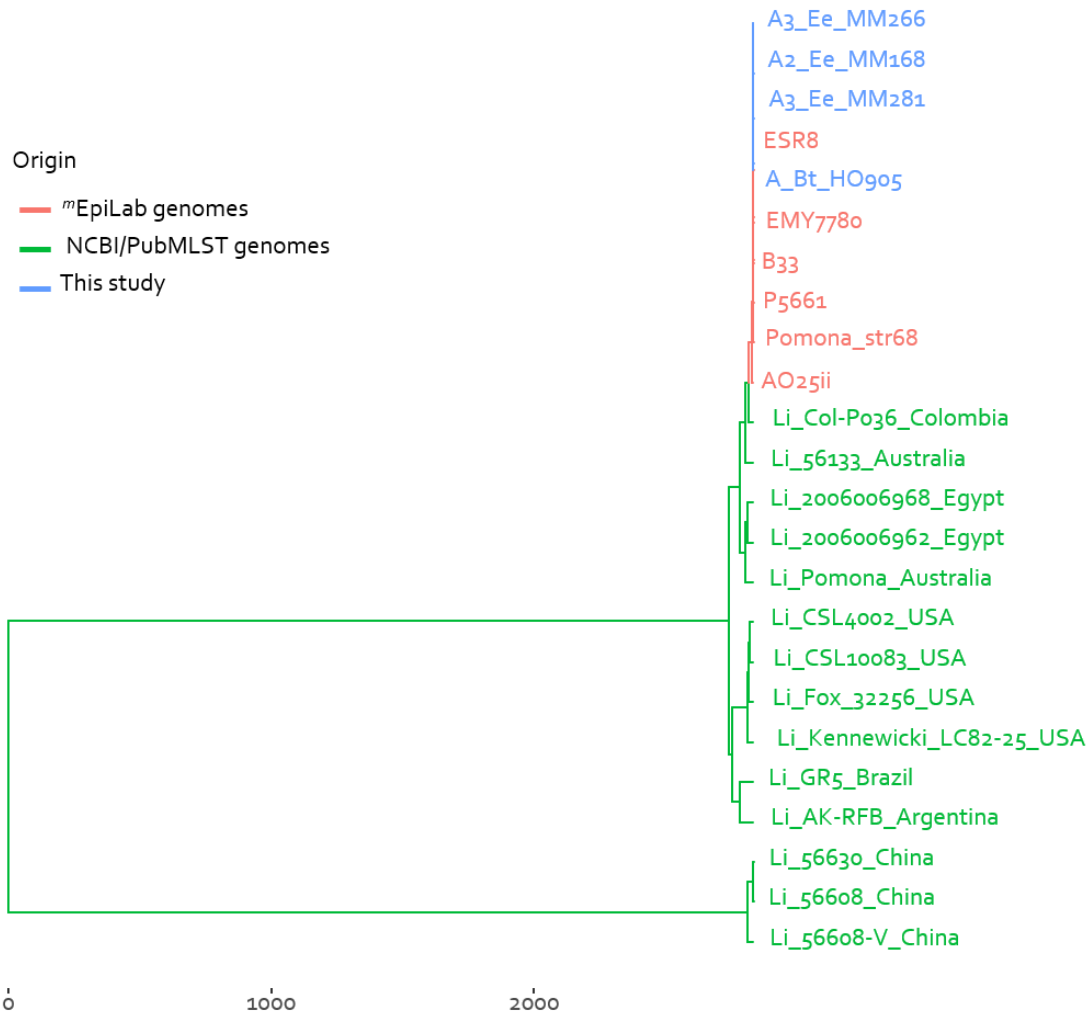

Figure 5-4 | Phylogenomic tree of the four Pomona isolates (MLST profile ST1.140 ST2.52 ST3.58, cgCT5) from this study compared with other NZ isolates and isolates publicly available sharing at least one MLST profile.

The two isolates of Hardjobovis from this study had only one SNP difference with each other (missense variant, Appendix 7), and 6 to 29 SNPs differences with other NZ isolates (Figure 5-5). Coverage was 95.93% and 95.95% for the two genomes from this study and ranged 89.15% – 95.86% for other genomes compared (detail available in Appendix 7).

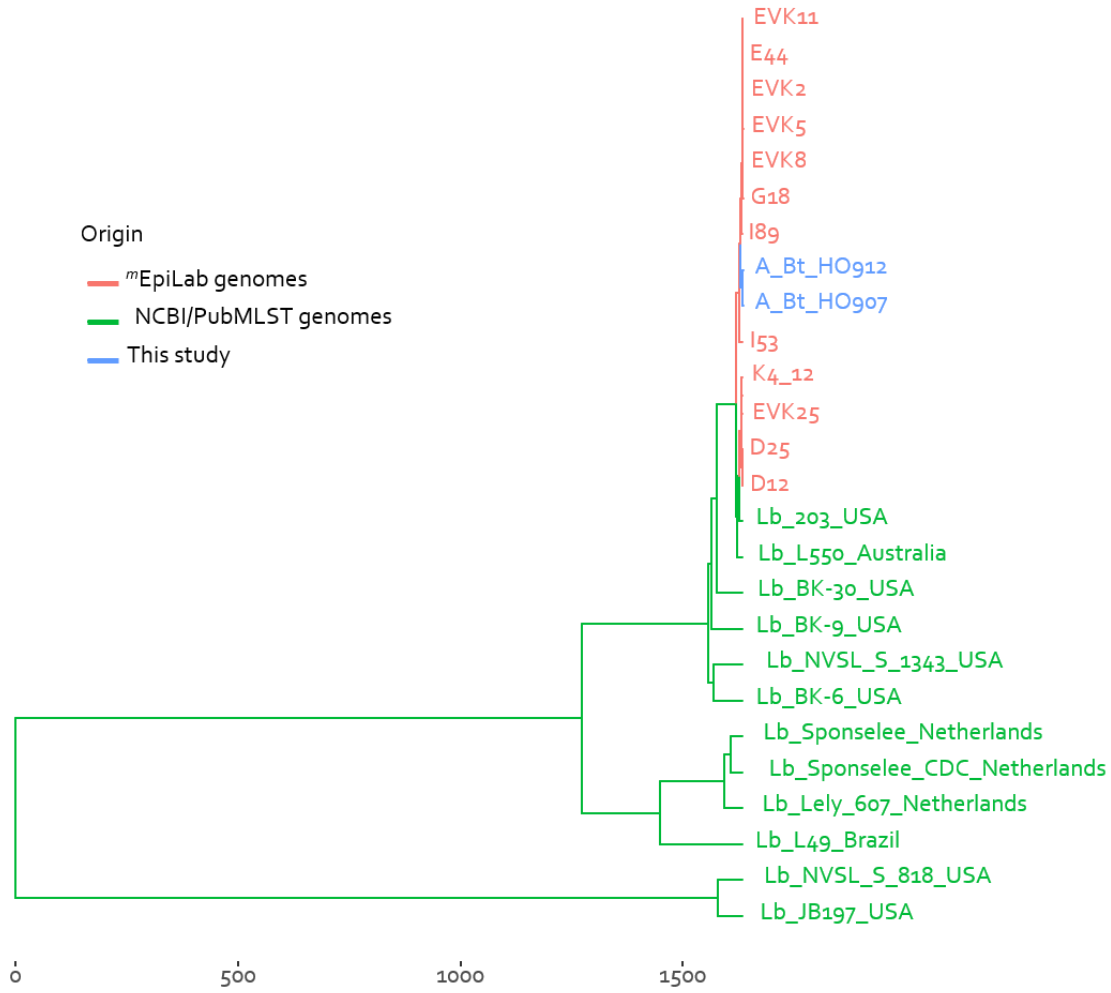

Figure 5-5 | Phylogenomic tree of the two Hardjobovis isolates (MLST profile ST1.152 ST2.175 ST3.145, cgCT72) from this study compared with other NZ isolates and isolates publicly available sharing at least one MLST profile.

Discussion

In this study, we identified four different genotypes of *Leptospira* circulating in animals living in the same environment. All genotypes had previously been identified to circulate in New Zealand. Within each genotype, genomes were compared with strains that are known to have circulated worldwide over as long as decades. The remarkable dearth of mutations at the whole genome scale confirmed, for the first time at this scale, the slow rate at which *Leptospira* genomes mutate.

We observed direct correspondence between single gene, multi-gene and whole-genome level classifications, suggesting that the intra-strain genetic diversity of *Leptospira* is extremely low. The low number or absence of SNPs between genomes of the same genotype, even at the whole genome scale was striking. *Leptospira* are bacteria

known to evolve slowly, and our results confirm this fact, for the first time at this level of discrimination. We did not expect a high level of variation between isolates but given the proportion of the genomes compared (> 95% for Ballum), and the barriers in space (~100 km between farms), and time (two years of sampling) that separated the populations studied, this low level of mutations was unexpected. Such slow mutation rates suggest that either mutations are deleterious and under strong negative selection, or that *Leptospira* have very efficient DNA repair mechanisms. The higher number of variants observed in laboratory adapted strains compared to wild strains of Hardjobovis is in favour of the first (Llanes et al., 2018).

Isolates of Ballum from this study were genetically most similar to the strain Muis 5 isolated in the Netherlands in 2008, but not Noumea 25, isolated in New Caledonia before 2013 (date not mentioned). This could relate to the origin of the invasive mice sampled in our study, the principal maintenance host for Ballum. Mice were introduced to NZ in several waves, starting with the first European vessels at the end of the 18th century (Veale et al., 2018). Genotyping studies of mice in New Zealand have confirmed the presence of three subspecies, *Mus musculus domesticus* (from western Europe, Near East and Northern Africa), *M. m. musculus* (from Eastern Europe and Northern Asia) and *M. m. castaneus* (from India and South-East Asia, Veale et al., 2018, King et al., 2016, King, 2016). The *M. m. domesticus* type predominates across the country (Veale et al., 2018). Assuming mice that colonised New Zealand brought Ballum with them and maintained it through time, a European ancestry for mice sampled in this study would explain the proximity with the Dutch strain. However, the southern part of the North Island presents a high level of haplotype diversity and *M.m. castaneus* haplotypes have been detected in the vicinity of Farm B (King et al., 2016). In the absence of any information regarding the genotype(s) of the mice sampled and the possible co-evolution phylogenetics of mice and *Leptospira*, we cannot prove the European origin of the Ballum strains isolated in this study. It is however possible that other strains of Ballum closer to the Asian isolates may be present in areas where *M. m. castaneus* is present (Southern South Island, Northern North Island; King et al., 2016, Veale et al., 2015).

We combined and compared several typing methods: in silico MLST from the contigs obtained by WGS, cgMLST and whole genome SNPs analysis, and when culture was unsuccessful, single-locus typing directly from kidney or urine samples. Identification of strains resulting from this last method had limited success, with only 1/4 *gyrB* and 10/18 (out of 58 positive *lipL32* PCR) *glmU* positive PCRs being successfully typed. This result is probably related to the very low bacterial load present in the initial samples. Indeed, no isolates were obtained from those samples, and the mean *lipL32* Ct

for these samples was 31.2, compared to 23.7 for the samples with a successful isolation submitted to WGS.

The presence of seven samples with both *lipL32* and *glmU* PCRs negative, but with leptospire-shaped organisms initially observed under the DFM and not isolated, lead to further investigations (Appendix 8). Six other samples were PCR negative but culture positive (five Ballum, one Pomona). Results of those investigations (Appendix 8) are in favour of a reduced sensitivity of PCR methods due to the matrix used (kidney).

Isolates typed with single-locus typing methods were assigned a serovar according to the knowledge of serovars previously isolated in NZ—*i.e.*, *L. borgpetersenii* svs Hardjobovis, Tarassovi, Balcanica and Ballum, and *L. interrogans* svs Pomona, Copenhageni, Australis and Canicola (Midwinter and Fairley, 1999)—but other strains could be present. Balcanica and Hardjobovis, both in the Sejroë serogroup, are serologically distinguishable only using the CAAT, a method cumbersome to apply, and Balcanica remained cryptic until DNA based methods were developed in the early 1980s to distinguish it from Hardjobovis (Robinson et al., 1982). Similarly, other serovars close antigenically could co-exist in NZ, and be distinguished only by MLST or cgMLST. The presence of *L. borgpetersenii* sv Arborea was suspected serologically in farmed red deer, but never isolated, and the authors concluded titres to Arborea were due to cross-reactivity (Subharat et al., 2011b). Although the single-locus and MLST typing methods could not distinguish Ballum from Arborea, results at the whole genome scale in this study indicated only one strain identified as Ballum was present in mice and hedgehogs. However, the SNP analysis revealed Ballum and other serovars such as Castellonis or Arborea are genetically very close, and better characterization of the genetic closeness between those serovars is needed. Improved knowledge of potential similarities between those serovars has the ability to better inform leptospirosis surveillance and control strategies, for instance by considering them as a similar epidemiological entity to better understand risk factors, or to develop a common vaccine. Arborea was recently responsible for the biggest human outbreak of leptospirosis (84 cases) ever recorded in Australia (Katelaris et al., 2019) and is also considered as an emerging serovar (Lau et al., 2015).

Early versions of the SNP analysis were conducted with contigs of ^{m}EpiLab genomes assembled using a different version of nullarbor (data not shown). The number of SNPs obtained with those contigs was greater than the number of SNPs with contigs assembled using the same bioinformatics pipeline as isolates from this study. For instance, the draft genome of H5 previously assembled showed 11 to 15 SNPs difference with our isolates, compared to 2 to 6 with the assembly done with the same pipeline. Although this observation does not impact the interpretation of results presented in this

study, it signals the need for caution when comparing the number of SNPs between genomes assembled with different tools. It is the case for NCBI/PubMLST genomes, for which raw reads were not available. We suggest raw or trimmed reads of *Leptospira* genomes be made more systematically available in public repositories alongside assembled genomes.

We had difficulties accessing samples for bacterial culture from livestock: sampling kidneys of the milking herd of dairy cattle was not an option, and clean urine samples were difficult to obtain. Despite the use of EMJH supplemented with STAFF which allowed a decrease in contamination rates, no isolates were obtained from cattle urine. On Farm B, ten cattle had a positive PCR on kidney or urine samples, but only one of them could be successfully sequenced and typed as Ballum, and no isolates were obtained. On Farm A', three isolates of Pomona and Hardjobovis were obtained from 20 cattle in the context of an abortion storm in the herd, with thus a bacterial load that was potentially higher in the kidneys at the time of sampling. The use of enrichment methods like DNA binding, which has been successfully applied to the non-cultivable spirochete *Treponema pallidum* (Pinto et al., 2016), could help in the future to overcome the issue of culture sensitivity and allow WGS without the need to culture and isolate a strain.

In this chapter, we used sequencing methods to type *Leptospira* strains detected in domestic and wild hosts sharing the same environment in two farms. Four different strains and profiles were found to circulate, *Leptospira borgpetersenii* profile Ballum in mice, hedgehogs, rats, and cattle; profile Hardjobovis in cattle and profile Balcanica in possum and deer, and *L. interrogans* profile Pomona in cattle and hedgehogs. Similar results using cgMLST, MLST and single-locus sequence typing suggest the diversity of strains currently circulating in NZ is sufficiently low for single-locus typing to be used to identify accurately infecting strains in leptospirosis cases identified in humans and animals without the need to cultivate and isolate the infecting strain. The within-strain genomic diversity was extremely low, which prevented infection transfer chains to be decrypted between species. Other methods to investigate within- and between-hosts transmission processes of *Leptospira* such as mathematical modelling could overcome this issue.

“In examining disease, we gain wisdom about anatomy and physiology and biology. In examining the person with disease, we gain wisdom about life.”

— Oliver W. Sacks

Chapter 6

Investigating infection in a community of maintenance hosts naturally exposed to *Leptospira*

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Chapter 6.
Reservoir dynamics

Abstract

Leptospirosis is a global emerging zoonosis due to bacteria of the genus *Leptospira*. In New Zealand (NZ) livestock have been considered as the main source of infection for people, but the epidemiology of the disease is changing. The role of rodents and hedgehogs as a source of *Leptospira* infection, long recognised internationally for the former, and more recently highlighted for the latter, is now also emerging in NZ. Mice (*Mus musculus*), ship rats (*Rattus rattus*), brown rats (*R. norvegicus*) and hedgehogs (*Erinaceus europaeus*) are considered maintenance hosts of *L. borgpetersenii* serovar Ballum, that is now one of the two most frequent serovars detected in notified human cases in NZ. There is a dearth of information on the impact of natural infection in those species, or on the bacterial load they harbour, but these factors shape their relative role as maintenance hosts.

We investigated the gross and microscopic lesions in a population of wild rodents and hedgehogs sharing the same farm habitat in NZ and with known *Leptospira* infection status based on previous MAT, qPCR, culture and WGS results, and assessed the *Leptospira* bacterial load in the kidneys of infected animals. Lesions compatible with leptospirosis were scored on a 3-point scale (0—no lesions, 1—mild, 2—moderate, 3—severe) and bacterial load was assessed visually with Warthin-Starry (WS) staining (absent, low, moderate, high load) and qPCR Ct values.

Kidneys of 207 mice, 32 hedgehogs and 20 ship rats were examined. Respectively 98, 14 and five of them were infected with *Leptospira* spp. While hedgehogs harboured different serovars (Ballum, Pomona, Balcanica), only Ballum was detected in mice and rats. No macroscopic lesions compatible with leptospirosis were observed and mild lesions were recorded in 51 mice (24.6%, 95% CI = 18.9, 31.1%), 14 hedgehogs (43.8%, 95% CI = 26.4, 62.3%) and one ship rat (5.0%, 95% CI = 0.1, 24.9%) of which 4 hedgehogs and 3 mice were negative by PCR, culture and WS. *Leptospira* bacterial loads were higher in kidneys of mice than in the other species. A portion of infected mice decreasing with age displayed no antibodies and no lesions with a low bacterial load—undetected by WS—and a qPCR Ct >27. Despite a similar low level of lesions due to infection across different host animal species, these results highlight interspecies and intraspecies heterogeneity in

Leptospira shedding, and suggest rats and hedgehogs are secondary hosts of serovar Ballum and mice the key host that should be targeted for control.

Introduction

Leptospirosis is an emerging infectious disease due to pathogenic bacteria belonging to the genus *Leptospira*. Despite livestock vaccination programmes, leptospirosis remains the most common occupational zoonosis in New Zealand (NZ). The epidemiology of this disease has changed considerably in the country over the last three decades (Thornley et al., 2002). Notably, a decrease was observed in the relative importance in human infections of *Leptospira borgpetersenii* serovar (sv) Hardjobovis and *L. interrogans* sv Pomona and the emergence of *L. borgpetersenii* sv Ballum, a serovar traditionally associated with rodents and hedgehogs and not included in current animal vaccines (El-Tras et al., 2018).

Despite numerous studies describing the isolation of leptospires from mice and rats, the description of potential lesions in those studies is often absent or macroscopic only, and "there is a dearth of information on clinical disease in wild rodents" (Adler, 2015, p. 113). Laboratory rats, and less frequently laboratory mice, have been widely used as models of chronic leptospirosis (Richer et al., 2015) but studies of natural infections in free-ranging wild rodents are rare (Tucunduva de Faria et al., 2007). In rodents, experimental infections have been mainly conducted on inbred laboratory strains in controlled environments, usually via the intraperitoneal route, which does not reflect the natural infection route (Zilber et al., 2016), nor the genetic diversity of wild rodent populations and the wide range of conditions they encounter in nature (Pedersen and Babayan, 2011). When infected with the same isolate of *L. interrogans* sv Copenhageni, four wild-type mice strains commonly used in laboratory experiments (A, CBA, BALB/c and C57BL/6) reacted differently and exhibited different responses, in terms of antibodies and lesions, but also in terms of bacterial load in their kidneys (Santos et al., 2010). Surprisingly, despite hedgehogs being frequently identified as an important source of *Leptospira* infection (Ayral et al., 2016, Babudieri and Farina, 1964, Chomel and Riley, 2005, Farina et al., 1963, Mailloux, 1973, Plesko et al., 1960, Van der Hoeden, 1958, Wolff and Bohlander, 1965), only one experimental infection in this species is described in the literature. This study concluded hedgehogs were highly susceptible to infection with serovar Pomona (Webster, 1957). The impact of infection on the host and the quantity of bacteria that they shed shape their role as maintenance or non-maintenance hosts. Specifically, the quantity of bacteria shed and the relative role of each of those species in the maintenance of Ballum has not been investigated.

The aims of this chapter were to describe the gross and microscopic lesions in a population of wild rodents and hedgehogs sharing the same farm habitat in NZ and with known *Leptospira* infection status based on serology, PCR, culture and WGS results and to assess the *Leptospira* bacterial load in the kidneys of infected animals. Lesions and bacterial load were then compared across species and put in perspective with other laboratory test results to help better define and understand *Leptospira* infection and patterns of shedding in a community of maintenance hosts. These findings have the ability to inform the potential impact of leptospirosis on livestock and human populations in NZ.

Materials and Methods

Origin of animals and samples

The present research was conducted in accordance with the New Zealand Animal Welfare Act 1999 and the Massey University Code of Ethical Conduct. The Massey University Animal Ethics Committee approved the procedures conducted on all animals sampled as part of these studies (Protocol 16/93).

Animals used in the present study were captured and sampled within the frame of two studies: a cross-sectional study in a dairy (A) and a beef and sheep farm (B) (refer to Chapter 3) and a longitudinal study on the same dairy farm (refer to Chapter 4). In brief, five sessions of captures were organised, four on Farm A (A1-A4, twice a year in spring and autumn starting spring 2016 and ending autumn 2018), and one on Farm B in Autumn 2017 (immediately prior to A2). Blood and kidney samples were collected on each animal. Although both farms are in the Manawatū-Whanganui region, Farm A is in a coastal, sandy and flat area, bordered with an exotic pine forest, while Farm B is in a hilly area and bordered with parcels of native bush. Details on the capture and sampling methods are given in Chapter 3 and Chapter 4. At the same time that blood and kidney were sampled for serology, culture and PCR, tissue samples including kidney, liver, lung and heart were sampled and placed in 10% neutral buffered formalin for fixation and subsequent histopathology.

Leptospira infection status determination

All animals included in this study had previously had their *Leptospira* infection status assessed by serology, PCR and culture. Briefly, the presence in the serum of

antibodies against *Leptospira* spp. had been determined by Microscopic Agglutination Test (MAT) using a panel representative of all serogroups described in animals in New Zealand, *i.e. L. borgpetersenii* svs Ballum, Hardjobovis, Tarassovi and *L. interrogans* svs Pomona and Copenhageni. All animals with a titre ≥ 48 were considered seropositive for the corresponding serovar. The direct presence of *Leptospira* or *Leptospira* DNA in kidney had been tested by culture and a real-time PCR targeting the *lipL32* gene. Seroprevalence was determined with results of the MAT and prevalence was determined with combined results of PCR and culture. Details on the laboratory methods used were set out in Chapter 3.

In addition, kidney samples positive by culture or *lipL32* PCR had been typed using either single-locus sequence typing on amplicons of a PCR targeting the *glmU* gene, or by multilocus sequence typing (MLST) on the contigs obtained after whole genome sequencing of the isolates. Further details on the laboratory methods used are set out in Chapter 5.

Renal histopathology

After formalin fixation for at least 48 hours, the kidney from each animal was sectioned longitudinally. For mice and ship rats the whole kidney was embedded in paraffin; for hedgehogs, a section including cortex, medulla and pelvis was sampled and embedded in paraffin. For each kidney, two 4-µm-thick slides were cut with a microtome, deparaffinised in xylene and re-hydrated in distilled water, and subsequently stained by haematoxylin-eosin (HE) and Warthin-Starry (WS) staining methods.

For HE staining, slides were stained on a Leica® Autostainer XL using the following protocol: slides were immersed in haematoxylin for 4 minutes, rinsed with tap water for 2 minutes to remove the haematoxylin, and then stained with eosin for 2 minutes. Slides were then dehydrated in graded alcohols, cleared in xylene and mounted.

For WS staining, slides were immersed in 1% silver nitrate solution ($AgNO_3$) at 56°C for 45 minutes, then at 60°C for a further 15 minutes. During this time, a developer solution of 2% $AgNO_3$, gelatin and hydroquinone was prepared. Slides were immersed in the developer solution and agitated until sections were golden brown, briefly rinsed with warm water, covered in 5% sodium thiosulphate for 5 minutes, rinsed with tap water for 5 minutes, dehydrated in graded alcohols, cleared in xylene and mounted. A kidney sample from an alpaca diagnosed with renal leptospirosis by histopathology including WS staining and PCR methods (*L. interrogans* sv Pomona) was used as a positive control for each WS staining batch. According to this control, the staining process was repeated

when necessary. Readers were blinded to animal identity and other results (serology, culture and PCR) when reading the HE and WS slides.

Histopathological lesions in kidneys compatible with *Leptospira* infection on HE staining were recorded and scored according to their severity and extent, based on the method described in (Agudelo-Flórez et al., 2013). Renal lesions considered consistent with *Leptospira* infection were interstitial nephritis, particularly lymphoplasmacytic inflammation, glomerulonephritis, interstitial fibrosis, protein casts, tubular dilation or atrophy (Agudelo-Flórez et al., 2013, Tucunduva de Faria et al., 2007, Faine, 1999). Briefly, a score of '0' was attributed to kidneys with no lesions, a score of '1' for those with mild multifocal lesions (affecting <30% of the section), a score of '2' for those with moderate lesions (affecting 30-50% of the section) and a score of '3' for those with severe lesions (affecting >50% of the section). Sections with non-specific lesions (e.g. congestion) or showing only minor focal lesions were given a score of '0'. Sections of liver, lung and heart were also stained and the presence of lesions recorded.

Bacterial load assessment

A thorough examination of each WS renal slide was performed for all animals, and sections were considered positive when black staining organisms with spirochete morphology were observed within tubule lumens. For all WS positive slides, a visual assessment of the bacterial load in the cortex, outer medulla, inner medulla, and renal papilla (when visible on slide) was recorded, with three different levels: low, moderate and high load. A low load corresponded to slides where only a few leptospires could be counted or where leptospires were present in approximatively less than 10% of renal tubule lumens. A moderate load corresponded to slides where leptospires were present in approximatively 10% to 50% of renal tubule lumens. Finally, a high load corresponded to slides where leptospires were present in approximatively more than 50% of renal tubule lumens (Figure 6-4). Sections of liver, lung and heart were also checked for the presence of leptospires.

For positive *lipL32* PCR reactions, we used the cycle threshold (Ct) values as a proxy of *Leptospira* bacterial load in the associated kidney samples and compared it with WS loads. In a positive PCR reaction, the Ct value is defined as the cycle number when the fluorescence of a PCR product can be detected above the background signal. Ct values are inversely proportional to the amount of target DNA in the sample tested.

Results

Numbers sampled

Of 279 animals sampled, renal samples for histopathology were available for 259 animals: 207 mice, 32 hedgehogs and 20 ship rats. Of these, 98/207 (47.3%) mice, 14/32 (43.8%) hedgehogs and 5/20 (25%) ship rats were positive for *Leptospira* infection by PCR and/or culture. Of these positive samples, the infecting *Leptospira* strain had previously (Chapter 5) been typed as *L. borgpetersenii* sv Ballum for 62/98 (63.3%) mice, 5/14 (35.7%) hedgehogs and 2/5 (40.0%) ship rats, as *L. interrogans* sv Pomona for 3/14 (21.4%) hedgehogs, and as a mixed infection with *L. borgpetersenii* svs Ballum/Balcanica in 1/14 (7.1%) hedgehogs. The infecting strain remained un-typed for the remaining 36 mice, five hedgehogs and three rats. Details of numbers captured and sampled per session are presented in Table 6-1.

Table 6-1 | Number of animals captured and sampled per species and trapping session.

		Trap.nights (TN)	Captures (#)	Capture rate /100TN	Unique individuals (#)	Sampled for MAT, PCR and/or Culture (#)	Sampled for Histo (#)
MOUSE (*Mus musculus*)							
Farm A	A1	718	40	5.6	32	24	23
	A2	720	177	24.6	103	79	66
	A3	864	39	4.5	25	22	22
	A4	864	88	10.2	71	67	65
Farm B	B1	648	70	10.8	37	33	31
	TOTAL	3814	414	10.9	268	225	207
RAT (*Rattus rattus*)							
Farm A	A1	683	2	0.5	2	1	1
	A2	781	14	3.3	8	3	3
	A3	648	2	0.3	2	2	2
	A4	885	5	0.6	5	5	5
Farm B	B1	750	24	5.7	18	11	9
	TOTAL	3747	47	1.3	35	22	20
HEDGEHOG (*Erinaceus europaeus*)							
Farm A	A1	683	9	2.2	9	4	4
	A2	781	8	1.9	8	8	8
	A3	648	5	0.8	5	5	5
	A4	885	14	1.6	14	14	14
Farm B	B1	750	5	1.2	5	1	1
	TOTAL	3747	41	1.1	41	32	32

Gross and histological lesions present

Independently of their infection status, macroscopic lesions compatible with leptospirosis were not observed while 51 mice (24.6%, 95% CI = 18.9, 31.1%), 14 hedgehogs (43.8%, 95% CI = 26.4, 62.3%) and one ship rat (5.0%, 95% CI = 0.1, 24.9%) presented histological renal lesions compatible with leptospirosis. All lesions were graded as mild (score 1, affecting <30% of the kidney), and consisted of multifocal minimal to moderate and predominantly monocytic (predominantly lymphocytes and histiocytes) infiltration of the tubular interstitium in the cortex and perivascularly (Figure 6-1).

Animals positive for *Leptospira* infection by PCR, culture or WS showed either no or only mild histopathological lesions (see Figure 6-1). When compared with PCR, culture and WS, half of the 118 animals positive on at least one of these methods displayed mild lesions (10 hedgehogs, 48 mice and one rat), while the other half (4 hedgehogs, 51 mice and 4 rats) displayed no lesions. Four hedgehogs and three mice had lesions compatible with leptospirosis recorded and were negative to all tests (Table 6-2). For hedgehogs (the only species harbouring several *Leptospira* serovars), no difference between the lesions present with different infecting strains was observed, with 4/5 hedgehogs infected with Ballum showing lesions, 3/3 infected with Pomona showing lesions, and 3/5 with un-typed *Leptospira* spp. infections showing lesions. The hedgehog showing signs of dual infection Ballum/Balcanica presented no lesions.

Table 6-2 | Presence of histopathological lesions compatible with leptospirosis among animals tested positive or negative for *Leptospira* spp. on Warthin-Starry (WS), PCR and/or culture. *The percentage per column is indicated between brackets*

Lesion Score†	PCR and/or Culture	WS	*Erinaceus europaeus*	*Mus musculus*	*Rattus rattus*	Total
0	negative	negative	14 (44%)	105 (51%)	15 (75%)	134 (52%)
		positive	0 (0%)	1 (0%)	0 (0%)	1 (0%)
	positive	negative	3 (9%)	35 (17%)	3 (15%)	41 (16%)
		positive	1 (3%)	15 (7%)	1 (5%)	17 (7%)
1	negative	negative	4 (13%)	3 (1%)	0 (0%)	7 (3%)
		positive	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	positive	negative	6 (19%)	4 (2%)	1 (5%)	11 (4%)
		positive	4 (13%)	44 (21%)	0 (0%)	48 (19%)

† No animals sampled had lesions with a score of 2 or 3.

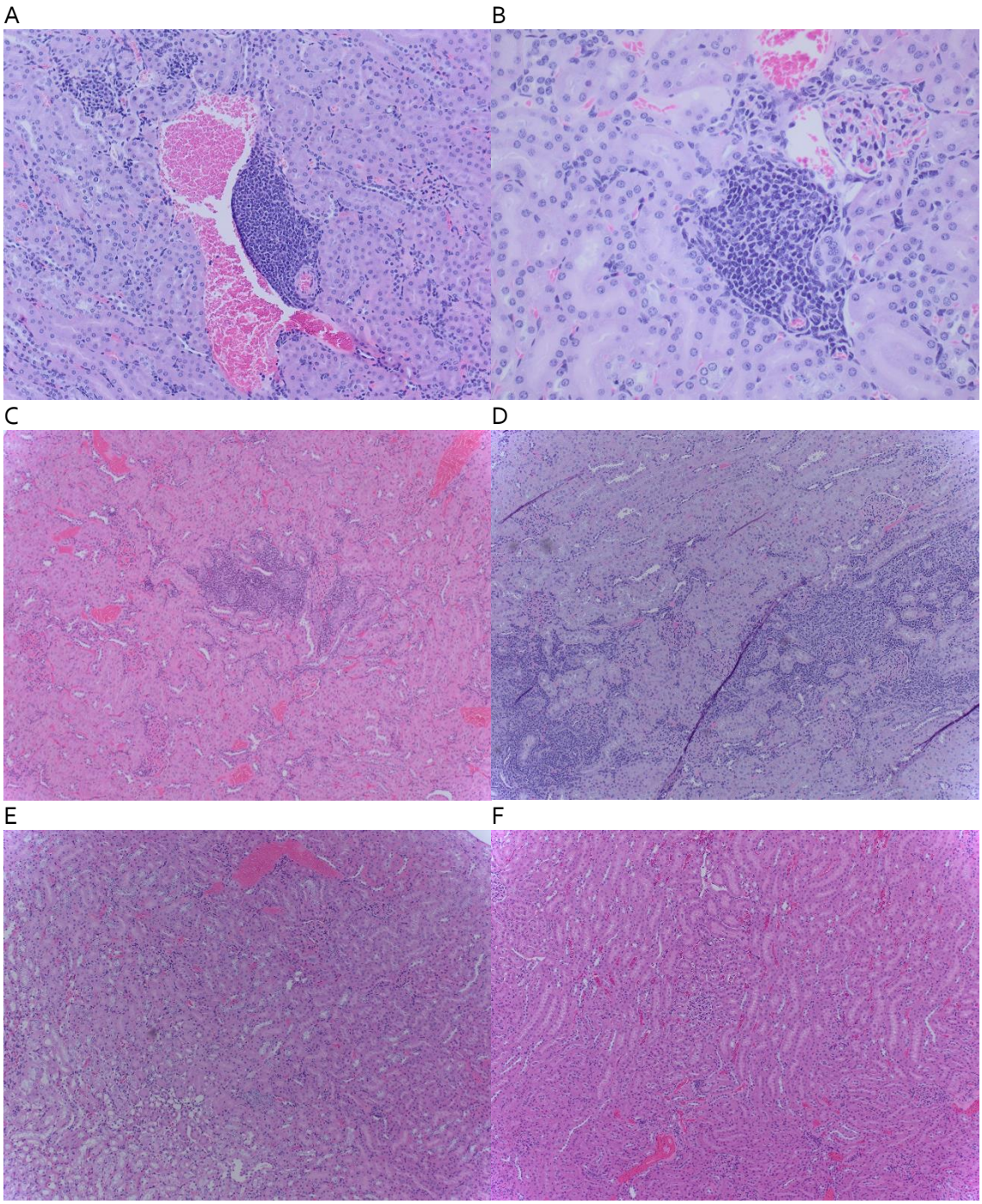

Figure 6-1 | Histopathological findings in the kidneys of animals naturally infected by *L. borgpetersenii* serovar Ballum (A-C, E, F), or *L. interrogans* serovar Pomona (D) on haemotoxylin and eosin (HE) staining. *(A) mouse with mild interstitial infiltration. HE. × 200. (B) mouse with mild interstitial infiltration. HE. × 400. (C) hedgehog with mild interstitial infiltration (infected with Ballum). HE. × 100. (D) hedgehog with mild interstitial infiltration (infected with Pomona). HE. × 100. (E) mouse without inflammatory foci. HE. × 100. (F) ship rat without inflammatory foci. HE. × 100.*

Presence of *Leptospira*

The prevalence of *Leptospira* infection (defined as the proportion of animals with a positive culture and/or PCR) is displayed in Figure 6-2. Adult mice and hedgehogs had a similar overall prevalence (respectively 56.6% and 54.6%, 95% CIs = 46.6, 66.2% and 32.2, 75.6%) that was higher than in juvenile or subadult animals sampled (Figure 6-2). Adult ship rats had a lower overall prevalence (22.2%, 95% CI = 6.41, 47.6%) than mice and hedgehogs, and only two subadults were sampled, one of them positive. Half of the ship rats tested came from Farm B where prevalence was overall lower (Table 6-3), with 21.2% [9.0, 38.9%] 7 of 33 mice tested positive, 1/11 rats and 0/1 hedgehog.

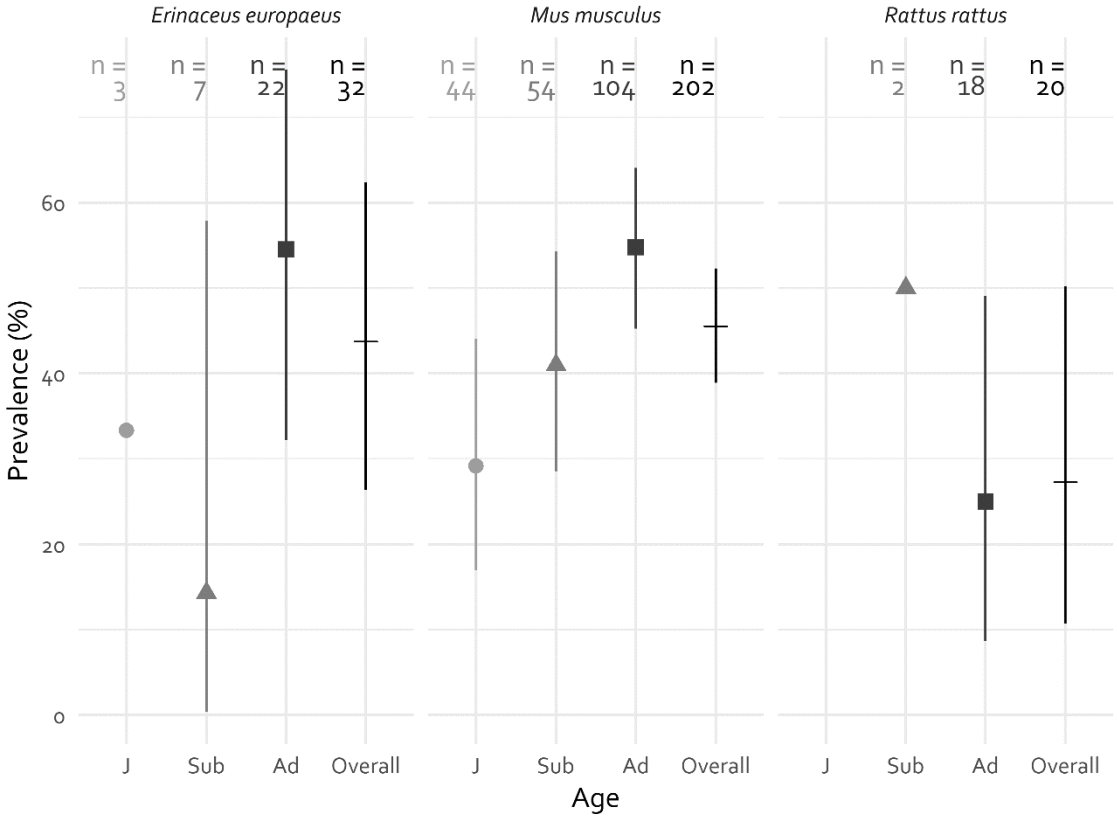

Figure 6-2 | Prevalence (%) for *Leptospira* spp. determined by kidney culture and/or PCR per species and age group *(J = juvenile; Sub = subadult; Ad = Adult)*

Table 6-3 | Prevalence for *Leptospira* spp. determined by kidney culture and/or PCR per species and session

Species	Session	#	Culture and/or PCR positive	Apparent prevalence	95% CI
Erinaceus europaeus	A1	4	1	25%	[1, 81]
	A2	8	3	38%	[9, 76]
	A3	5	4	80%	[28, 99]
	A4	14	6	43%	[18, 71]
	B1	1	0	0%	[0, 98]
Mus musculus	A1	23	20	87%	[66, 97]
	A2	79	25	32%	[22, 43]
	A3	22	20	91%	[71, 99]
	A4	67	30	45%	[33, 57]
	B1	33	7	21%	[9, 39]
Rattus rattus	A1	1	0	0%	[0, 98]
	A2	3	1	33%	[1, 91]
	A3	2	2	100%	[16, 100]
	A4	5	2	40%	[5, 85]
	B1	11	1	9%	[0, 41]

Agreement between PCR, Culture and WS

There was substantial agreement between the results of PCR, culture, MAT and WS staining (Cohen's $\kappa_{WS\text{-}PCR}$ = 0.61; Cohen's $\kappa_{WS\text{-}Culture}$ = 0.76; and Cohen's $\kappa_{WS\text{-}MAT}$ = 0.65). Sixty mice (29.0%, 95% CI = 22.9, 35.7), five hedgehogs (15.6%, 95% CI = 5.3, 32.8) and one ship rat (5.0%, 95% CI = 0.1, 24.9) had a positive WS result. All of these were also positive by culture and/or PCR except one mouse, which tested negative by PCR and for which culture results were not available (Table 6-4). No leptospires were seen in examined sections of liver, lung, and heart.

Bacterial load in kidney

Leptospira bacterial loads in the kidney as visually assessed on WS staining were higher for mice than for rats or hedgehogs. Hedgehogs and rats had only low loads recorded, while the majority of loads recorded in mice were moderate or high (Figure 6-3 and Figure 6-4). Although leptospires were present in different parts of the kidney, they were more often present within the tubules in the outer medulla (Figure 6-3). The bacterial load also appeared to increase as mice aged, with a high load recorded in the

outer medulla in 1/3 juvenile mice, 6/11 subadult mice and 28/46 adult mice, but numbers were insufficient to detect a significant difference. The bacterial load assessed visually in the outer medulla showed a strong correlation with the PCR Ct value (Figure 6-5).

Among the animals with a positive PCR, the mean PCR Ct value for animals positive by WS was 22.1 (SD = 3.7) but was 32.0 (SD = 3.2) for animals negative by WS, and except one rat, all animals that were WS negative had a PCR Ct value above 27.

Table 6-4 | Agreement between Warthin-Starry, PCR and Culture to detect *Leptospira* spp. in naturally infected hedgehogs, ship rats and mice

WS	PCR	Culture	*Erinaceus europaeus*	*Mus musculus*	*Rattus rattus*	Total
Negative	Negative	Negative	18	96	15	129
		Positive	1	8	0	9
		NA	0	12	0	12
	Positive	Negative	4	24	3	31
		Positive	4	6	1	11
		NA	0	1	0	1
Positive	Negative	Negative	0	0	0	0
		Positive	0	1	1	2
		NA	0	1	0	1
	Positive	Negative	0	4	0	4
		Positive	5	51	0	56
		NA	0	3	0	3

NA =Not Available

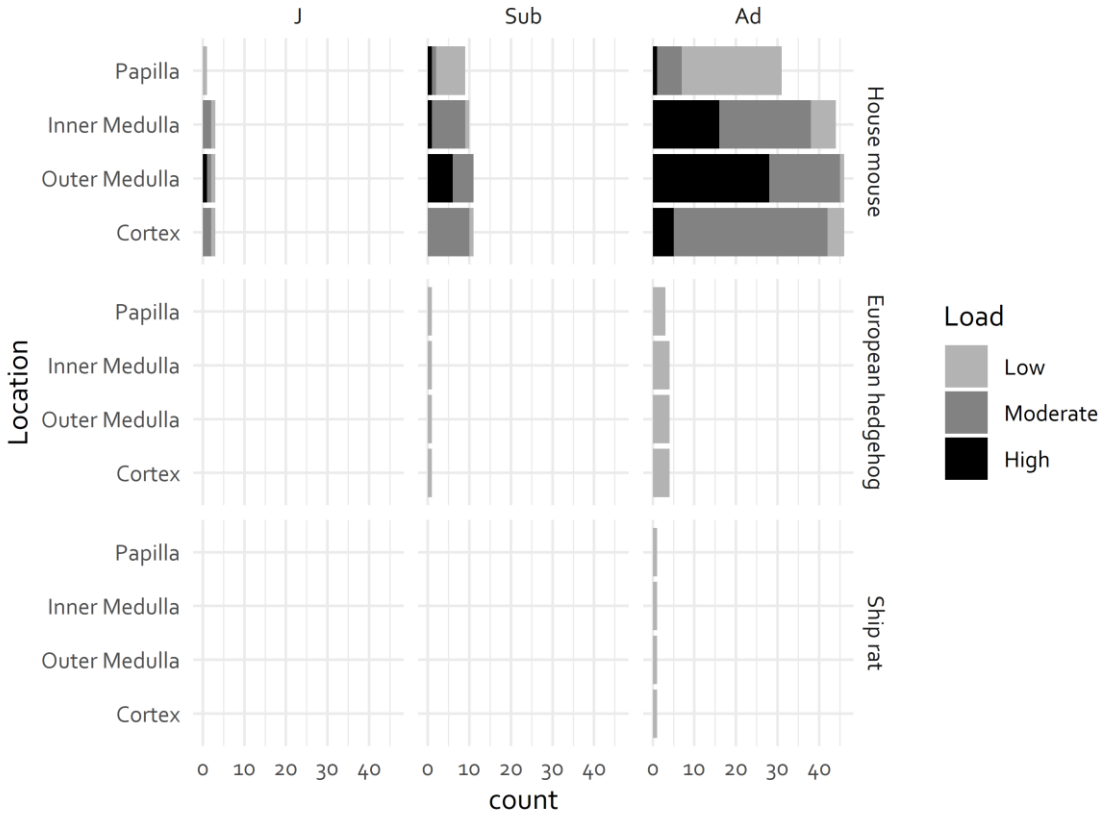

Figure 6-3 | Presence and load of *Leptospira* spp. in different locations of kidneys stained by Warthin-Starry. *The count of observations in papilla is lower because papilla was not always visible on the section*

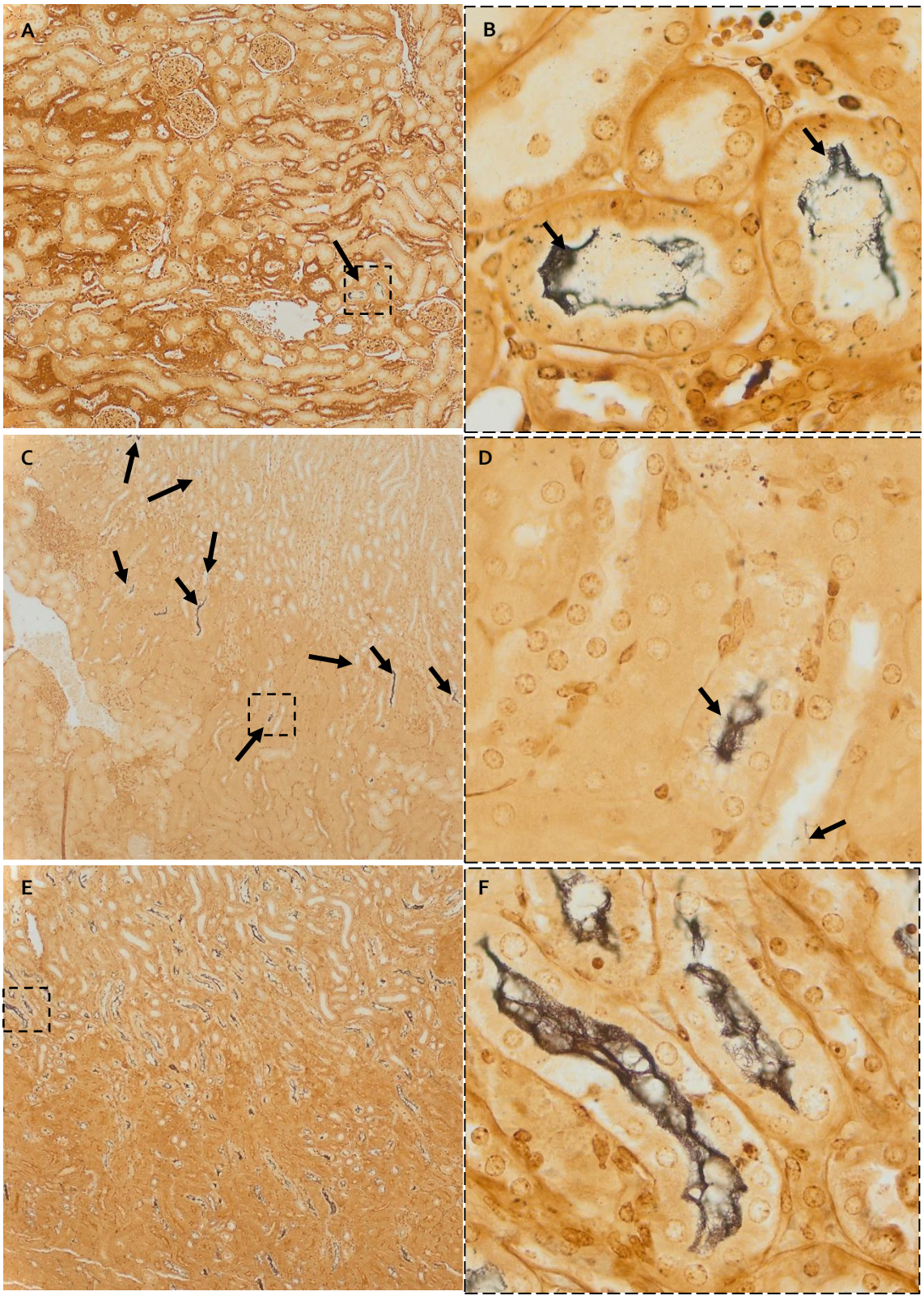

Figure 6-4 | Histopathological examination and bacterial load assessment of kidney sections of wild animals naturally infected with *Leptospira* spp. using Warthin-Starry (WS) silver stain. *(A-B) Low load of leptospires visible in approximatively less than 10% of tubules lumen. (C-D) Moderate load of leptospires visible in 10 to 50% of renal tubules. (E-F) High load of leptospires visible in more than 50% of tubules. (A, C, E) Low magnification × 100. (B, D, F) High magnification × 1000*

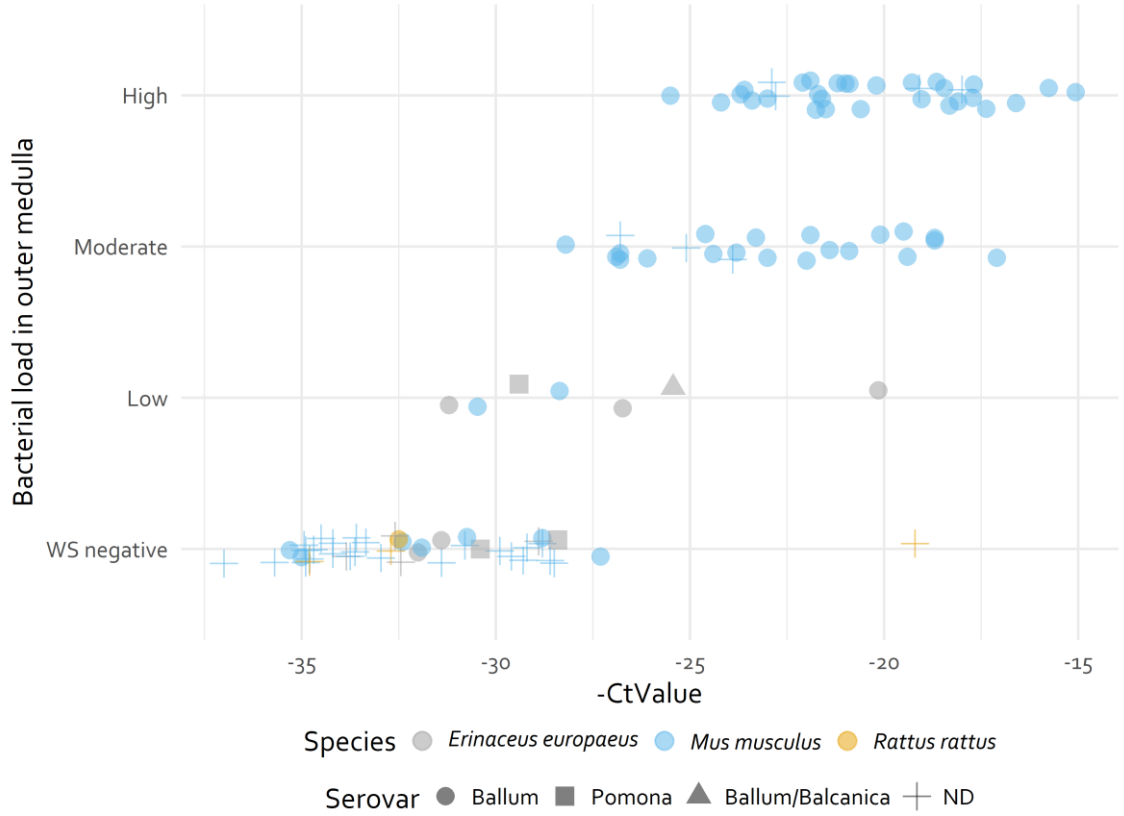

Figure 6-5 | Agreement between renal bacterial load assessed by real-time PCR and visually by Warthin-Starry (WS) staining in the outer medulla of wild animals naturally infected by *Leptospira* spp. (*i.e.* positive by PCR or culture). *As Ct values are inversely proportional to the amount of target DNA in the sample tested, -Ct is presented instead so that the bacterial load increases along the x-axis. ND = Not Determined*

Distribution of antibody titres

The seroprevalence for Ballum increased with age across species (Figure 6-6). While seroprevalence for Ballum was higher than prevalence in adult hedgehogs (72.7%, 95% CI = 49.8, 89.3%, see Appendix 9, Table S 9-2), seroprevalence was lower than prevalence for adult mice (45.2%, 95% CI = 35.4, 55.3%) and rats (16.7%, 95% CI = 3.6, 41.4%), due to rodents seronegative but positive by culture or PCR. Sixty-two percent of juvenile, 48% of subadult and 26% of adult mice that were positive by culture and/or PCR were 'silent shedders', they displayed no detectable antibodies against Ballum and, with the exception of two mice and two hedgehogs, exhibited no lesions, but also exhibited only undetectable or low loads of bacteria in their kidneys, as determined by WS (Figure 6-7). These seronegative and low shedding mice were present through all sessions (Figure 6-8), while seropositive mice had high titres (≥ 192) in all sessions and moderate bacterial loads in spring sessions (A1, A3) compared to high loads in autumn sessions (A2, A4, B1).

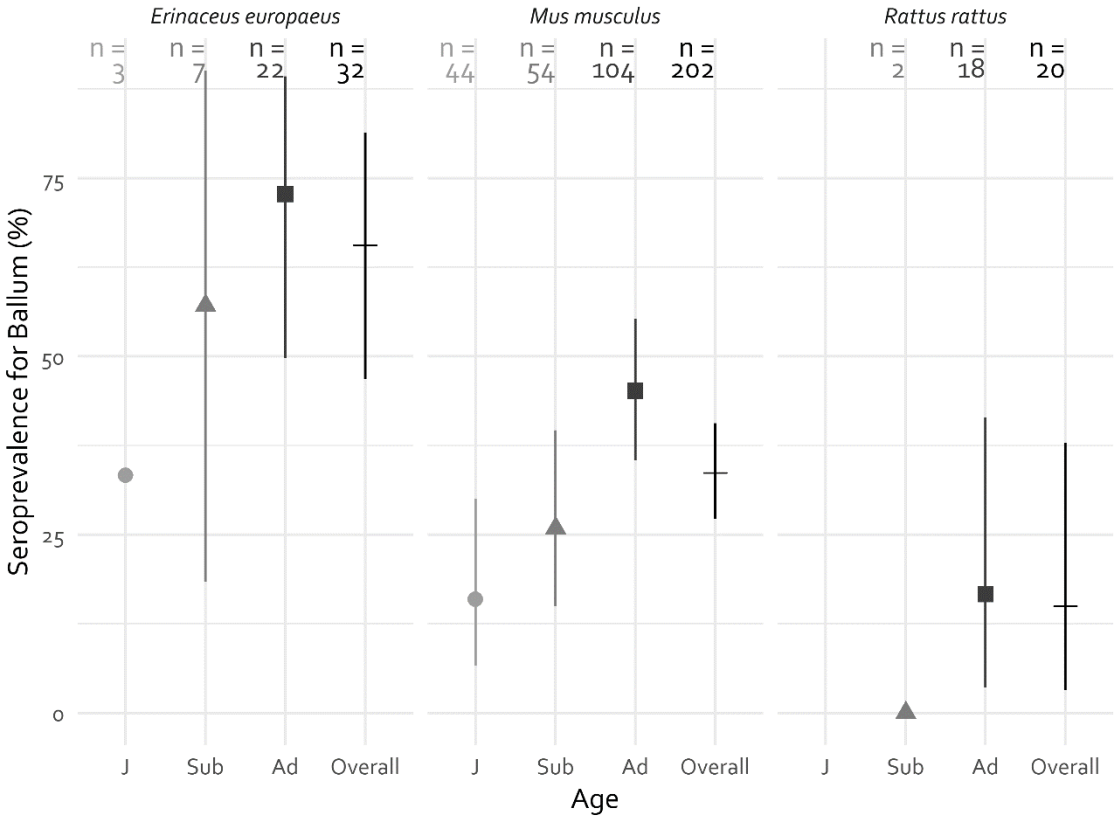

Figure 6-6 | Seroprevalence for *L. borgpetersenii* serovar Ballum across age groups and species *(J = Juvenile; Sub = Subadult; Ad = Adult)*

Discussion

In this study, we collated and placed in context the results of serological, microbiological and histopathological analyses investigating the infection by *Leptospira* spp., particularly *Leptospira borgpetersenii* sv Ballum, in a community of maintenance hosts, mice, ship rats and hedgehogs. In addition to being the first study to describe the renal lesions in NZ wild mammals with known *Leptospira* infection status, this study highlighted that despite a similar low level of lesions associated with infection across different host animal species, *Leptospira* bacterial loads were higher in the kidneys of infected mice than in ship rats or hedgehogs. These results suggest mice act as a key maintenance host and hedgehogs and ship rats are secondary maintenance hosts for Ballum, and therefore could form what Fenton et al. (2005) call an 'apparent multi-host maintenance community'. An 'apparent multi-host maintenance community' is a specific case where a pathogen spills over from a "key" maintenance host to a non-maintenance host in a sustained way. As a result, prevalence is high in both maintenance and non-maintenance hosts, which gives the appearance of a true multi-host maintenance community (Fenton and Pedersen, 2005). This could be the case between mice, rats and

hedgehogs since despite similar prevalence, only the former harbour high concentrations of leptospires in their kidneys.

The level of lesions assessed by histopathology was similarly low for all *Leptospira*-infected animals despite differences observed in bacterial load across species, underlining the inadequacy of pathology to assess maintenance/non-maintenance host status. Maintenance hosts have historically been associated with a 'chronic, largely asymptomatic infection', as has often been described in the literature on leptospirosis (Zuerner, 2015), but we suggest that a paradigm shift in this approach may be needed, as this study and others suggests that not all asymptomatically infected species necessarily act as maintenance hosts. Asymptomatic chronic infection in humans has for instance been described (Ganoza et al., 2010).

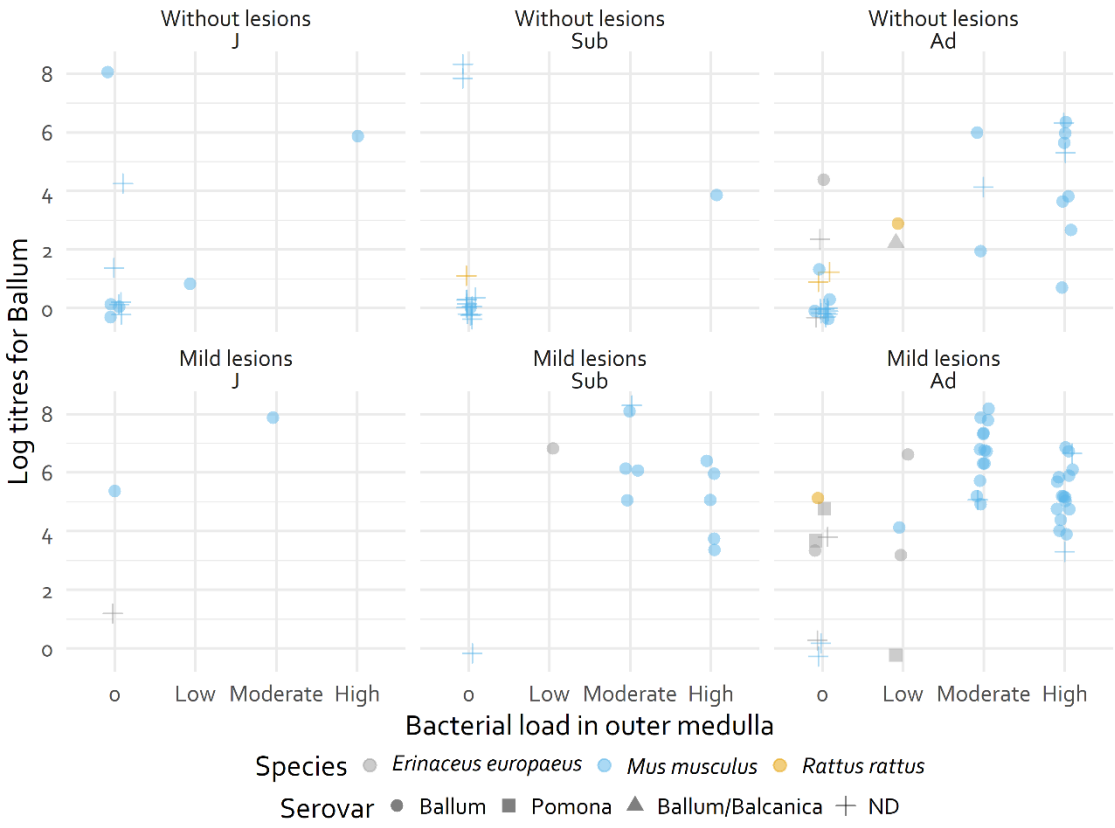

Figure 6-7 | Distribution among age and lesion groups of Ballum log-titres and bacterial load assessed visually by Warthin-Starry (WS) staining in the renal outer medulla of wild animals naturally infected with *Leptospira* spp. *Animals positive by culture and/or PCR with no leptospires detected by WS were attributed a load "o".*

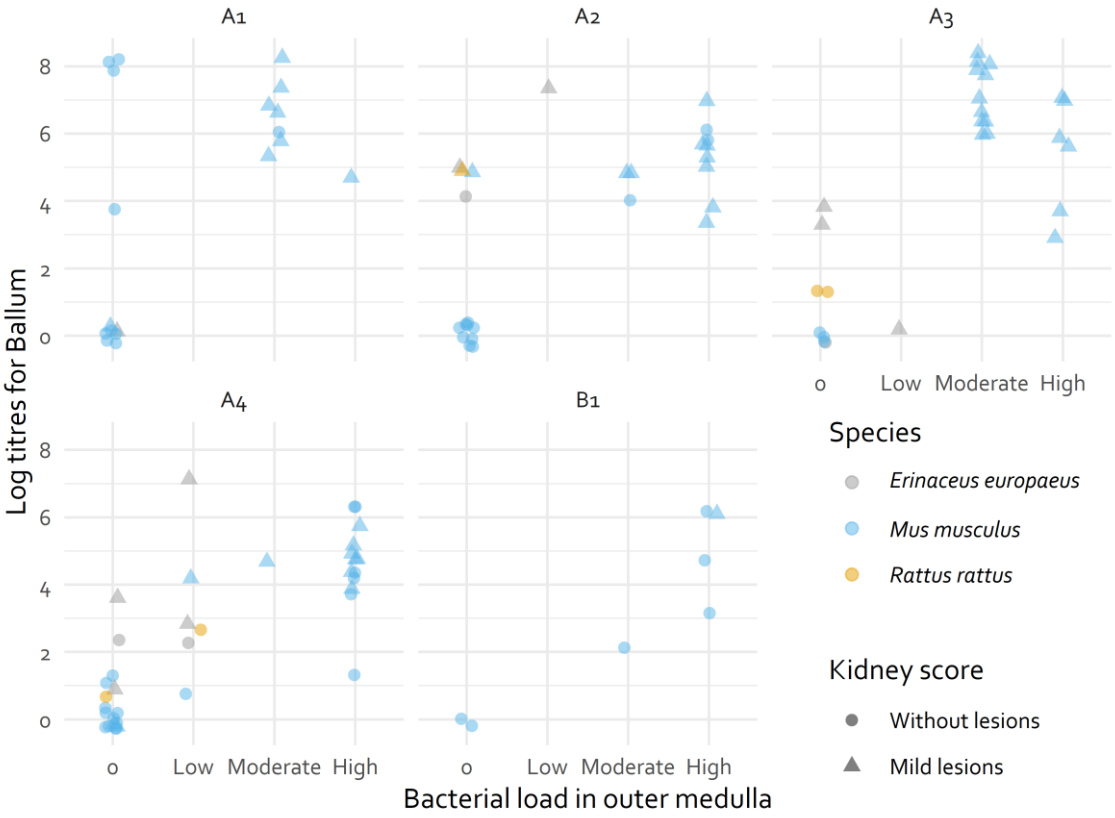

Figure 6-8 | Distribution per session of Ballum log-titres and bacterial load assessed visually by Warthin-Starry (WS) staining in the renal outer medulla of wild animals naturally infected with *Leptospira* spp. *Animals positive by culture and/or PCR with no leptospires detected by WS were attributed a load "0". A1 and A3 occurred in spring while A2, A4, and B1 occurred in autumn.*

Historically, Ballum has been linked to the house mouse (*Mus musculus*), considered globally to be the main maintenance host of this serovar. It was first isolated in a farm of the eponymous town in Denmark in 1943 from a field-house mouse (Borg Petersen, 1944). Several other isolations of Ballum were made in other house mice in Europe and worldwide (Wolff et al., 1949, Borg Petersen, 1944), especially in laboratory mice colonies (Yager et al., 1953). The first human case described was infected via a skin scratch from a laboratory mouse, and subsequent investigations of that laboratory mouse colony showed that 28/30 of the mice, all asymptomatic, were shedding this serovar (Borst et al., 1948). Several other species were shown to also harbour this serovar (Farina and Babudieri, 1957, Brown and Gorman, 1960, Yager et al., 1953, Ferris et al., 1961, Michna and Ellis, 1974, Higa and Fujinaka, 1976). Due to this diversity of hosts from which Ballum has been isolated and its wide geographical distribution, it is considered to be a 'generalist pathogen' (Hathaway, 1978). Nevertheless, these isolations were often made in animals trapped alongside house mice or sharing the same habitat, and

infections in other species were already considered unusual and often "closely associated with large [highly infected] house mouse populations" (Brown and Gorman, 1960). It is therefore possible those other species were secondary or spillover hosts of Ballum.

In NZ, Ballum was first isolated from calves, and subsequently, introduced hedgehogs (*Erinaceus europaeus*), ship rats (*Rattus rattus*) and brown rats (*R. norvegicus*) have also been shown to harbour this serovar (Ris et al., 1973, Brockie and Till, 1977, Hathaway and Blackmore, 1981a). Prior to the current work, only two studies investigated mice alongside other wild species in the country (Brockie, 1977, Hathaway et al., 1981), but mice share the habitat of the other hosts described in NZ. The possibility that hedgehogs are only secondary hosts of Ballum is illustrated by the fact that, to our knowledge, all mention of Ballum in hedgehogs occurred in New Zealand, except for one strain of Ballum (Kipod 88) isolated from an urban hedgehog in Tel-Aviv in 1957 (Van der Hoeden, 1958). In their original home range, the overlap between hedgehog and mouse habitats is limited to urban areas, but this is not the case in NZ where those invasive species are present virtually everywhere except in high altitude (King and Barrett, 2005). The absence of *Leptospira* isolates from rats or hedgehogs in other studies (Kakrada, 1999, Smith, 1964) is also an indication their role in the maintenance of Ballum could be less important than previously thought.

Interestingly, we found no difference in the level of lesions or bacterial load in hedgehogs infected with Pomona and Ballum, despite hedgehogs being described as clinically affected by Pomona, but maintenance hosts of Ballum in the literature (Webster, 1957, Brockie and Till, 1977). Susceptibility to disease and infection with Pomona was described in an experimental study (Webster, 1957), while maintenance of Ballum was suggested following the isolation of this serovar and the presence of antibodies in several hedgehogs captured in the wild with no macroscopic lesions (Brockie and Till, 1977). The experimental study conducted by Webster in the 1950s, used "some thirty hedgehogs [caught] in a suburban garden" in New Zealand and a local strain of Pomona isolated during an ovine outbreak, (Webster, 1957). "Every hedgehog contracted a clinical leptospirosis", and Webster described a "classical Weil's disease" in juvenile and sub-adult individuals, "almost invariably" fatal ("recurrent febrile peaks, icterus, meningitis, 'butterfly' lungs, and typical degenerative lesions in spleen, liver and kidneys"). Adult individuals would usually survive, develop high antibody titres and excrete leptospires in their urine "over a period" and some pregnant females reportedly aborted (Webster, 1957). Precise numbers of animals and details on the infecting route and dose, time of onset, duration of symptoms, antibody presence or *Leptospira* excretion were not given in the article.

When titres to Ballum were put in perspective with the bacterial load and the presence/absence of lesions across age categories, a portion of the population 'refractory' to infection emerged in mice (Figure 6-7). This portion of the population of naturally infected mice, was more predominant in adult mice, and these mice displayed no antibodies or lesions while harbouring low loads of bacteria in their kidneys. Soupé-Gilbert (2017) demonstrated that after experimental infection with Ballum, laboratory mice showed an increasing load of bacteria in their urine as early as 7 days post-infection (DPI) that plateaued after 63 DPI. No serological tests were conducted in that study. There are three possible reasons for the 'refractory' mice observed. They may represent animals with an early stage infection before they build an immune response and develop lesions. There is little information available in the literature on the timing of the serological response of mice to Ballum. In mice or rats infected with serogroup Pyrogenes (formerly '*L. Australis B*') or *Leptospira interrogans* sv Copenhageni antibodies appeared after 3-7 days (Zilber et al., 2016, Faine, 1962). In rats infected with *L. interrogans* sv Copenhageni lesions of chronic interstitial nephritis appeared after four weeks (Tucunduva de Faria et al., 2007). The presence of seronegative low shedders in the current study could also be due to a difference in the transmission route, but experimental studies investigating the impact of the transmission route on renal colonization and serological response showed for other serovars that all animals infected rapidly developed antibodies (Zilber et al., 2016, Birnbaum et al., 1972b). On the contrary, the absence or low serological response following infection with Ballum was noted even with intraperitoneal inoculation at the highest dose with this serovar (Hathaway et al., 1983). Lastly, it is also possible that mice show a different response to infection according to the immune status of their mother, and display a long lasting passive protection passed from their mother, as demonstrated experimentally by Birnbaum et al. (1972a, 1974) on mice infected with sv Grippotyphosa. Offspring of mice infected intraperitoneally with this serovar were conferred protection against shedding for a period of over a year and developed only low and short-lasting titres (Birnbaum et al., 1972a) which led to a cyclic shedding status among mice two-generations apart (Birnbaum et al., 1974). A modelling approach to distinguish these possibilities could help understand better the within-species transmission process.

In contrast to the usual presentation in histopathology manuals or to previous studies (e.g. Sterling and Thiermann, 1981, Cianciolo and Mohr, 2016), despite lesions being limited to the renal cortex, bacteria in the present study were principally located in the outer medulla. This unusual location for leptospires has already been noted in wild house mice infected with Ballum in Argentina (Rossetti et al., 2004), although the percentage of infected mice with lesions was higher in their study (68%, 13/19 *vs.* 48%,

48/99 in this study). Interstitial nephritis is not pathognomonic of an infection by *Leptospira*, and lesions observed are not necessarily linked to *Leptospira* and have been attributed to other pathogens like *Klosiella muris* (Songer (1983) in Rossetti et al., 2004), but are non-specific lesions seen with many different causes of renal injury. In addition, the temporality of lesions and infection is difficult to estimate, and the presence of lesions could be related to old, cleared infections. However, in this study, lesions were mostly seen in mice with a higher bacterial load, and only seven uninfected animals had lesions detected. It is also possible the apparently uninfected animals with lesions were in fact infected (false negatives), but the bacterial load was too small to be detected by PCR, or by culture or by WS staining.

In this chapter we used the Ct value of a real-time *lipL32* PCR as a proxy for *Leptospira* concentration in the kidney. This PCR was initially developed and validated for human diagnostics (Galloway and Hoffmaster, 2015) and limitations were discussed in Chapter 5 and Appendix 8 concerning the sensitivity of this PCR on kidney as a matrix. The standard curve built with *Leptospira* dilution mixed in a matrix of kidney DNA showed inhibition was likely at a Ct above 33 (Appendix 8). It was also not possible to weigh and dilute kidney samples in a precise 1:1 ratio of PBS (Phosphate Buffered Saline) when sampling in the field, and despite using the same volume of kidney slurry for each DNA extraction, the amount of kidney tissue used per PCR assay was imprecise and likely inconsistent. Particularly, entire mice kidneys and entire to half rat kidneys were used with a roughly equivalent volume of PBS while 1 cm^3 portions of hedgehog kidneys were dilacerated and diluted in 1 mℓ of PBS. As pre-made 1 mℓ aliquots of PBS were used, it is therefore likely the kidney concentration in the slurry used for culture and PCR was greater for hedgehogs than for rats than for mice. For these reasons, we did not attempt to calculate a concentration in genome equivalent (GE) per gram of kidney, but compared the Ct value with a paired visual assessment of the bacterial load assessed visually by WS in the other kidney (Figure 6-5). The agreement between both methods (qPCR Ct and WS) suggests confidence in the results presented. Despite a measurement bias towards a lower amount of *Leptospira* DNA in mice kidney, the observation of higher loads and lower Ct values in this species compared to rats and hedgehogs remains robust. The use of Ct values alone to estimate the bacterial load in kidneys of species with an expected low bacterial load however must be done with caution. WS slides showed leptospires were often concentrated in a limited number of tubules rather than disseminated in all tubules throughout the kidney and the sampling method probably impacts the probability to detect leptospires. This was observed by Rahelinirina et al. (2019) with PCR performed on either one or four kidney lobes of cattle in Madagascar, the result being more likely to be positive using four lobes. The same is probably true for

WS, with a higher probability to detect low bacterial loads when the number of slides or area of kidney examined increases.

It is also possible our sample was biased, with the most affected hedgehogs not being captured in our traps. However, this is considered less likely, as by CMR methods, we had previously shown the opposite, that the capture probability and home range were affected by infection status in mice, infected mice being more likely to be trapped than uninfected mice (Chapter 4). A variation in the capture probability of infected animals between species could hinder the comparison of observed prevalence and seroprevalence between species.

Another important consideration highlighted by the results of this study is the implication of a variable bacterial load for the assessment of prevalence. The bacterial load impacts the sensitivity of laboratory tests. We demonstrated that the result of the WS was directly related to the PCR Ct value, with a positive threshold for WS around a Ct = 27. Polymerase chain reaction and culture were also more likely to show disagreements when the WS was negative, *i.e.* the bacterial load was low. This aspect is often overlooked in epidemiological studies, and test sensitivity often given as a unique value, although a test sensitivity depending on the bacterial load would better describe the physiological and analytical processes taking place. Mice had a higher bacterial load in their kidney than hedgehogs and rats, although the small number of positive rats limits the comparison. The sensitivity of laboratory tests used to assess the status is thus likely to be lower for hedgehogs and rats, and low shedders in general. Several hedgehogs in particular were seropositive but negative by WS, culture or PCR, which could indicate false negative with a low bacterial load. Prevalence might be underestimated in hedgehogs, ship rats and species with a low bacterial load compared to mice.

A variable bacterial load among infected animals also has epidemiological implications in terms of quantity of bacteria shed in the environmental reservoir and each animal's contribution to the maintenance of the pathogen. We assumed that the bacterial load in the kidneys assessed by PCR or WS reflected the load of bacteria shed in urine. Using a similar PCR method, Costa et al. (2015b) found a significant positive correlation between the DNA concentration of *Leptospira* in paired samples of urine and kidney from brown rats. The amount of bacteria shed in the environment is dependent on the concentration of *Leptospira* in the urine but also on the volume of urine shed on average by the different species, and this volume was not assessed in this study. Studies modelling the amount of Ballum shed in the environment, integrating the abundance of each species and the volume of urine they shed would be necessary to further investigate the relative role of each species in the maintenance of Ballum.

In summary, the present chapter reports the results of histopathological examination of kidney tissues from wild mice, ship rats and hedgehogs naturally infected with *Leptospira* spp., and compares them with MAT, PCR and culture results previously recorded. Mild interstitial nephritis was the only lesion observed across those three species, but the bacterial load assessed by two different techniques (visually by WS or by real-time PCR) identified mice were shedding Ballum at higher levels than ship rats and hedgehogs. We suggest the role of rats and hedgehogs in maintenance and transmission of Ballum may be of lower importance than that of mice. Within mice, the description of a subgroup of low shedders, with undetectable antibodies and no visible lesions suggests heterogeneity in the maintenance function that requires further investigations.

“For millions of years, mankind lived just like the animals. Then something happened which unleashed the power of our imagination. We learned to talk and we learned to listen. Speech has allowed the communication of ideas, enabling human beings to work together to build the impossible. Mankind’s greatest achievements have come about by talking, and its greatest failures by not talking. It doesn’t have to be like this. Our greatest hopes could become reality in the future. With the technology at our disposal, the possibilities are unbounded. All we need to do is make sure we keep talking.”

— Stephen Hawking

Chapter 7

General discussion

Chapter 7.
General discussion

Introduction

In the introduction of this thesis, the increase in the proportion of human cases due to Ballum was highlighted, as well as the need to understand better the transmission pathways of this serovar. Ballum is associated with wild maintenance hosts, in a context where the focus of research and control measures had historically always been on serovars linked to domestic livestock species, Hardjobovis and Pomona.

The review of the literature in Chapter 2 identified that Ballum may spill over to livestock and that there is a scarcity of information available on the epidemiology of this serovar in New Zealand. Except for pilot studies related to this work, only one study was undertaken on wild species since the early 1980s. In addition, there was a 35-year gap in information on Ballum in livestock species due to serological tests being limited to Hardjobovis and Pomona in the studies conducted between 1982 and 2016. Recent studies in cattle herds showed a seroprevalence for Ballum higher than in the 1970s—6 to 13% *vs.* close to zero—and detected Ballum DNA in dairy cows by PCR and sequencing methods (Yupiana, 2019, Mannewald, 2016). The literature review also highlighted the lack of information on the ecology of invasive rodents in farm habitats, where the risk of spillover to cattle is present.

The aim of this thesis was to investigate whether wild mammals are an important source of *Leptospira* infection in livestock. Initially, a cross-sectional study was conducted in two different farm systems, a dairy and a beef and sheep farm, and both wild mammals and sympatric domestic animals present on farm were sampled. The presence and abundance of wild mammal species was investigated and serology (MAT), culture and PCR were used to assess the circulation of various serovars of *Leptospira*. This study led to the selection of mice, hedgehogs and ship rats as a model system to further investigate the reservoir dynamics of Ballum in a community of hosts, with a focus on the most abundant species, house mice. The combination of laboratory test results was used to estimate the true prevalence in this species using two different methods, latent class modelling and occupancy modelling. The similarity between the frequency distributions of *Leptospira* serovars among the species sampled was measured using a proportional similarity index (PSI).

Secondly, a longitudinal study was implemented in the dairy farm. Capture-Mark-Recapture methods were used on mice to develop a spatially explicit capture recapture (SECR) model and investigate the dynamics of Ballum prevalence and mice densities over time.

During those two studies, blood samples were taken and submitted to MAT. We also collected kidney and urine samples, for PCR, culture and histopathology. It was necessary to identify and type the strains circulating in the different species, and we used molecular methods for this purpose. Isolates and kidney or urine samples collected on both farms during the two studies were submitted to different sequencing techniques, with an increasing resolution. Single-locus typing targeting the *glmU* gene, MLST (covering 3 sets of 6-7 genes), cgMLST (545 genes) and whole-genome SNP analysis (>3000 genes) were applied to the PCR-positive kidney and urine samples or to the isolates obtained from cultures and sequenced by WGS. Specifically, with the high resolution offered by WGS—covering more than 99% of the bacterial genome—we investigated whether strains circulating within a species were closer genetically compared to strains circulating in other species. Knowing whether strains evolve in parallel in sympatric species or on the contrary are more similar locally than at a larger geographical or time scale can theoretically inform transmission pathways and potentially allow the identification of a common source of infection. Such type of source attribution study had not been reported to date for *Leptospira*.

Finally, the bacterial load and effects of infection on hosts were investigated using renal histopathology, and all results were combined to investigate the characteristics of infection and shedding in the community of maintenance hosts comprised of mice, ship rats and hedgehogs naturally exposed to Ballum.

Relevance and implications of findings

Summary of findings

Titres against Ballum were found in all species investigated during the cross-sectional study (Chapter 3), except possums. Ballum seroprevalence ranged between 4 and 20% in domestic animals depending on the age group and farm, and 0 to 50% in wild animals. Titres to other serovars were also detected in some species: Hardjo and Pomona titres were present with a high seroprevalence in sheep, cattle and dogs, but not in wild species, to the exception of hedgehogs for Pomona and possums for Hardjo, likely a cross-reaction with Balcanica. Hedgehogs had titres to all serovars tested. Mice and

rats had a high seroprevalence for Ballum, but not for other serovars. Latent class and occupancy modelling on mice results highlighted the underestimation of prevalence when prevalence is calculated from a single laboratory test, mainly because of the presence of 'silent shedders', *i.e.* seronegative mice with leptospires detected in their kidneys.

The SECR model built in Chapter 4 offered an insight on the dynamics of mice populations over time. Prevalence as measured by PCR and culture showed extreme variations over time, with the highest prevalence in Spring sessions, 83%, 95% CI [61, 95%] to 86% [65, 97%], and the lowest in Autumn sessions, 31% [21, 43%] to 37% [26, 50]. However, density estimates varied conversely, with 3.6 to 8.9 mice/ha in Spring and 27.1 to 55.8 mice/ha in Autumn, and as a result, the density of shedding animals was more constant over sessions, with 2.9 to 7.6 shedding mice/ha. Interestingly, in the final SECR models, the status of infection negatively affected the probability of capture and positive mice had a higher home range.

Genomic methods applied to the samples and isolates obtained from the two farms in the cross-sectional study, and from the dairy farm in the longitudinal study allowed for the typing of *Leptospira* strains circulating in the different species in both farms. Despite a higher resolution power of WGS, in terms of proportion of genome included in the comparison, the results of cgMLST and whole-genome SNP analysis added little information to MLST or single-locus typing. The genotype Ballum was identified in mice, ship rats, hedgehogs and beef cattle. Pomona was identified in cattle and hedgehogs, Hardjobovis in cattle only, and Balcanica in possums and red deer. One hedgehog showed signs of a dual infection with Ballum/Balcanica. No new strain was identified. Contrary to the use of MLST and single locus typing, whole genome SNP analysis ascertained that the strain identified as Ballum was a unique strain and not Arborea. This serovar belongs to the same MLST and cgCT as Ballum and was suspected in farmed deer positive by serology (Subharat et al., 2011b).

However, although sequencing techniques allowed distinguishing the different genotypes circulating in different species even at a low-resolution power, they were limited for within-strain comparisons. Even the whole genome SNP analysis that covered > 99% of the assembled genomes faced the issue of extremely similar genomes within a serovar, with 0 to 6 SNPs differences between NZ isolates of Ballum. Source attribution was not possible with such a low genomic diversity.

Histopathology results combined with serological and microbiological analyses confirmed that all three species investigated—mice, ship rats and hedgehogs—presented a similar low level of lesions. However, the bacterial load in kidneys assessed by visual scoring on Warthin-Starry staining and with the PCR Ct value was higher in mice than in

rats or hedgehogs, suggesting mice could be a key maintenance host while rats and hedgehogs be secondary maintenance hosts and therefore could form an apparent multi-host community rather than a true multi-host community.

Wild mammals as a source of *Leptospira* infection for livestock

Concomitant sampling in wild animals and sympatric livestock during the cross-sectional study developed in Chapter 3 underlined the presence of a serological overlap between domestic and wild species for Ballum in all species, and for Pomona in livestock, working dogs and hedgehogs. This result is at odds with investigations led in NZ in the late 1970s where this overlap was virtually non-existent. Seroprevalence for Ballum in livestock exposed to Hardjobovis or Pomona was around 1% (Hellstrom, 1978) and infections in livestock with Ballum were considered sporadic, . Despite being unable to isolate Ballum from livestock species, we found Ballum by PCR in the kidney of one beef cattle, as did Yupiana et al. (2019) in 13/4000 urine samples from dairy cattle tested by PCR. We also isolated Pomona from healthy hedgehogs, which is at odds with the description of a clinical disease in hedgehogs experimentally infected with Pomona (Webster, 1957), and with the results of Hathaway (1978) and Brockie (1977) who only isolated Ballum in this species. Webster described the presence of leptospires by dark-field microscopy in the urine of two hedgehogs trapped in a farm that presented high titres to Pomona (1957), but no isolation or typing was attempted, and only Pomona was included in the serology panel, so the nature of the infecting strains described by Webster is open to question. Neither the studies led in the 1970s nor the present work offer a representative view of the situation throughout NZ, as they were all limited to specific areas in the North Island. These results would need to be substantiated by additional studies, particularly in the South Island. With this caveat in mind, our results indicate the amount of Ballum leptospires available to livestock at a given point in time and space—what Plowright et al. (2017) call the 'pathogen pressure'—may have increased in the last decades.

Changes in agricultural practices observed the last four decades in NZ could lead to an increased risk of spillover for livestock. We demonstrated in Chapter 4 that mice densities can be as high around pastures as densities described in forest habitats in NZ. It is not possible to know if the densities of wild hosts have evolved in pastoral habitats during the last decades, but densities of dairy cattle have increased, and with them, dairy cattle exposure to strains found in wild hosts. In 1980, 2.97 million dairy cattle, 5.16 million beef cattle, 68.77 million sheep and 104,000 deer were present in the country for a population of 3.2 million people, while in 2016, 6.62 million dairy cattle, 3.53 million

beef cattle, 27.58 million sheep and 835,000 deer were raised for a population of 4.7 million people (source StatsNZ archive.stats.govt.nz).

The impact of livestock vaccination against Hardjobovis and Pomona on susceptibility to infection by other strains has not been studied *per se* in natural conditions in NZ. Hathaway suggested there could be "competitive exclusion" between strains to explain the nidality of different *Leptospira* strains found in different species in the same ecosystem (Hathaway, 1978, Hathaway, 1981c). With the long-term use of vaccination, he predicted Hardjobovis and Pomona would fade out. This competitive exclusion would disappear and this would make Hardjobovis- and Pomona-free livestock more susceptible to other non-vaccine strains. This relies on the assumption that vaccine programmes are complete and efficacious and there is no wild reservoir. Vaccines for dogs in Europe and the USA initially included serovars Icterohaemorrhagiae and Canicola but after several decades of vaccination, other serovars like Grippotyphosa and Pomona (USA) or Grippotyphosa and Bratislava (Europe) emerged in the canine populations and had to be added to the vaccines (Arent et al., 2013, Ellis, 2010). The uptake of vaccination to prevent livestock shedding was particularly strong in the early 1980s, when the vaccines began to be available, and it is estimated that 95% of dairy herds are vaccinated (Yupiana, 2019, Yupiana et al., 2017). Most of the pig farmers also comply, since vaccination is enforced in the swine industry (Lawrence et al., 2018). Vaccination of sheep, beef cattle and deer is thought to be marginal, around 10% (Sanhueza, 2016). Except for observations on changes in the relative importance of non-vaccine serovars following long-term vaccination programs, very little is known about within-host competition between strains. The disappearance of this competitive exclusion due to vaccination could explain the increase observed in Ballum seroprevalence in dairy cattle over time, but not in beef cattle and sheep that still show a high prevalence of Hardjobovis and Pomona alongside titres to Ballum (Vallée, 2016, Mannewald, 2016). Moreover, recent studies suggest mixed-infections are more common than previously thought, but are not detected by the usual methods of typing (Rahelinirina et al., 2019, Moseley et al., 2018).

Key and secondary maintenance hosts

In Chapter 6, we collated results of MAT, PCR, culture and typing with histopathology examination to better describe the infection by *Leptospira* spp., in particular *Leptospira borgpetersenii* sv Ballum, in the community of maintenance hosts formed by mice, ship rats and hedgehogs. Despite a high prevalence and low level of lesions in all three species, we found higher bacterial loads in mice kidneys compared to

rats and hedgehogs. The capacity for rats and hedgehogs to void Ballum in the environment and therefore participate in the transmission of this serovar appears limited compared to mice. We therefore proposed mice could be the key maintenance host while rats and hedgehogs are secondary maintenance hosts and form with mice what Fenton et al. (2005) call an 'apparent multi-host maintenance community'.

Distinguishing this apparent multi-host maintenance community from the situation where animals form a true multi-host maintenance community has important implications for transmission and potential control or intervention measures. Control measures targeted at secondary hosts and overlooking the key maintenance host(s) are likely to be ineffective. In the case of mice and Ballum, controlling infection in rats without controlling that in mice could even increase the transmission of Ballum, as they benefit from the removal of the former (Goldwater et al., 2012). Further studies investigating the presence of Ballum in rat and hedgehog populations in areas where mice are absent (*e.g.* on NZ offshore islands where rats and/or hedgehogs are present but where mice were never introduced or were successfully eradicated) could give insights on the capacity of those host species to maintain Ballum by themselves.

Intra-species transmission occurs via different routes including vertical, direct (sexual, via fighting), or indirect transmission (via the environment). Modelling studies have highlighted the variation in the relative importance of those different routes in different host species and for different strains of *Leptospira* (see below p. 180). An experimental study on Ballum (among other serovars) in mice showed that in experimental settings, the contamination of naive mice housed with infected ones happened only after sexual maturity (Hathaway et al., 1983). However, the high prevalence in juvenile and subadult mice tested in this study in natural farm environments highlight the possibility of vertical or environmental transmission in natural settings, and the relative importance of each route remains to be investigated.

However, inter-species transmission is more likely to happen via a contaminated environment, as vertical and sexual transmission are not possible routes, and direct contacts are rare, with the exception of predator-prey interactions. The prey-to-predator transmission of *Leptospira* has been demonstrated in a few species of carnivores (Shophet and Marshall, 1980, Reilly et al., 1970). But rats and hedgehogs are not predators of mice and even if scavenging and attacks have been documented between rats and mice the latter are not part of their diet (King and Barrett, 2005). So, controlling mice populations, and limiting contacts of other species with contaminated environments, or acting on this environment to reduce the viability of leptospires present could potentially be sufficient in controlling leptospirosis due to Ballum.

Livestock as bridge hosts between wild maintenance hosts and humans?

Risk factors for leptospirosis due to Pomona and Hardjobovis are reasonably well understood, and are related to direct contact with livestock and their urine, either on farm or in slaughterhouses (Sanhueza, 2016, Dreyfus et al., 2012a). Through the activity of milking and calf feeding, dairy farmers are more at risk of infection from livestock than other farmers who have less frequent contacts with their animals. Moreover, dairy farms are mostly located in flat, lowland or irrigated areas where the risk of exposure to *Leptospira* is higher (Sanhueza et al., 2015, Sanhueza et al., 2017). This is to be related to a longer survival of *Leptospira* in a low-declivity environment where water is more likely to stagnate, and therefore a higher level of exposure for livestock grazing in those areas.

Farmers account for half of the notified cases attributed to Ballum (Nisa et al., 2019a, Sokolova, 2019), and risk factors for this serovar are not so well understood as those for Pomona and Hardjobovis. Results presented in this thesis identified a seroprevalence for Ballum in cattle higher than previously reported, in accordance with recent results (Yupiana, 2019, Mannewald, 2016), but although some cattle had PCR positive samples genotyped as Ballum, attempts to isolate this strain from livestock were not successful in this thesis (Chapter 5). These results are supported by a previous study, where Ballum was identified in none of the 23 *Leptospira* isolates obtained from 399 sheep and 146 beef cattle sampled in a NZ abattoir—all were either Hardjobovis or Pomona (Fang, 2014). This suggests shedding levels of Ballum in cattle are low and/or intermittent, and the main route of contamination for farmers might not be through direct contact with cattle urine as for Hardjobovis and Pomona. If transient shedding of Ballum occurs after cattle are infected, identifying the window of time when cattle are shedding after infection, and determining when infection mainly occurs during the farm management seasonal cycle—and potential differences with different management options like autumn calving—would be of importance to better mitigate the risk of contracting leptospirosis by direct contact. The higher seroprevalence recorded in the younger cattle mobs in our study and in (Yupiana, 2019) suggests calves could represent a higher risk than adult livestock for farm workers. The epidemiology of notified human cases underlines the importance of occupations other than farm or meat workers in Ballum cases, and a higher proportion of women than in other serovars (Sokolova, 2019). Other sources of infection, and particularly indirect (environmental) contamination should also be investigated.

A need to adapt leptospirosis surveillance and control

The work presented in this thesis was motivated by the increase in the proportion of human cases due to Ballum, and the lack of recent, local information on this serovar in both wildlife and livestock, for which virtually no information was available for almost four decades (Chapter 2). Surveillance of human cases allow to monitor and identify changes in the occurrence of leptospirosis and the relative importance of the infecting serovars, but the identification of change is not in real time. Such a change in proportion of predominant serovars was recently described in humans in American Samoa, where a recent seroprevalence study described the emergence of three serovars, undetected in a previous seroprevalence study (Lau et al., 2012). The cause of the change remained unknown and there was no information on potential animal hosts for one of the serovars, LT 1163 (Lau et al., 2012). Being a zoonosis, leptospirosis would benefit from a more thorough surveillance involving not only human cases, but also domestic and wild hosts (Boadella et al., 2011). Although a continuous monitoring system for *Leptospira* infection in livestock and wildlife would be costly to implement and run, we recommend epidemiosurveillance of *Leptospira* serovars circulating in both wild and domestic mammals, for instance with surveys conducted on healthy animals at a frequency sufficient to detect a change in the main circulating serovars or the introduction of a new, exotic serovar. The introduction of a new serovar would indeed have important repercussion in the agricultural and public health sectors. In fact, *Leptospira* serogroup Australis was ranked in the top 3 and top 10 exotic pathogens in terms of risk for the human and livestock sectors respectively (McKenzie et al., 2007). An early detection and warning would allow to reduce the costs of control measures (OIE 2010).

Our results underlined the inadequacy of MAT alone for the surveillance and investigation of *Leptospira* infection in maintenance hosts, especially mice. The description of 'silent shedders' (Chapter 4) is not new: Hathaway (1978) mentioned 39/44 (89%) of isolations from brown rats, 8/12 (67%) isolations from ship rats and 2/2 isolates from mice were from seronegative animals, and for Brockie (1977) these numbers were respectively 4/8 isolates from brown rats, 3/4 from ship rats and 8/9 from mice. The proportion of 'silent shedders' in hedgehogs was smaller but also previously described: 1/9 isolates from seronegative hedgehogs in this study, 1/7 in (Brockie and Till, 1977), 0/5 in (Hathaway et al., 1981). This finding is not limited to known maintenance hosts, and low or no titres associated to a shedding state have also been described in cattle (Moinet et al., 2018), squirrels or opossums (Shotts et al., 1975). More generally, as noted by Hathaway (1978), titres to Ballum have frequently been described as much lower than

titres to other serogroups, for instance in possums (Hathaway, 1978), or in the cattle sampled in Chapter 3 and Appendix 3 and it is possible the antibody half-life is particularly short for this serovar. A rapid titre decay rate could explain the high seroprevalence and important number of individuals with a titre of 24 found in replacement stock in Chapter 3, (Moinet et al., 2019) and in (Yupiana et al., 2019b), for which seroprevalence for Ballum was respectively 12% [5, 23%] and 20% [11, 32%] in R1 and R2, 33% [23, 43%] in R1, and 73% [58, 85%] in R1 after vaccination with the same cut-off at 48, and the proportion of individuals with a titre of 24 was respectively 28% in R1 and 32% in R2, 32% (29/92, data not shown) in R1, and around 20% in R1 after vaccination. The half-life of Pomona and Hardjobovis antibodies has been estimated in sheep (Vallée et al., 2015), but the half-life of Ballum antibodies remains to be studied.

Paradoxical reactions, where the highest MAT titres are detected for a serogroup different from the infecting one are common, and hinder the identification of the infecting serovar (Levett, 2001). Potentially paradoxical reactions have been reported for Ballum, with Ballum isolated from the liver, kidney and urine of a dog showing clinical and serological signs of infection with Canicola (Grabinski 1967 cited in Michna and Ellis, 1974). In other instances, investigations into potentially new serovars exotic to NZ like Canicola or Arborea detected by serology in human or animal cases failed to effectively isolate those serovars and these could be paradoxical reactions too (Frazer et al., 2012, Subharat et al., 2011b). The use of serology alone in surveys targeting Ballum should therefore be avoided, and the use in conjunction of other methods allowing for typing, such as genotyping should be preferred.

The Ministry of Health definition of a confirmed case of human leptospirosis includes the "detection of leptospiral nucleic acid from a clinical specimen" (Ministry of Health, 2012). Despite the recommendation to undertake both nucleic acid testing (NAT) and MAT, cases are often confirmed with one test only. Between 2013 and 2017, the proportion of cases confirmed by MAT (alone or with NAT) decreased from 80% to 63% while the proportion of cases confirmed by NAT alone increased from 20% to 37% (Synthetized from ESR 2014, 2015, 2016, 2017, 2019). If this trend continues, the information on the infecting serovar will stop being available for epidemiologists and change in the relative importance of endemic serovars will be more difficult to objectify. The absence of Ballum or Tarassovi in the MAT panel of serological surveys conducted on cattle between the 1980s and the 2010s led to the silent evolution of these serovars. To avoid the same issue in the future, incentives should be provided for genotyping to be conducted on positive NAT whenever possible. This implies using PCR primers that allow the distinction of all circulating serovars (Nisa et al., 2019b). In New Zealand, all confirmed cases should be referred to WorkSafe for occupational investigations

(Ministry of Health, 2012) and the information on the infecting serovar would be of interest to identify the possible source of infection.

The present work identified mice, rats and hedgehogs as overlooked sources of Ballum infection for domestic animals and people. Control and preventive methods against human leptospirosis tend to reflect the epidemiology of Pomona and Hardjobovis rather than the epidemiology of Ballum. Livestock vaccination against Hardjobovis and Pomona to prevent shedding has been a mainstay of leptospirosis control (Ryan et al., 1982). Through this work and the research conducted by Yupiana (2019), there is evidence of livestock shedding Ballum at a very low prevalence, probably in low concentrations, and it is unsure if the adaptation of vaccines to cover this serovar is worth the investment given the current knowledge. More information should be gathered for this serovar on shedding patterns of livestock, especially young stock—for instance with an experimental study—and risk factors for humans—a case control study is currently underway—before deciding to develop a vaccine against Ballum in livestock species.

Indeed, contact with wild hosts or with a contaminated environment might be the main source of infection, and therefore the main area to target for control. A recent study analysing the risk factors associated with Ballum in human cases notified between 1999 and 2017 in NZ identified occupation as a main factor. There was an odds ratio (OR) of 0.05, 95% CI [0.02, 0.13] for meat workers compared to farm workers, and an OR of 2.61 [1.64, 4.14] for 'other' occupations (neither farm nor meat workers) compared to farm workers (Sokolova, 2019). In other words, other (supposedly non-risk) occupations and farmers are more at risk of contracting Ballum than meat workers. This suggests the source of contamination is not at the meat works and may not be related to direct contact with farm animals but could be due to indirect exposure. Farmers should therefore be reminded that leptospirosis can be transmitted through a contaminated environment and not only by contact with their livestock.

Leptospirosis control programs like LeptoSure® targeting dairy farmers (Cranefield and Keown, 2019) or the Quality Assurance programme led by the NZ Pork Industry Board for pig farmers, maintain awareness in the agricultural sector. There is a need to also raise awareness for people involved in predator control operations (see discussion p.96 on that aspect) and 'non-risk' occupations. However, as stated by Sokolova (2019), the non-risk occupation group encompasses a very diverse set of occupations and further work would be needed to refine the risk factors within this group. It is likely water-based leisure activities are also a risk, as in numerous other developed countries (Mwachui et al., 2015, Guillois et al., 2018, Nardone et al., 2004, Lau et al., 2010) and should be targeted for prevention. New Zealand outdoors and beautiful

scenery attract an increasing number of tourists, 3.86 million visitors in 2018 (source http://archive.stats.govt.nz) and possible cases of leptospirosis in this population are not collected in NZ. New Zealand was listed as one of the main destinations of Australian tourists returning with leptospirosis acquired internationally (Lau et al., 2010).

Awareness among general practitioners should also be increased on leptospirosis risks for people not in contact with livestock, because under-diagnosis and under-reporting is probably more important for people in occupations not usually considered at risk. Previous studies estimating the rate of under-diagnosis used data on the risk of influenza-like illness associated with *Leptospira* seropositivity and focused on populations of meat-workers with serological data for Pomona and Hardjobovis (Dreyfus et al., 2015a) or used the number of cases notified to ESR in the 'other' occupation group—an estimate already potentially biased by under-reporting—because no data on influenza-like symptoms was available in this group (Sanhueza et al., 2019). In the human cases notified to ESR between 1999-2017, the number of hospitalised cases was higher than the number of non-hospitalised cases since 2009, and the proportion of hospitalisations was the highest (61%, 95% CI = 55, 67%) within the 'other' occupation group (Sokolova, 2019). Using hospitalisation as an indicator of severity, this suggests diagnosis may be biased towards the most severe cases in this 'other' occupation group and therefore indicates a high level of under-diagnosis.

Specific recommendations to farmers

Several recommendations can be given to farmers to limit the risk of contamination due to Ballum:

- Continue vaccination of their livestock against Hardjo and Pomona but be aware that the risk of shedding other serovars, albeit lower, is not null. Twenty-six percent of vaccinated dairy herds showed evidence of at least one animal shedding *Leptospira* in a recent cross-sectional study (Yupiana, 2019).
- Limit contacts with a contaminated environment. Grazing of livestock in flooded pastures should be avoided, and in those areas, interventions should be aimed at increasing water drainage efficiency to limit *Leptospira* survival in the environment. In that aspect, regenerative farming practices that increase soil drainage in pastures (Batemana and Munoz-Rojasa, 2019) seem to be an interesting option to consider. The impact those practices may have on *Leptospira* survival and subsequent livestock or human exposure has, to our knowledge, not been studied. A recent systematic review looking at how land

management practices can impact different diseases found a scarcity of studies on the subject and none for *Leptospira* (Lugassy et al., in prep).

- Understand the biology, ecology and behavior of rodents and hedgehogs and how control strategies might impact them. While rodents can be attracted to artificial food resources like palm kernel used as supplementary feed for milking cows, it will attract mostly rodents for which the food is within their home range. This is what was observed in the CMR study in this thesis (Chapter 4) where traps around the palm kernel shed did not attract more mice than other traps in the two grids deployed. Ruffino et al. observed a similar pattern with ship rats using locally available resources rather than moving to adjacent habitats with higher-quality resources, and suggested rat mobility was restricted by intraspecific interactions (2011). This means poison baits should not be limited to specific areas but spread over the area to cover all individual home ranges. This also means that, counterintuitively, the exposure to rodents potentially shedding leptospires may not necessarily be higher around supplementary feed.
- Limit suitable habitats for wild hosts near pastures and farm buildings, and secure water reserves. House mice favour sites with long grass (Whitehead et al., 2014) and trimming grass around pastures—*i.e.* where livestock has no access—could be an efficient way to reduce available shelter and limit mice densities. Specific efforts should be made to prevent rodents and hedgehogs accessing water reserves intended for livestock, and all the more for human consumption. Fencing all pastures against rodents and hedgehogs is not a realistic measure, but the use of deterrents in key areas could be investigated. Artificial light has for instance been shown to impact mice foraging behaviour, who avoid illuminated areas (Farnworth et al., 2016) and could be used around farm buildings, animal feed or drinking troughs.
- Monitor rodents and hedgehog numbers (*e.g.* with kill-trap numbers or tracking tunnels) and implement pets control measures when densities are low. When mice are seen during the day, it is a sign numbers exceed natural carrying capacity and already too late for an efficient control (J.C. Russell, pers. comm.). Results of the longitudinal study presented in Chapter 4 suggest the densities of mice decrease through winter and were lower in early spring while the prevalence of Ballum was highest, before young uninfected individuals are recruited in the population. Control strategies targeting the adult mice during winter are likely to be the most efficient for both mice and Ballum control. Most

rodent control measures have been devised for rats in NZ and are not well adapted for mice but targeting only rats is likely to have undesirable consequences on mice populations. Due to their high remanence in the environment and the development of resistance, the Department of Conservation (DoC) stopped using anticoagulants like brodifacoum on the mainland and recommend 1080 (sodium fluoroacetate) for rats, but its efficiency on mice appears limited (Jones, 2019). Efficient traps and effective toxins suitable for mainland use have been identified as priority research needs for mice and hedgehogs (Jones, 2019). Internationally, rodent control at low densities is recommended and chemical control even forbidden at high densities to prevent secondary poisoning (Coeurdassier et al., 2014).

- Facilitate the development of a 'landscape of fear' as a mitigation strategy to the use of poison. This term was coined by ecologists to describe the impact predators have on their prey's behaviour, and on their use of their environment. The presence of domestic predators of rodents like cats and dogs can deter foraging activities in rodents (Mahlaba et al., 2017) and therefore their ability to spread leptospires. However, mammalian predators can also be infected by Ballum and participate in the dissemination of the bacteria (van de Pol, 2016, Shophet, 1979a), and only decrease the seeing of rodents, not their densities (Parsons et al., 2018). Moreover, in the specific context of NZ, most mammal predators, including domestic cats, also have a dire impact on native birds, and choosing to foster mammalian predators would come with a cost in terms of conservation. Fostering native bird predators like the morepork (*Ninox novaeseelandiae)* could be an alternative. Installing perches for raptors is for instance one of the actions recommended for a sustainable control of water voles in areas where this species impact the pastoral sector (Coeurdassier et al., 2014, Delattre and Giraudoux, 2009). Other birds like weka (*Gallirallus australis*) could also be used as potential control predators. Behaviours of predation on animals as large as brown rats and stoats have been described for this species (Beauchamp and Miskelly, 2017), and in Russell peninsula where the abundance of weka increased following a reintroduction programme, populations of mice, brown rats and hedgehogs appeared to decline simultaneously (Ough Dealy, pers. comm.). The swamp harrier (*Circus approximans*), the kingfisher (*Todiramphus sanctus*) and the falcon (*Falco novaeseelandiae*) are other native birds known to prey on mice (King and Barrett, 2005).

Chosen methodology and impact on conclusions

Study design

Farm selection, timespan of study. Animals sampled in this thesis originated from two farms in the Manawatū-Whanganui region. For convenience, those farms were selected near Massey University (less than 1h driving), from a pool of farms that had previously been involved in studies on livestock diseases, and on which leptospirosis due to Hardjobovis and Pomona had been identified in livestock and humans (Benschop et al., 2017, Yupiana et al., 2019b, Vallée et al., 2015, Dreyfus et al., 2018). The absence of a random selection introduces a selection bias, as the two farms selected are not representative of New Zealand dairy and beef and sheep farms. There is a bias for farms prone to leptospirosis, that are likely to provide better conditions for the spread and survival of all serovars of *Leptospira*, thus the prevalence observed in the two farms should be interpreted as prevalence in an endemic area.

The timespan chosen for the longitudinal study described in Chapter 4 (2 years) is relatively short, and the frequency of sessions (twice per year) is also limited. The choice of such a study design is to be linked with limited resources available for fieldwork (both human and financial) and with the inherent limited timeframe of a PhD. Rodent population are known to experience a high interannual variability, with density peaks linked to seed mast events (King and Barrett, 2005). Other than food resources, interannual variations in climate can also influence both mice population dynamics and Ballum epidemiology. The short timespan in this study does not incorporate this variability, results are therefore likely to be a reflection of the specific conditions of the year. Sampling over a longer time scale would be necessary to encompass those interannual variations. But at the same time, mice are short-lived species and an annual time scale is not adapted to have a comprehensive picture of how densities and prevalence evolve within a population over time. We conducted two sessions per year, at the end of winter, before the recruitment of new individuals in the population, and in autumn, when mice born during summer are present in the population. This is a minimum, and resolution would have increased with a higher number of sessions. We described in Chapter 4 the dynamics of density and prevalence in Spring and Autumn, and it is possible maxima and minima in those parameters are reached at another period of the year. It is common when investigating mice population dynamics to implement four sessions throughout the year (King and Barrett, 2005, Bridgman et al., 2018, Choquenot and Ruscoe, 2000). Even if increasing the number of sessions would have

been possible given the funding and time constraints, this study implied the removal of all individuals trapped during the second phase of each session and increasing session frequency would likely have impacted the population dynamics, with an increased external recruitment (migration of mice from areas surrounding the trapping areas).

Trapping and sampling methods. Live-trapping was used in this study, which is different from previous studies where snap-traps (Fabri, 2016, van de Pol, 2016), a mix of live-traps and snap-traps, or night-shooting were used (Hathaway, 1981c, Brockie, 1977, Brockie and Till, 1977). Samples were taken immediately before (blood) or after (kidneys) euthanasia and kidneys were cultured immediately after sampling. In previous studies, kidneys were cultures in the laboratory, several hours after death (Fabri, 2016, van de Pol, 2016, Brockie, 1977, Brockie and Till, 1977), or at variable times, either immediately after death for animals live-trapped, or the following morning for animals killed in snap-traps or shot during the night (Hathaway, 1981c). Postmortem changes in kidney samples impact the survival of leptospires, and the delay between death and culture can impact the success of isolation, as well as the temperature at which samples are stored. "Leptospires survive well at 4°C but are rapidly killed in tissues held at 20°C or even more rapid at 30°C to 40°C" (Hartskeerl et al., 2006, p. 35). As described in Chapter 5, the rate of isolation in this study was high, with 66% (67/102) of PCR positive samples being successfully isolated. This high isolation rate could be due to the virtually non-existent delay between sampling and culturing and could impact the comparison with other studies where this delay was longer, especially for species harbouring a lower number of viable organisms like hedgehogs and ship rats. For instance, the isolation rate was 19% [13, 27%] in PCR-positive livestock kidneys sampled at an abattoir (Fang, 2014, Chapter 5). It is therefore possible a part of the increase observed in prevalence between studies from the 1970s and the present work is due to the methods used, and not a real increase. However, the increase in seroprevalence for Ballum observed by MAT in cattle between the 1970s and 2010s indicates the exposure to this serovar has increased, so the possibility for a real increase in prevalence in wildlife should not be ruled out.

During sessions A2 and B1, a field volunteer forgot to sample kidneys in formalin for histology. As a result, 10 mice and two ship rats could not be included in the analysis presented in Chapter 6. One seronegative rat was culture negative but PCR positive, two seropositive mice were PCR and culture positive and another mouse was seropositive but PCR negative. All others were negative for all tests. Session specific and overall prevalence and seroprevalence were calculated with all animals for which a PCR/Culture or MAT result was available to prevent any bias. It is unfortunate one of the omitted rats was positive, as sample size was small for this species. This omission might impact the

overall proportion of animals with/without lesions but is unlikely to change the conclusions drawn in this chapter.

Impact of phase I and II on samples. Trapping sessions were partitioned in two phases, a first phase of capture-mark-recapture allowing for the densities to be estimated, and after five days, a second phase of sampling, where captured animals were removed from the population and sampled, to test for the presence of *Leptospira* in captured individuals. During the first two sessions on Farm A, and the session on Farm B, this protocol was trialled on all captured species, but the rate of recapture for rats was low, and null for hedgehogs, and prevented us from getting samples for laboratory tests, so we decided to narrow this protocol to mice only in the subsequent sessions. Such a protocol has never been applied to the study of *Leptospira* infection in populations of wild rodents. A protocol close to ours, and, to our best knowledge, the only other example of CMR applied to *Leptospira* epidemiology, used CMR before and after kill-trapping to investigate the impact of culling on *Leptospira* carriage in urban brown rats (Lee et al., 2018). However, they were sampling urine throughout the CMR phase and were therefore able to access the infection status of released individuals. Given the results obtained in Chapter 4, and even if it comes at the expense of a more complicated organisation in the field, we would recommend a protocol using a random removal/sampling throughout the CMR session to estimate densities of infected rodents. Indeed, the protocol we devised used a CMR followed by removal/sampling to estimate densities of infected rodents, and our results indicate the serological status of animals is not random over the time of trapping. We may therefore have a sampling bias leading to a possible overestimation of the seroprevalence with this protocol, as seropositive mice were trapped earlier on average. Besides, the secr package used for the spatially-explicit capture recapture model allows removed individuals to be accounted for in the density modelling. Alternatively, non-lethal methods of sampling allowing a determination of the status while releasing the animal could be preferred, as done by (Lee et al., 2018), but their implementation may impact other aspects of a research project. For instance, sampling urine rather than kidneys could affect the isolation of *Leptospira* and subsequent identification of the infecting strain, which was another goal of our research project (see Chapter 5), as *Leptospira* isolation success rates are better for kidney than urine (Tulsiani et al., 2011b). Moreover, in terms of ethics, the quantity of blood that can be safely taken on an animal intended to be released is much lower than the volume that can be drawn after euthanasia and depends on individual weight of the animals. It should not represent more than 10% of their body weight, which hinders the use of serology in the case of mice, where volumes taken (*ca.* 0.1 mℓ of blood) are not sufficient to perform MAT for all five serogroups present in NZ. All in all, and as often in projects

involving wild animals, a compromise had to be found between the best scientific approach and the time, funds and resources available.

Species targeted. It was not possible to sample all species present in the pastures. The present work investigated the wildlife-livestock interface in two farm settings and attempted to capture a picture as broad as possible of *Leptospira* infection in animal species present in the farm. We sampled dairy cattle, beef cattle, sheep, working dogs, house mice, rats, hedgehogs and possums, as well as a few feral cats and mustelids (weasels and ferrets) captured as by-catch in the possum traps, and a couple of red deer shot by a hunter present on Farm B during our trapping session. The panel of mammal species sampled is broad, but not exhaustive: rabbits (*Oryctolagus cuniculus*) were observed in both farms, and sambar deer (*Rusa unicolor*) on Farm A, and other more elusive species not targeted by our traps might also have been present. According to the hunter who provided red deer samples, wild goats (*Capra hircus*) were also present on Farm B but feral pigs (*Sus scrofa*) were not. Although previous studies conducted on wild cervids or carnivores concluded those species were not a likely source of *Leptospira* in NZ (Hathaway, 1978, Hathaway and Blackmore, 1981b, Daniel, 1966, Daniel, 1967), they were conducted over 30 to 50 years ago (see also Chapter 2), and the situation may have changed. This is what happened with sheep, considered as spillover host of Pomona in the 1980s (Blackmore et al., 1982), but now considered to be able to maintain this serovar (Vallée, 2016). In a project concomitant to this thesis, the contact frequency of livestock with wildlife was assessed by camera trapping in both farms. Direct and indirect contact with red or sambar deer were observed in both farms, and indirect contact with possums on Farm B (Oosterhof, 2017). Given that titres to Ballum have been found in farmed deer (Mannewald, 2016, Flint, 1988, Subharat, 2010), it is possible wild deer are also involved in Ballum epidemiology. In another project, sequences indicative of *L. interrogans* sv Copenhageni and *L. borgpetersenii* have been found in mustelids from the Manawatū region (Edwards, 2019) and, in Europe, mustelids have been described as major carriers of *Leptospira* (Ayral et al., 2016, Moinet et al., 2010). Of particular concern is the difference between the seroprevalence described in cats for Ballum, 31.1% (56/180) in 2016 in the frame of a pilot study (van de Pol, 2016), compared to the previous estimate of 1.77% (4/225) in 1979 (Shophet, 1979b). Also, as briefly described in Chapter 2, the studies investigating *Leptospira* infection in NZ in species other than rodents, hedgehogs and possums were limited in their sample size or the panel of serovars tested, and the question whether those other species play a role in the maintenance and dissemination of leptospires still remains open. Although the absence of information about *Leptospira* in non-captured species is unlikely to impact significantly the findings of this thesis concerning the captured species, the possibility they could also be involved in the

maintenance of this pathogen should not be overlooked. Therefore, surveillance of *Leptospira* infection levels in other wild populations not targeted in this study would be desirable.

Despite leptospirosis being known as a multi-host system, very little is known on the effect of community composition on *Leptospira* infection (Lugassy et al., in prep). It is possible species present but not sampled not only participate in Ballum epidemiology, but also influence the incidence of Ballum in other sympatric species. The dilution effect has been thoroughly studied, described and debated in other pathogens systems such as borreliosis (Randolph and Dobson, 2012), but a recent systematic review of the literature identified only one study investigating the link between biodiversity and human incidence of leptospirosis (Lugassy et al., in prep). This study found a negative correlation between human incidence and both the total species richness and the terrestrial mammal species richness in island nations (Derne et al., 2011), indicating a possible dilution effect. Other effects to relate to the community composition such as species abundance or predation/competition effects on *Leptospira* prevalence have been described in some studies. In Australia, proximity with colonies of fruit bats (*Pteropus conspicilliatus* spp.) was associated with a low abundance of rodents but a high prevalence of *Leptospira* (Tulsiani et al., 2011a). The introduction of an additional rodent species (ship rat) on Futuna island was associated with a general increase of the biomass of *Leptospira*-carrying rodents (Theuerkauf et al., 2013). The abundance of small mammals or rodents was associated with an increased prevalence in some studies (Lovera et al., 2017, Hathaway and Blackmore, 1981a), but not in others (Millán et al., 2018). In the case of (Hathaway and Blackmore, 1981a), the prevalence of Ballum infection was correlated with abundance for brown rats, but not for ship rats, but the presence/absence of mice was not assessed and could confound those results, as competition exists between mice and ship rats (King and Barrett, 2005). The importance of community composition and interactions between species on the results presented here is unknown.

Laboratory tests

Serovar panel in MAT test. MAT is a standard serological method for leptospirosis diagnosis (WHO 2003) and was used to test sera for antibodies to *Leptospira borgpetersenii* svs Hardjobovis, Ballum and Tarassovi and to *Leptospira interrogans* svs Pomona and Copenhageni. The OIE Terrestrial Manual specifies that "antigens selected for use in the MAT should include representative strains of the serogroups known to exist in the particular region as well as those known to be maintained elsewhere by the

host species under test" (OIE 2014). All serovars formerly isolated in NZ are included in the panel we used except *L. borgpetersenii* sv Balcanica. This serovar belongs to the same serogroup as Hardjobovis (Serogroup Sejroë), all locally know serogroups were therefore covered in the present work. However, the species we investigated are known to maintain strains in other serogroups in other parts of the world or strains suspected to be present but never isolated from animals—like Australis (Thompson, 1980, Ayral et al., 2016, ESR 2015, 2019), Grippotyphosa (Havlik and Hubner, 1958, Reilly et al., 1968) or Canicola (Chereshky et al., 1993, Frazer et al., 2012, ESR 2014, 2016)—were not included in the panel. The typing of isolates described in Chapter 5 did not uncover new serovars, but the possibility that other strains not covered in the panel are circulating should not be overlooked. The saprophytic strain Patoc I (*L. biflexa*) is known to cross-react with a variety of serogroups (Hartskeerl et al., 2006). The Leptospirosis Reference Centre in Amsterdam includes it in its MAT panel, and widens the number of serogroups tested if titres to this saprophytic serovar are the only obtained (Goris, pers. comm.), and this strategy could be adopted to help detect strains exotic to NZ that could be present in animals.

lipL32 PCR on kidney matrix. We used the real-time PCR developed by Stoddard (Stoddard, 2013, Stoddard et al., 2009) and optimized by (Galloway and Hoffmaster, 2015) on the kidneys sampled as a screening test for *Leptospira* infection. This PCR targeting the *lipL32* gene was developed and validated for human diagnosis on blood, sera and urine samples. Despite its wide use in the veterinary literature (Vieira et al., 2019, Benavidez et al., 2019), it has not been validated for tissue samples such as kidneys. We found that the sensitivity was lower than described in (Galloway and Hoffmaster, 2015), with samples positive by culture and negative by PCR on several instances (Appendix 8). Galloway et al. (2015) described a lower limit of detection (LLOD) of 5×10^1 Genome Equivalent (GE)/µL for Ballum in culture, and respectively 1×10^1, 1×10^3 and 1×10^4 leptospires/mℓ of blood, serum and urine. The standard curves we made using *Leptospira* DNA spiked into extracted kidney DNA indicated a LLOD 10 times higher (Appendix 8). The lower sensitivity likely affects the detection of low-level shedders, and therefore the estimation of prevalence.

It is important to note that the *lipL32* gene is present only on pathogenic leptospires, but *Leptospira* from the intermediate clade are also described in human and animal cases (Zakeri et al., 2010). The PCR method we used would need to be complemented by another PCR targeting a less specific gene—for instance *16s rRNA*—to detect a potential infection with intermediate leptospires.

Delay between _lipL32_ PCR and _glmU_ PCR. Because funds were limited, it was decided to perform single-locus typing only on samples that did not have associated WGS data, or

that had WGS data showing signs of contamination. A two-stage PCR diagnosis was used to type the infecting strain of *Leptospira*:

1. a first PCR to screen for presence/absence of pathogenic leptospires (*lipL32* real-time PCR),
2. for samples positive to the *lipL32* that did not have an isolate typed by WGS, a second PCR (conventional *glmU* PCR) and sequencing of the amplicons.

The sensitivity of the conventional *glmU* PCR was low (49 positive samples by *lipL32* PCR were negative by *glmU* PCR) and subsequent success of sequencing and typing affected. The delay between both PCRs, and the possible denaturation of DNA in frozen samples in the meantime could explain this low sensitivity, as the concentration of DNA was low for most of these samples. The *lipL32* PCR was conducted up to 3 months after each field session. The *glmU* PCR was conducted in one batch on the same extracted DNA kept at -20°C in June 2018, once all other results had been obtained, *i.e.* up to 18 months after DNA extraction.

Analytical methods

LCA/Occupancy model and bacterial load for true prevalence. We described a difference in the bacterial load in kidney samples, and concluded the amount of shedding varied, with mice shedding more leptospires than rats and hedgehogs, except for a subgroup of 'refractory mice' that were shedding in low quantities (Chapter 6). In Chapter 3, we had modelled the true prevalence in mice including the sensitivity as a single parameter, without accounting for individual heterogeneity in bacterial load in positive kidneys. Our estimates of true prevalence and PCR sensitivity are therefore likely biased by the proportion of mice shedding at low level (the higher this proportion, the lower the sensitivity), and should not be extrapolated to other species. Using an iterative approach and testing the same sample multiple times could overcome this issue, with an impact on the overall budget necessary for laboratory testing. This approach was used by (Lachish et al., 2012) to estimate the true prevalence of avian malaria in birds, using the parasitic load as an extra parameter. They carried out six qPCRs replicates per sample, and modelled that using three or more replicates made the probability of a false negative diagnosis negligible (< 0.03%; Lachish et al., 2012). At the population scale, low-level shedders probably have a negligible role in the dissemination of leptospires in the environment and under-detection of these individuals (*i.e.* misclassification as negative animals) does not affect the overall results of our study. However, differentiating between low-shedding exposed animals and negative unexposed animals in models of

Leptospira transmission has important consequences in the determination of the portion of the population susceptible to infection.

Bioinformatics methods used for WGS. Results of WGS described in Chapter 5 showed a surprisingly low level of within-strain variation. At this low variation level, the SNP-fallacy phenomenon can appear and bias the results. When the number of SNPs is low, it becomes difficult to differentiate between real SNPs and SNPs due to a sequencing or an assembly error. We observed differences in the overall number of SNPs when contigs were assembled with different versions of the assembly tool. When first using the Nullarbor pipeline version 1.0, the number of SNPs was greater (*ca.* 20, data not shown). The extremely low (or even null) number of SNPs observed in the alignment of strains from the same genotype assembled with the Nullarbor pipeline version 2.0 suggests this assembly tool is robust. It also implies the assembly tool used to build contigs used in a phylogenetic tree has important consequences. Ideally, all sequences being compared should be assembled with the same tool, but in practice, raw reads are almost never available in international databases (*e.g.* NCBI, PubMLST) where only contigs or scaffolds are present. Comparisons with other sequences published internationally might therefore be biased by the assembly tool used, and contigs assembled using the same tool might artificially show less variations compared to contigs assembled with a different tool. Efforts should be made to keep raw reads of *Leptospira* available.

WGS of *Leptospira* isolates is a relatively new method and the number of genome assemblies available for comparison still relatively low (310 genomes for *L. interrogans* and 44 for *L. borgpetersenii* in NCBI at the time of writing). The number of sequences available for other pathogens like *E. coli* (> 18,000 genome assemblies in NCBI) or *Campylobacter jejuni* (> 1,600 in NCBI) is much bigger in comparison. The phylogenetic results displayed in Chapter 5 compared our sequences of Ballum with eight international sequences and identified a possible European origin. These results would need to be confirmed by an increased number of isolates with a genome assembled using the same method.

Conclusions and suggestions for future research

The results presented in this thesis provided some answers on the role of wild mammals as a source of *Leptospira* infection for livestock and humans and provided knowledge on the serovars circulating in different species sharing the same farm habitat, on the temporal dynamics of Ballum infection in mice, on genotypes present, and on the reservoir dynamics in a community of maintenance hosts. This thesis also raised light on areas that require further investigations as below:

Dynamics of Ballum infection in livestock species

The literature review (Chapter 2) investigated the literature available on the different barriers that Ballum had to cross to spillover from wild maintenance hosts to livestock and eventually humans. This thesis focused on the distribution and abundance of wild hosts and the infection dynamics within those hosts, and the cross-sectional study forming Chapter 3 also investigated the level of exposure and competence of domestic animals. Despite a seroprevalence relatively high for Ballum (up to 20% in the younger group of replacement heifers), and molecular signature of this serovar in some samples, we were not able to isolate Ballum, and concluded the level of shedding probably limited the competency of cattle to transmit *Leptospira*. The young stock were probably exposed to Ballum early in their life, as most of calves are born at the end of winter, between July and September, when we measured the lowest densities, but also the highest prevalence in mice. It is also uncertain whether limiting exposure to Ballum in young cattle is an efficient way of preventing leptospirosis in farm workers. Delaying the time of infection rather than completely preventing it would mean animals infected later in their life might be more likely to shed when entering the milking herd, and therefore increase the exposure rate for farm workers. Depending on the duration of shedding, challenging young animals by natural infection could represent an approach to control leptospirosis (Blackmore et al., 1981). Experimental studies investigating the level and duration of shedding after exposure to Ballum in calves are needed to understand better the risk of contracting leptospirosis by contact with young stock.

Survival of Ballum in the environment

Another "barrier" to spillover reviewed in Chapter 2 was the presence of Ballum in the environment. We underlined in Chapter 6 the existence of species and individual heterogeneity in the release of Ballum from wild hosts. The survival of Ballum in the environment after release was not in the scope of the present thesis but it emerges as a key element to understand the inter-species transmission or the exposure level of humans and appears like a priority for further research. Bacteria of the species *L. borgpetersenii* have a shorter genome than bacteria belonging to *L. interrogan*s and this difference is thought to be linked to a difference in the transmission process. Survival outside of a mammalian host is suspected to be lower for *L. borgpetersenii* that is lacking some key genes to adapt to the external environment (Bulach et al., 2006). However, this contradicts several findings in the specific case of Ballum in NZ that would need further research: Ballum found in a majority of environmental samples analysed by

shotgun metagenomics (Nisa et al., 2019b); Ballum exposure in a variety of species in the same environment that is difficult to explain through direct transmission (Chapter 3).

As underlined in Chapter 2, interactions between *Leptospira* and other bacteria influence its survival in the environment. Given the artificial nature of pastures in NZ, where all species of grass are imported, and pastures often limited to two species of grass, usually perennial ryegrass and white clover (Charlton and Stewart, 1999), the study of interactions between *Leptospira* and other microorganisms in the soil under the specific NZ situation could help shed light on their maintenance in the environment.

Modelling transmission pathways

Aside from research in microbiology targeting the characteristics of Ballum survival in the environment, epidemiological modelling methods could be used to better understand the transmission pathways of Ballum within and among reservoir hosts. Natural intraspecies transmission of *L. borgpetersenii* sv Ballum is thought to happen by venereal transmission (Faine, 1999) and experimental studies of natural transmission within family groups of laboratory mice have shown that Ballum was primarily transmitted sexually, after the onset of sexual maturity (Hathaway et al., 1983). The description of young infected individuals in our study and the exposure and participation of other mammal species in the maintenance of Ballum contradict this sexual transmission, and modelling methods could be used to identify the most important transmission routes. Very few attempts to model leptospirosis transmission in rodents have been done, and those show conflicting results, maybe due to differences in transmission routes between the different species of *Leptospira* investigated. Minter et al. (2018) used modelling to study the importance of vertical, sexual and environmental transmission of *L. interrogans* serovar Copenhageni in a unique host population, brown rats (*Rattus norvegicus*) in Brazil. They found that a significant proportion of rats left the nest already infected, and sexual maturity did not impact the risk of infection, suggesting vertical and environmental transmissions were more important than sexual transmission. Holt et al. (2006) built a similar model for *Leptospira* spp. in the African multimammate mouse (*Mastomys natalensis*) and also highlighted the importance of environmental transmission. On the contrary, experimental studies on the transmission of *L. borgpetersenii* sv Balcanica in possum (*Trichosurus vulpecula*) led to the conclusion that transmission was unlikely to be environmental (Day et al., 1997b) but happened during the breeding season (Day et al., 1998). Caley et al. (2001) built on those results to model the impact of tubal ligation as a population control tool on the epidemiology of Balcanica. A density-dependent transmission model was the most appropriate,

congruent with a sexual transmission, and an increased frequency of oestrus in tubally ligated females was linked to an increased transmission rate of Balcanica. The transmission pathways of *Leptospira* within reservoir hosts thus appear complex and not thoroughly investigated.

Similarly, no studies have been published on the transmission pathways of *Leptospira* spp. between different maintenance hosts species. The calculation of a species-specific reproductive number (Ro_i) using the technique developed by (Fenton et al., 2015) could be used to quantify the contribution of each host species to Ballum persistence and a first foray into this calculation building on results of this thesis is already under way.

Improving the sensitivity of typing methods

As described earlier in this chapter (p. 176) and in Chapter 5, the typing of *Leptospira* strains infecting animals was hindered by low bacterial loads in some species or individuals. Genomic analysis is currently dependent on successful bacterial isolation, and leptospires are widely renowned for their difficulty to grow (Rahelinirina et al., 2019). Therefore, better isolation and enrichment methods or the development of culture-independent methods for WGS would improve how genomics could be applied to leptospirosis research. Direct whole genome sequencing from human samples of *Treponema pallidum*, the cause of syphilis and another spirochete, was recently described (Grillová et al., 2019), and the development and validation of similar techniques for leptospires would greatly improve the molecular epidemiology of this organism.

Investigating the phylogeny of *Leptospira*

The limited amount of genetic variation between draft genomes of the same strain described in Chapter 5 impacts on the use of sequencing methods for the comparison of similar strains at a local scale and in a short period of time. These limitations can become an advantage at the international scale and an increased timeframe, opening the possibility to investigate *Leptospira* phylogeography, and, in the case of Ballum or other rodent-associated strains of *Leptospira*, investigate the co-evolutionary phylogenetics of rodents and *Leptospira*. Determining the phylogeny of eukaryote hosts is complex (Veale et al., 2018) and the possibility to use *Leptospira* evolution as a proxy for its host could be investigated to better understand the patterns of dispersal of these rodent species introduced in most of the world.

Of particular interest is the closeness between Ballum and other serovars like Castellonis or Arborea revealed in Chapter 5. Indeed, the reference strain of serovar Arborea (strain Arborea, LRC, Amsterdam, The Netherlands), isolated from a wood mouse (*Apodemus sylvaticus*) in 1955, was submitted to WGS and the genome draft included in the SNP analysis of Ballum isolates. Compared to our isolates, it showed little more variations than the other isolates of Ballum published internationally, and even less than Strain 56604 isolated from a rat in China (see Figure 5-3 in Chapter 5). Arborea has recently been described as an emerging pathogen in Australia (Lau et al., 2015, Slack et al., 2010, Slack et al., 2006a). It is a particular concern in fruit-picking occupations, since the largest known outbreak of leptospirosis in the country (84 cases), documented on raspberry harvesters in 2018, was due to this serovar (Katelaris et al., 2019). Further investigation of the relatedness between those two strains would likely help epidemiologists understand better the emergence of both serovars. Serovars Icterohaemorrhagiae and Copenhageni are more distant than Ballum and Arborea genetically but one indel in a gene related to the LPS biosynthesis is sufficient to explain the difference (Santos et al., 2018).

The high similarity of genomes from the same genotype challenges the current methods, developed for bacteria exhibiting a high level of mutations and recombinations. The development of bioinformatic tools specific to *Leptospira* for the genomic comparison of close strains, at the pangenome scale, would be needed.

"It's not the mountain we conquer but ourselves"

— Sir Edmund Hillary

Appendices

Appendix 1.
List of publications and presentations related to this thesis

Pilot studies

FABRI, N. D. 2016. *Leptospirosis in wildlife in New Zealand - a pilot study.* Minor research project thesis, Utrecht University, Netherlands. Available: https://dspace.library.uu.nl/handle/1874/369535

VAN DE POL, M. 2016. *Leptospirosis in wildlife and cats - Pilot study.* Minor research project thesis, Utrecht University, Netherlands. Available: https://dspace.library.uu.nl/bitstream/handle/1874/325382/Leptospirosis%20research%20scientific%20report%20final%20NZ.docx?sequence=2

Undergraduate and Master students

EDWARDS, M. P. 2019. *A novel strain of Leptospira in New Zealand: a retrospective PCR-based study of archived animal samples (2010-2017) in New Zealand.* Master of Science in One Health M.Sc. thesis, The University of Edimburgh.

EL ATTAR SOFI, O. 2017. *Evaluation of renal lesions in wild mammals in relation with leptospirosis. Summer project report*. unpublished report: National Veterinary School of Toulouse.

OOSTERHOF, H. 2017. *An assessment of Leptospiral infection in livestock associated with contact frequency with wildlife in the Manawatu region in New Zealand.* Minor research project thesis, Utrecht University, Netherlands. Available: https://dspace.library.uu.nl/bitstream/handle/1874/354152/Final%20report%20H.%20Oosterhof.pdf?sequence=2

Publications

LUGASSY, L., ALOUT, H., AMDOUNI-BOURSIER, L., BERREBI, R., BOËTE, C., BOUÉ, F., BOULANGER, N., COSSON, J.-F., DURAND, T., GARINE, M., LARRAT, S., MOINET, M., MOULIA, C., PAGES MARTINEZ, N., PLANTARD, O., ROBERT, V. & LIVOREIL, B. 2019. What is the evidence that ecosystem components or functions have an impact on infectious diseases? A systematic review protocol. *Environmental Evidence,* 8. Available: https://doi.org/10.1186/s13750-019-0147-5

MOINET, M., WILKINSON, D., NISA, S., HAACK, N., OOSTERHOF, H., ABERDEIN, D., RUSSELL, J., VALLÉE, E., WILSON, P., COLLINS-EMERSON, J. M., HEUER, C. & BENSCHOP, J. 2017. Is wildlife a source of *Leptospira* infection in livestock in New Zealand? *Proceedings of the Society of Dairy Cattle Veterinarians & Large Animal Veterinary Technicians of the NZVA Combined Annual Conference "Better together"*, pp. 26-29. Wellington: NZVA Society of Dairy Cattle Veterinarians. Available: http://www.sciquest.org.nz/node/140337

MOINET, M. & YUPIANA, Y. 2017. Leptospirosis in New Zealand: an international perspective. *VetScript*, p12. Available: http://www.sciquest.org.nz/node/134676

Presentations

BENSCHOP, J., COLLINS-EMERSON, J., ABERDEIN, D., HAACK, N., MOINET, M., NISA, S., RUSSELL, J. C., VALLÉE, E., WESTON, J., WILKINSON, D. A., WILSON, P., YUPIANA, Y. & HEUER, C. 2018. Emerging sources and pathways for leptospirosis. *In:* NZVA (ed.) *Proceedings of the Food Safety, Animal Welfare & Biosecurity, Epidemiology & Animal Health Management, and Industry branches of the NZVA*, pp. 71-72. Hamilton. Available: http://www.sciquest.org.nz/node/144134

MOINET, M. Ecology tools and concepts applied in epidemiology: 2 examples applied to mice (*Mus musculus*) as maintenance hosts of *Leptospira*. New Zealand Ecological Society Conference, 25-29 November 2018 Wellington, NZ.

MOINET, M., NISA, S., COLLINS-EMERSON, J., BENSCHOP, J. & WILKINSON, D. A. Can sequencing methods help decipher transmission pathways of *Leptospira* within a community of hosts in New Zealand ? MICRObes across 'SCOPEs - NZMS annual conference, 2019a Palmerston North, NZ. New Zealand Microbiological Society. Student best oral presentation award from the New Zealand Microbiological Society (3rd place)

MOINET, M., NISA, S., COLLINS-EMERSON, J., BENSCHOP, J. & WILKINSON, D. A. Merits and limitations of sequencing methods in the eco-epidemiology of *Leptospira*. ILS Bisannual conference, 2019b Vancouver, Canada.

MOINET, M., NISA, S., HAACK, N., ABERDEIN, D., RUSSELL, J. C., VALLÉE, E., COLLINS-EMERSON, J., HEUER, C. & BENSCHOP, J. 2019c. Flown under the radar for too long: *Leptospira* from wildlife. *In:* NZVA (ed.) *Proceedings of the Epidemiology & Animal Health Management Branch and Food Safety, Animal Welfare & Biosecurity Branch of the NZVA*, pp. 43-46. Wellington: New Zealand Veterinary Association. Available: http://www.sciquest.org.nz/node/155846

MOINET, M., OOSTERHOF, H., NISA, S., HAACK, N., WILKINSON, D. A., ABERDEIN, D., RUSSELL, J. C., VALLÉE, E., WILSON, P., COLLINS-EMERSON, J., HEUER, C. & BENSCHOP, J. *Leptospira* infection at the wildlife/livestock interface in New Zealand: results from a cross-sectional study. 2018 One Health Aotearoa Symposium, 12-13 December 2018a Wellington, New Zealand. One Health Aotearoa.

MOINET, M., WILKINSON, D., ABERDEIN, D., VALLÉE, E., WILSON, P., COLLINS-EMERSON, J., HEUER, C. & BENSCHOP, J. 2017a. Are Wildlife Workers and Conservationists in New Zealand at Risk of Leptospirosis? Proceedings of the joint WDA- NZVA Wildlife Society joint conference, 25-30 November 2017, Christchurch, NZ. *Kokako,* 24, 13-14. Available: http://www.sciquest.org.nz/node/158549

MOINET, M., WILKINSON, D., NISA, S., HAACK, N., OOSTERHOF, H., ABERDEIN, D., RUSSELL, J., VALLÉE, E., WILSON, P., COLLINS-EMERSON, J., HEUER, C. & BENSCHOP, J. Is mammalian wildlife a source of *Leptospira* infection in livestock in New Zealand? International Leptospirosis Society Scientific Meeting, 27 Nov-1 Dec 2017b Palmerston North, New Zealand. 72.

MOINET, M., WILKINSON, D., NISA, S., HAACK, N., OOSTERHOF, H., ABERDEIN, D., RUSSELL, J., VALLÉE, E., WILSON, P., COLLINS-EMERSON, J. M., HEUER, C. & BENSCHOP, J. 2017c. Is wildlife a source of *Leptospira* infection in livestock in New Zealand? *Proceedings of the Society of Dairy Cattle Veterinarians & Large Animal Veterinary Technicians of the NZVA Combined Annual Conference "Better together"*, pp. 26-29. Wellington: NZVA Society of Dairy Cattle Veterinarians. Available: http://www.sciquest.org.nz/node/140337

MOINET, M., YUPIANA, Y., NISA, S., HAACK, N., WILSON, P., VALLÉE, E., WILKINSON, D., RUSSELL, J. C., HEUER, C., ABERDEIN, D., COLLINS-EMERSON, J. & BENSCHOP, J. Of mice, cattle and men: using conceptual frameworks to understand the eco-epidemiology of *Leptospira borgpetersenii* serovar Ballum in New Zealand. 3rd European Leptospirosis Society Scientific Meeting on leptospirosis and other rodent borne haemorrhagic fevers, 24-26 May 2018 2018b Alghero, Italy.

MOINET, M., YUPIANA, Y., NISA, S., HAACK, N., WILSON, P., VALLÉE, E., WILKINSON, D., RUSSELL, J. C., HEUER, C., ABERDEIN, D., COLLINS-EMERSON, J. & BENSCHOP, J. Using disease ecology conceptual frameworks to understand the eco-epidemiology of

Leptospira borgpetersenii serovar Ballum in New Zealand. New Zealand Ecological Society Annual Conference, 2018c Wellington, NZ.

MUYLAERT, R. L., DE AZEVEDO, T. S., MOINET, M., RIBEIRO, M. C. & HAYMAN, D. T. Seasonality and city structure affect the risk of leptospirosis in humans within southeastern Brazil. New Zealand Ecological Society Annual Conference, 2018.

NISA, S., COLLINS-EMERSON, J., WILKINSON, D. A., YUPIANA, Y., MOINET, M., BENSCHOP, J. & HEUER, C. 2019a. Long-term changes of human exposure to *Leptospira* in New Zealand. *In:* NZVA (ed.) *Proceedings of the Epidemiology & Animal Health Management Branch and Food Safety, Animal Welfare & Biosecurity Branch of the NZVA*, pp. 47-50. Wellington: New Zealand Veterinary Association.

NISA, S., HEUER, C., MOINET, M., YUPIANA, Y., BENSCHOP, J. & WILKINSON, D. A. Long-term changes of human exposure to *Leptospira* in New Zealand. ILS bisannual conference, 8-12 July 2019b Vancouver, Canada.

Appendix 2.
Is wildlife a source of *Leptospira* infection in livestock in New Zealand?

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Is wildlife a source of Leptospira infection in livestock in New Zealand?

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In New Zealand, livestock are the main source of infection for human leptospirosis which is considered to be an occupational risk with farm and abattoir workers prominant amongst notified cases. In the 1970-80s, 95% of the human cases of leptospirosis in New Zealand were caused by *Leptospira interrogans* serovar (sv) Hardjobovis and *L. interrogans* sv Pomona (Carter *et al.* 1982). The routine vaccination of dairy cattle and pigs alongside increased awareness was associated with a rapid reduction of human notified cases from 677 in 1979 to 325 in 1981 and 179 in 1982 (Marshall and Chereshsky 1996).

Today livestock are still considered as the main source of leptospirosis infection, but the dominant serovars in human cases have changed. Between 1990-92 and 1996-98 while the notifications of Hardjobovis and Pomona infections decreased, the incidence of *L. borgpetersenii* sv. Ballum increased significantly from 0.2 to 0.6 cases per 100,000 (Thornley *et al.* 2002). Compared to the crude annual incidence of respectively 0.2, 2.3 and 1.6 per 100,000 described by Thornley *et al.* in 1990-92 for Ballum, Hardjobovis and Pomona respectively (Thornley *et al.* 2002), the average incidence of cases attributable to these serovars for the last decade (2007-16) was 0.4, 0.7 and 0.3 per 100,000 per year (ESR 2008-2017). Although the absolute number of cases attributed to Ballum has not increased *per se* (the annual incidence rather fluctuates between 0.2 and 0.6 (ESR 2008-2017)), the concurrent decrease in the other serovars now makes it one of the most common. Around 30% of the cases notified at the *Leptospira* Reference Laboratory at ESR in 2015 and 2016 (ESR 2016, 2017) were associated with Ballum. This serovar is usually linked to house mice (Mus musculus) and ship rats (*Rattus rattus*) worldwide and introduced hedgehogs (*Erinaceus europaeus*) in New Zealand. A recent study (Yupiana *et al.* see article in these proceedings) suggests that livestock infections may also be changing; *Leptospira* shedding has been observed in the urine of vaccinated dairy cattle, and was correlated with increasing serological titres to Tarassovi, a serovar commonly associated with pigs (Adler 2015).

These changes in leptospirosis epidemiology may be linked to exposure of livestock to non-vaccine serovars through contact with other animal reservoirs. In this article we summarise the current understanding of the role of wild mammalian species as reservoirs of Leptospira infection in New Zealand and present preliminary findings of a cross-sectional study conducted in the Manawatu-Whanganui region to show that the same serovars are present in wildlife and sympatric livestock.

There is a dearth of up-to-date information in the literature concerning wildlife-related serovars. Most of the knowledge available on *Leptospira* infection in New Zealand wildlife comes from a thorough investigation which took place between 1974 and 1978 (Hathaway 1978). This work, as well as others (Caley and Ramsey 2001, Day *et al.* 1998, Day *et al.* 1997a, Day *et al.* 1997b, de Lisle *et al.* 1975), focused primarily on the invasive brush-tailed possum (*Trichosurus vulpecula*) as a maintenance host for *L. borgpetersenii* sv Balcanica. This serovar belongs to the same serogroup as Hardjobovis (Hebdomadis) and the two are virtually indistinguishable by standard serological methods such as the microscopic agglutination test (MAT).

In the 1950s experimental infections of hedgehogs with sv Pomona showed they were highly susceptible to this serovar and characteristic lesions associated with high agglutination-lysis titres for Pomona were also described in two naturally infected individuals, and urinary shedding in one of them (Webster 1957). In the 1970s surveys of healthy hedgehogs in dairy farms reported 40% to 56% seroprevalence for Ballum and isolation of this serovar from the kidneys of 6% to 19% of them (Brockie and Till 1977, Hathaway *et al.* 1981). Six urban hedgehogs were also reported clear of infection (Brockie and Till 1977). Hedgehogs were subsequently proposed as maintenance hosts for Ballum.

In 1951, Kirshner and Gray reported 8/53 Norway rats (*Rattus norvegicus*) from Auckland and Dunedin seropositive for *L. icterohaemorrhagiae* (most probably a cross reaction with antibodies against *L. interrogans* sv Copenhageni). They also tested 47 ship rats but found no seropositive animals. Neither did Blakelock and Allen (1956) at the same period in Wellington on 183 ship and Norway rats. Serovars Hardjobovis and Ballum were not tested in those studies. In the 1970s, Brockie (1977) and Hathaway *et al.* (1981) reported shedding of *L. interrogans* sv Copenhageni by Norway rats and shedding of Ballum by Norway rats, ship rats and mice. The majority (67 to 89%) of Ballum shedding rodents were seronegative. The last available serological survey of ship and Norway rats in 1999 found 24% and 29% of them,

respectively, seropositive for sv Ballum (Kakrada 1999).

Other New Zealand wild species have typically been considered as insignificant hosts for the maintenance and propagation of leptospirosis, and have been little studied. Studied species for which an absence of serological titres have been reported include feral pig (*Sus scrofa*), rabbit (*Oryctolagus cuniculus*), hare (*Lepus europaeus*), stoat (*Mustela erminea*), ferret (*Mustela putorious furo*), weasel (*Mustela nivalis*), Sika deer (*Cervus nippon*), Fallow deer (*Dama dama*), white-tailed deer (*Odocoileus virginianus borealis*), Chamois (*Rupicapra rupicapra*), Himalayan tahr (*Hemitragus jemlahicus*) (Anon 1983, Daniel 1967, Hathway and Blackmore 1981, Hathaway *et al.* 1981). Occasional titres were found against Hardjobovis and Ballum in wallabies (probably dama wallabies[1], *Macropus eugenii*); against Pomona and Ballum in feral cats (*Felis catus*); against Pomona, Hardjobovis, Balcanica and Ballum in feral goats (*Capra hircus*), against Pomona and Copenhageni in hunted deer[2] (*Cervus elaphus*) and more recently against Canicola, Hardjobovis and Pomona in fur seal (*Arctocephalus forsteri*) and Pomona in sea lions (*Phocarctos hookeri*) (Anon 1983, Inglis 1984, Mackereth *et al.* 2005, Mist 1984, Roe *et al.* 2010, Schollum and Blackmore 1981). Those results were interpreted as sporadic infections through contact with known maintenance species of these serovars (livestock, rodents or other wild species).

Many peculiarities of New Zealand's infrastructure and environment likely influence the ecology of leptospirosis: the relatively narrow range of mammalian species present, almost all exotic (King and Barrett 2005), the even fewer *Leptospira* serovars circulating in the country (Marshall and Manktelow 2002), the importance of livestock as a source of infection for humans (Cowie and Bell 2012), and the widespread implementation of effective vaccination programs in cattle and pigs for a subset of serovars.

Unlike many other countries, mice in New Zealand have virtually no competitors and are present in forests and pastures throughout the country (King and Barrett 2005). Hedgehogs, which avoid pastures in their native geographical ranges due to the risk of predation by badgers (Young *et al.* 2006), are thriving in New Zealand pastures. More information is needed to assess if these ecological factors affect leptospirosis epidemiology.

Thus, to better assess the role of wild mammalian species in New Zealand leptospirosis, we designed and conducted a cross-sectional study on both wildlife and sympatric[3] cattle.

Traps were set for 20 trap-nights on a dairy farm targeting rodents, hedgehogs and possums. Trapped wild animals and an age-stratified random sample of cattle were blood sampled. Sera were tested by MAT against *L. borgpetersenii* sv Hardjobovis, Ballum and Tarassovi and *L. interrogans* sv Pomona and Copenhageni. The positive threshold was set at a titre of 1:48 or higher. Results are presented in the table (below).

Table 1. Seroprevalence of *Leptospira* in our multi-species study

Species	Seroprevalence				
	Hardjo	Pomona	Ballum	Copen	Tarassovi
Cattle	84/203	70/203	26/203	15/203	4/203
Mouse	7/95	1/95	30/95	3/95	3/95
Ship rat	1/4	0/4	1/4	0/4	0/4
Hedgehog	6/12	2/12	7/12	1/12	3/12
Cat	0/3	0/3	0/3	0/3	0/3

In 1981 Hathaway described an epidemiological scenario for New Zealand leptospirosis where "despite high prevalence of endemic infection of Hardjobovis and Pomona in cattle and pigs respectively and Ballum and Balcanica in wildlife, [there was] virtually no evidence of interspecies transmission". Today, whether due to changes in vaccination practices, ecological factors, species distributions or diagnostic techniques, we can see that this is no longer the case. Serovars found in wildlife species in our study were commonly found in livestock in the same environment supporting the concept of inter-species disease transmission, or "spillover". Furthermore, there is no information available for cattle on the agreement between serological titres for Ballum and current infection, but they could, as described in rodents, be shedding this serovar despite low or no detectable titres. New Zealand leptospirosis should thus be regarded as a complex multi-host, multi-serovar disease model, where our understanding of the dynamics of infection will be greatly increased by further study in humans, livestock, wild species and the environment.

While the efforts to control Hardjobovis and Pomona in cattle and pigs can be successful and should be maintained, intervention strategies should take into account other serovars. All but two studies (Harland *et al.* 2013, Subharat 2010) reported on animal leptospirosis in New Zealand the last decade were focused only on serovars Hardjobovis and Pomona (Dorjee *et al.* 2008, Dreyfus 2013, Fang 2014, Ridler *et al.* 2015, Roe *et al.* 2010, Sanhueza *et al.* 2013, Vallée *et al.* 2015) and information on other serovars, their prevalence and their impact on domestic species is needed. A number of recent and current projects (Massey University, unpublished) are addressing other serovars.

Having a better understanding on which serovars circulate and how transmission occurs between species is crucial if leptospirosis is to be efficiently controlled. While infection in a wild reservoir is difficult to control, the interactions with livestock remain central to reducing incidence in New

[1] The report only mentions the origin of the samples, the Rotorua area, where only this species is established.
[2] The species is not mentioned but only feral populations of red deer (Cervus elaphus) and in lower densities fallow deer (Dama dama) have been described in the sampling area (Nelson).
[3] occupying the same geographical range

Zealand as adapted vaccination, pest control and protective intervention measures are all readily accessible options for leptospirosis disease control in the farm environment.

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Appendix 3.
Flown under the radar for too long: *Leptospira* from wildlife

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Flown under the radar for too long: *Leptospira* from wildlife

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Outline

Leptospirosis in New Zealand has traditionally been associated with livestock rather than rodents. It was first described as the 'dairy farm fever' and is indeed the most common occupational zoonosis in the country, with more than two thirds of the notified cases being farm or meat workers.

Leptospires are difficult bacteria to isolate and grow, and since they have been first described, serological typing has been a mainstay for their surveillance in human and animal populations. Until 1989, leptospires were classified only according to their pathogenicity, with two species described (pathogenic *L. interrogans* vs saprophytic *L. biflexa*), and their antigenic serological properties, with more than 300 serovars (sv) and 30 serogroups described (Levett 2001). The more recent development of molecular tools allowed for a better understanding of the genetic diversity of those bacteria, and the development of a genotypic classification. Under this classification, there are – at the time of writing – 35 described genomospecies in the genus *Leptospira*, with three distinct clades, saprophytic, intermediate and pathogenic, and two sub-clades in the latter, virulent and avirulent pathogenic *Leptospira* (Thibeaux *et al.* 2018).

Of this wide variety of species and serovars described worldwide, only two species and eight serovars, from five serogroups, have been isolated in New Zealand: *Leptospira borgpetersenii* sv Hardjobovis, Ballum, Balcanica and Tarassovi and *L. interrogans* sv Pomona, Copenhageni, Australis and Canicola. The last two have been isolated from humans only (Midwinter 1999).

The microscopic agglutination test (MAT) has been, and still remains the reference test for serology (World Organisation for Animal Health (OIE), 2014). However, this typing method is far from perfect. There are common cross-reactions within a serogroup, or even among serogroups, especially at the early stage of infection. As a result, serovar Balcanica cannot be distinguished from Hardjobovis (both in serogroup Sejroë). Also described are paradoxical reactions (serological response to a serovar previously exposed to rather than the infecting one, or to a completely different organism), or the possible presence of chronically infected animals showing low or no titres to the serovar they are shedding (Hartskeerl *et al.* 2006, Levett 2001). The presence of vaccinal titres or maternal antibodies in young animals can also hinder the interpretation of this test.

Work conducted in the 1970s underlined the importance of serovars maintained by livestock; Hardjobovis and Pomona. Only a few species of wild mammals harboured other 'atypical' serovars, like Ballum maintained by ship rats, brown rats, hedgehogs and mice, and Balcanica maintained by possums (Moinet *et al.* 2017). Those strains were rare among the notified human cases, and the rare titres for those atypical serovars found in livestock were interpreted as cross-reactions (Hathaway 1978, Hellstrom 1978). So, in the early 1980s, vaccination of dairy cattle and pigs against Hardjobovis and Pomona was promoted. Good hygiene

practices and correct use of PPE were encouraged too. The number of human cases notified subsequently dropped from 677 in 1979 to 325 in 1981, and 179 in 1982 (Marshall and Manktelow 2002). Leptospirosis was then "assumed to be largely done and dusted" (Collins-Emerson 2017) and sources of funding for *Leptospira* research 'dried up' (Anonymous 1983). Because the MAT panel usually involves the maintenance of live cultures of all the strains to be tested, and can be cumbersome and time-consuming, most of the serosurveys conducted on livestock after 1983 only included Hardjobovis and Pomona in their panel.

Although these atypical serovars were unimportant a few decades ago, they cannot be overlooked any more. Indeed, serovar Ballum represented a third of the 63 and 85 human cases notified in 2015 and 2016 (The Institute of Environmental Science and Research Ltd 2016, 2017). In this presentation, after describing the reasons why those atypical serovars flew under the radar, current evidence of spillover between wild maintenance hosts and livestock or humans will be presented, with a focus on serovar Ballum.

We conducted a cross-sectional study on one farm in the Manawatu-Whanganui region to identify and isolate the serovars of *Leptospira* circulating at the wildlife-livestock interface. Traps were set for 10 trap-nights in March 2018 on a dairy farm, targeting house mice (*Mus musculus*), ship rats (*Rattus rattus*) and hedgehogs (*Erinaceus europaeus*). Trapped wild animals and a random sample of one-year-old (R1) heifers were blood sampled. Sera were tested by microagglutination test for five serogroups. Cattle urine and wildlife kidneys were sampled for culture and qPCR targeting the *lipL32* gene.

At a titre cut-point of 48, thirty of 92 R1 heifers (33% [CI 23-43%]) were seropositive for Ballum, 43 (47% [CI 36-57%]) for Hardjobovis (suspected post-vaccinal titres), one for Tarassovi [CI 0-6%] and none for Pomona or Copenhageni. Six were PCR positive (7% [CI 2-14%]), putatively for serovar Ballum, but technical issues prevented us from typing these samples. Concomitant results from wildlife as well as results from other trapping sessions will also be presented, alongside genotyping results of cultures.

These results add to the growing body of evidence that serovars maintained by wild species can spill over to livestock, and that specific control measures targeting serovar Ballum should be implemented.
Indeed, a sero-survey conducted nationwide in 2009-2010 on beef cattle, sheep and farmed deer identified a seroprevalence for this serovar of respectively 14% [CI 12-16%], 10 % [CI 9-12%], and 7% [CI 5-8%] (Mannewald 2016). Yupiana *et al.* found 13/4000 (0.3% [CI 0.2-0.6%]) of adult vaccinated dairy cows, all seronegative, shedding Ballum (Yupiana *et al.* 2017). The higher seroprevalence in our survey could be related to the younger age in our sample (heifers vs adult animals).

The presence of 'house' mice in pastures is an oddity, related to the peculiar natural history of New Zealand (and other islands), where no other competing rodents are present except ship and brown rats, which are also invasive. Mice are especially cryptic and only become visible at very high densities. Hedgehogs are more easily visible, and probably help in disseminating the bacteria over a larger range. As such, they could be used as epidemiological sentinels (Halliday *et al.* 2007).

In this context of an increased rate of contact between mice and livestock (when compared to other countries), these results underline the importance of a continuous effort for monitoring all strains of *Leptospira* circulating and for detecting new hosts as early as possible before they constitute a new maintenance population.

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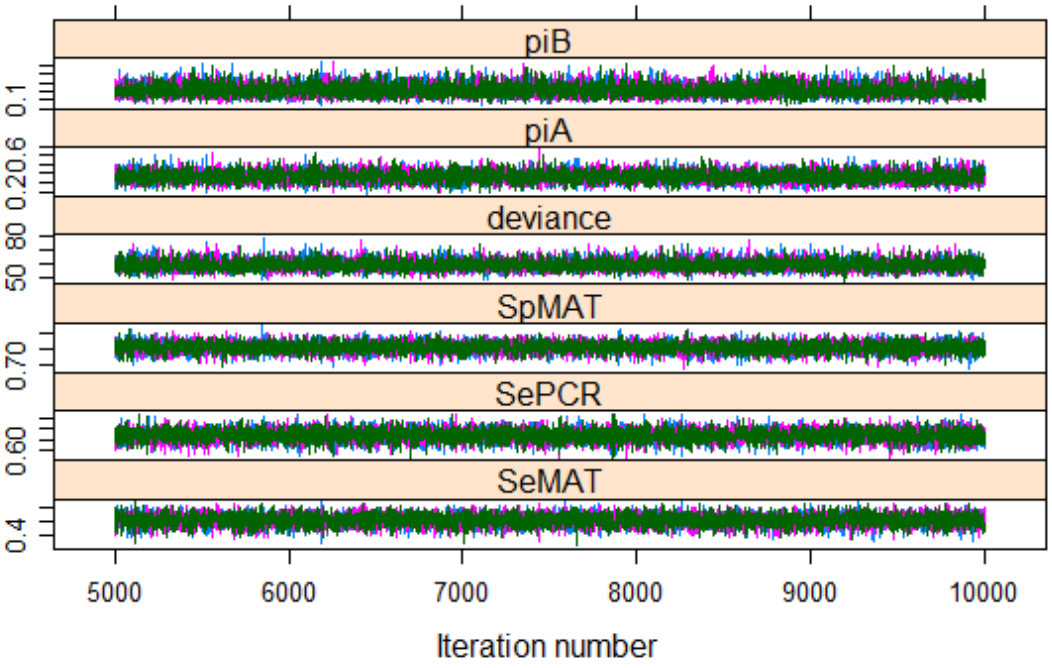
Appendix 4.
R script for Latent Class Modelling (Chapt.3)

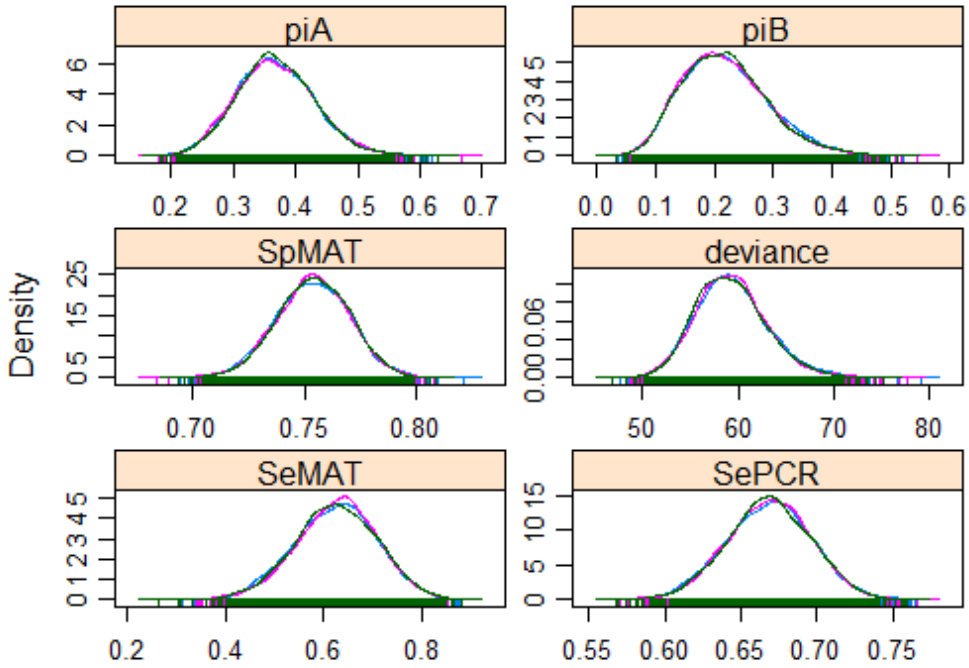
```
#load libraries
library(readxl)
library(epiR) # for epi.betabuster()
library(R2OpenBUGS) # for bugs()
library(coda) # for bugs(codaPkg=TRUE) and to read coda files/make graphs
library(lattice)# for graphs with coda files
library(dplyr) # for bind_rows and other
library(stringr) # for strg_replace
library(tibble) # for add_column
library(ggplot2) # for forest plot

###determination of priors Beta distribution (BetaBuster)
Priors <- read_excel("Priors.xlsx")
Pmode<-Priors$mode
Pconf<-Priors$conf
Pgreaterthan<-Priors$greaterthan
Px<-Priors$x

a<-c()
b<-c()

for ( i in seq_along(Priors)) {

  tmp<-epi.betabuster(mode = Pmode[i], conf = Pconf[i], greaterthan = Pgreate
rthan[i], x = Px[i], conf.level = 0.95, max.shape1 = 1000, step = 0.001)
  a<-c(a,tmp$shape1)
  b<-c(b,tmp$shape2)
}
result <- data.frame(a,b)
write.table(result,"clipboard",sep="\t", row.names = FALSE)
#and ctrl+V in excel spreadsheet
```

```
## LCM

#initial model
model <- function() {
  # Priors
  #Dairy
  piA ~ dbeta(2.04, 6.47)

  #Beef
  piB ~ dbeta(1.36, 5.12)

  #PCR
  SePCR ~ dbeta(171.39, 92.75)
  #SpPCR ~ (0.97+0.03)

  #MAT
  SeMAT ~ dbeta(6.47, 2.04)
  SpMAT ~ dbeta(466.48, 173.16)

  # Likelihood
  #Dairy
  X1[1:4] ~ dmulti(p1[1:4], n1)
  p1[1]<-piA*SePCR*SeMAT                          #11
  p1[2]<-piA*SePCR*(1-SeMAT)                      #10
  p1[3]<-piA*(1-SePCR)*SeMAT  +(1-piA)*(1-SpMAT) #01
  p1[4]<-piA*(1-SePCR)*(1-SeMAT) + (1-piA)*SpMAT #00

  #Beef
  X2[1:4] ~ dmulti(p2[1:4], n2)
  p2[1]<-piB*SePCR*SeMAT                          #11
  p2[2]<-piB*SePCR*(1-SeMAT)                      #10
  p2[3]<-piB*(1-SePCR)*SeMAT  +(1-piB)*(1-SpMAT) #01
  p2[4]<-piB*(1-SePCR)*(1-SeMAT) + (1-piB)*SpMAT #00

}
model.file <- file.path(tempdir(), "model.txt")
write.model(model, model.file)

data <- list(X1=c(15, 8, 3, 48), X2=c(5, 1, 0, 27), n1=74, n2=33)
params <- c("piA","piB","SePCR", "SeMAT","SpMAT")
inits <- function() { list(piA=0.16,piB=0.08,SePCR=0.65, SeMAT=0.84,SpMAT=0.7
3) }

out <- bugs(data, inits, params, model.file, n.iter=10000)
all(out$summary[,"Rhat"] < 1.1)  #if false, increase n.iter

## [1] TRUE

print(out, digits=5)

## Inference for Bugs model at "model.txt",
## Current: 3 chains, each with 10000 iterations (first 5000 discarded)
## Cumulative: n.sims = 15000 iterations saved

##              mean      sd     2.5%      25%      50%      75%    97.5%
## piA       0.36761 0.06227  0.25239  0.32420  0.36500  0.40862  0.49591
## piB       0.21803 0.07213  0.09766  0.16570  0.21220  0.26370  0.37620
## SePCR     0.66862 0.02770  0.61380  0.65010  0.66910  0.68740  0.72150
## SeMAT     0.62596 0.08240  0.45880  0.57127  0.62825  0.68300  0.78140
## SpMAT     0.75376 0.01632  0.72110  0.74280  0.75400  0.76510  0.78500
```

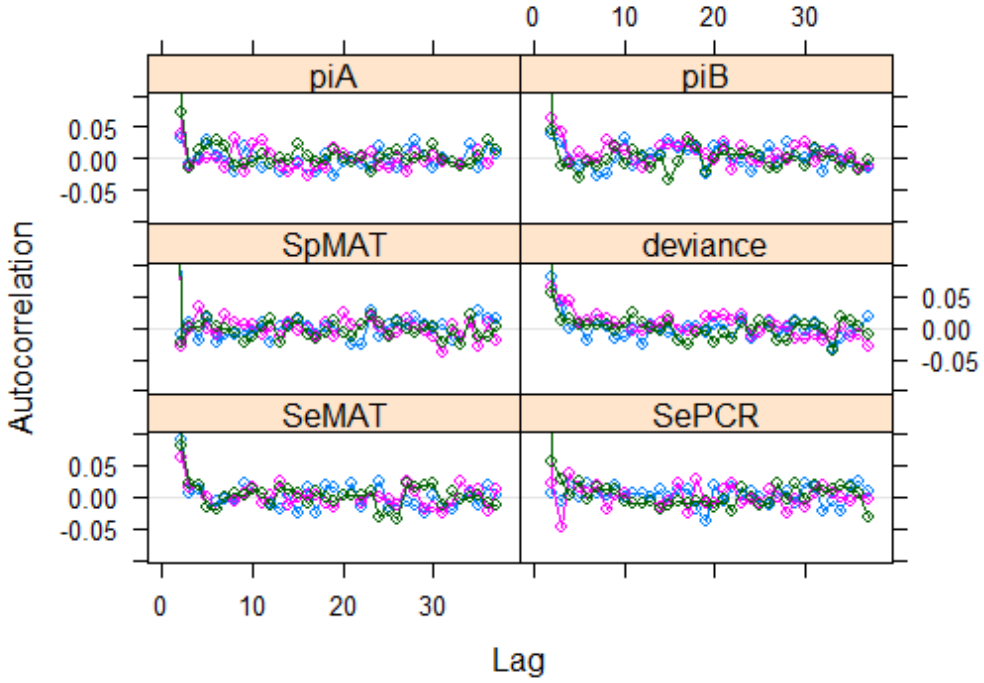
```
## deviance 59.29163 3.75102 52.61000 56.68000 59.06000 61.55000 67.47050
##             Rhat n.eff
## piA      1.00104 14000
## piB      1.00121  6500
## SePCR    1.00092 15000
## SeMAT    1.00130  5000
## SpMAT    1.00116  7700
## deviance 1.00128  5200
##
## For each parameter, n.eff is a crude measure of effective sample size,
## and Rhat is the potential scale reduction factor (at convergence, Rhat=1).
##
## DIC info (using the rule, pD = Dbar-Dhat)
## pD = 2.56200 and DIC = 61.85000
## DIC is an estimate of expected predictive error (lower deviance is better)
.

##Diagnostics
out <- bugs(data, inits, params, model.file, codaPkg=TRUE, n.iter=10000)
out.coda <- read.bugs(out)

xyplot(out.coda) #check if simulation values stabilize
```




```
densityplot(out.coda) #inspect if the density plot is well-defined
```




```
acfplot(out.coda) #check if the auto-correlation of the time series converge
to zero
```

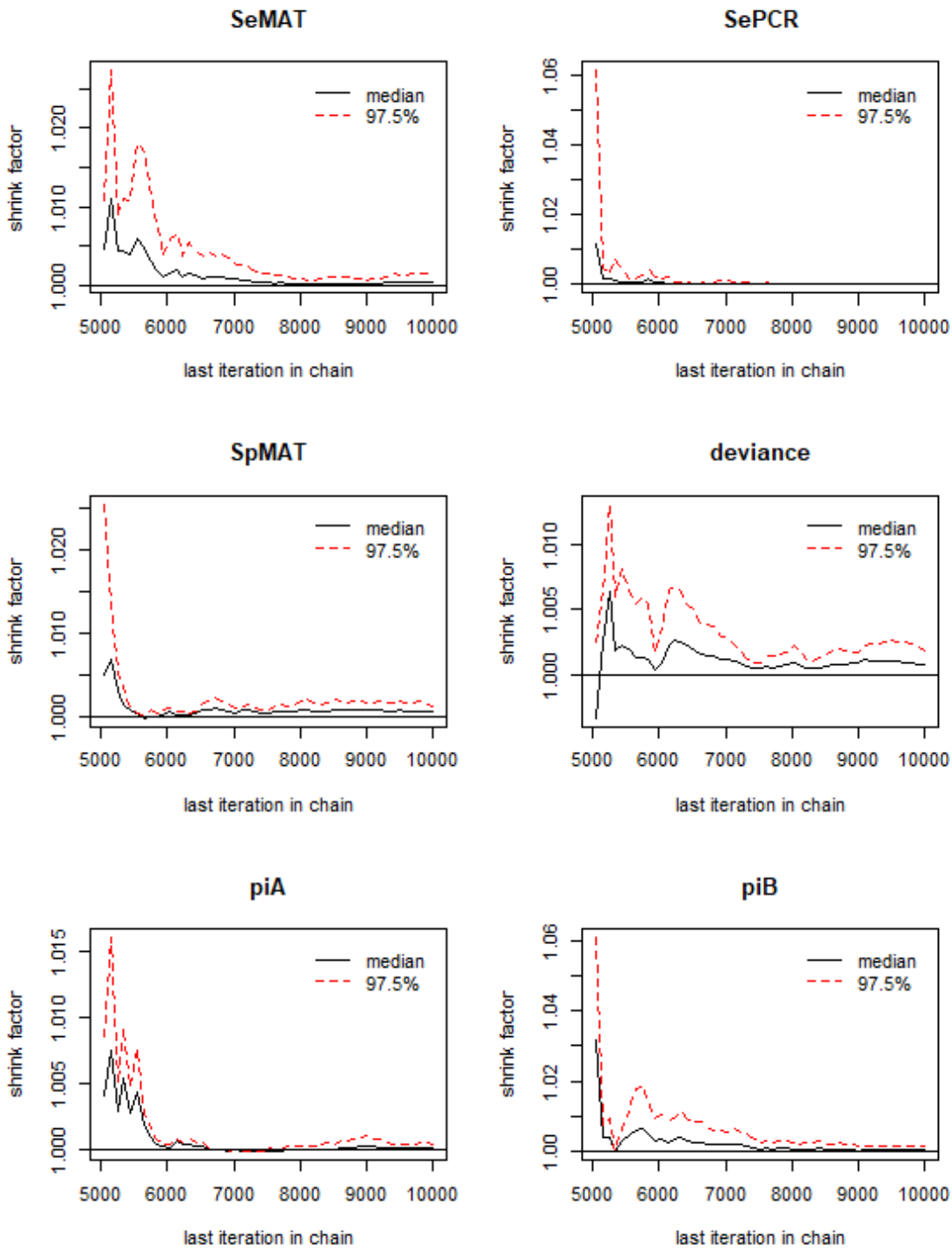



```
gelman.diag(out.coda) #shrink factors should be below 1.05
```

```
## Potential scale reduction factors:
##
##          Point est. Upper C.I.
## SeMAT             1          1
## SePCR             1          1
```

```
## SpMAT              1          1
## deviance           1          1
## piA                1          1
## piB                1          1
##
## Multivariate psrf
##
## 1
```

```
gelman.plot(out.coda) #Gelman-Rubin-Brooks plot for visual confirmation of th
e shrink factor convergence
```



```r
#retrieve point estimates and 95% credible intervals
out.summary <- summary(out.coda, q=c(0.025, 0.975))
out.summary$stat
```

```
##                Mean         SD     Naive SE Time-series SE
## SeMAT     0.6259558 0.08240187 0.0006728085   0.0007279397
## SePCR     0.6686218 0.02770335 0.0002261969   0.0002336840
## SpMAT     0.7537568 0.01631514 0.0001332126   0.0001323422
## deviance 59.2916307 3.75101593 0.0306269168   0.0339350688
## piA       0.3676077 0.06227121 0.0005084423   0.0005417730
## piB       0.2180274 0.07212884 0.0005889295   0.0006331803
```

```r
out.summary$q
```

```
##                 2.5%      97.5%
## SeMAT     0.45879750  0.7814025
## SePCR     0.61379750  0.7215025
## SpMAT     0.72110000  0.7850000
## deviance 52.61000000 67.4705000
## piA       0.25239500  0.4959075
## piB       0.09765975  0.3762000
```

```r
Run<-cbind(out.summary$stat,out.summary$q)
write.csv(Run,file = "Run_initial.csv", row.names = TRUE)
```

Appendix 5.
Occupancy model in E-Surge (Chapt.3)

Mice were considered as 'sites' and laboratory tests as 'detection occasions'

Assumptions

- All positive tests were regarded as true (no false positive)
- Detection histories were independent
- No change of status during testing

States:
Exposed to *Leptospira* (ψ)
Unexposed to *Leptospira* ($1-\psi$)
Dummy-state ('dead', needed in GEPAT)

Events:
Tested positive (p)
Tested negative ($1-p$)

We defined for model (ψ, p) the initial state (Π), transition (Φ) and event (B) matrices:

$\Pi = [1-\psi \quad \psi]$

$\Phi = \begin{bmatrix} 1 & 0 \\ 0 & 1 \end{bmatrix}$ (a specific case of static (single-season) model with no change of state)

$B = \begin{bmatrix} 1 & 0 \\ 1-p & p \end{bmatrix}$

Syntax in GEPAT (GEnerator of PAttern matrices)

$Initial\ state = [* \quad \pi]$

$Transitions = \begin{bmatrix} * & - & - \\ - & * & - \\ - & - & * \end{bmatrix}$ (the last row and columns are for the 'dead' state)

$Event = \begin{bmatrix} * & - \\ * & \beta \\ * & - \end{bmatrix}$ (the last row and columns are for the 'dead' state)

Syntax in GEMACO (GEnerator of MAtrices COnstraints)

Model	(ψ, p)	(ψ_{Farm}, p)	(ψ, p_{Method})	(ψ_{Farm}, p_{Method})
Initial state	i	i + g(1,2)	i	i + g(1,2)
Transition	i	i	i	i
Event	i	i	t	t

Default parameters in the IVFV menu (Initial Values or Fixed Values of parameters) were used, and the option to compute Hessian confidence intervals selected.

Appendix 6.
Supplementary data for Chapter 4

Table S 6-1 | Breakdown of shedding and serological status by age and session in mice captured.

		Adult			Juvenile			**Total**
		NT	Non-shedder	Shedder	NT	Non-shedder	Shedder	
	NT	4	0	1	4	0	1	**10**
A1	seronegative	0	2	5	0	1	1	**9**
	seropositive	1	0	10	0	0	2	**13**
	NT	13	4	1	11	1	0	**30**
A2	seronegative	0	21	2	0	25	7	**55**
	seropositive	0	1	12	0	2	3	**18**
	NT	3	0	0	0	0	0	**3**
A3	seronegative	0	2	3	0	0	0	**5**
	seropositive	0	0	12	0	0	5	**17**
	NT	3	1	0	1	0	0	**5**
A4	seronegative	0	15	10	0	16	5	**46**
	seropositive	0	3	13	0	2	2	**20**
Total		**24**	**49**	**69**	**16**	**47**	**26**	**231**

NT = Not tested

Table S 6-2 | Conditional Likelihood SECR models initially built. *Only top 12 ranked models with an AICc weight (AICcwt) > 0 are listed, along with a null model describing factors affecting the probability to detect a mouse when home range centre and trap coincide (g_0) and the spatial scale over which this probability declines (σ).*

Initial models	npar	logLik	AIC	AICc	dAICc	AICcwt
g_0~session + age σ~session	17	-1349.55	2733.09	2735.98	0.00	0.29
g_0~session + age σ~session + age	18	-1348.96	2733.92	2737.16	1.18	0.16
g_0~year + age σ~session	12	-1355.96	2735.92	2737.36	1.38	0.14
g_0~session + sex + age σ~session	18	-1349.50	2735.00	2738.24	2.26	0.09
g_0~session + age σ~session + sex + age	19	-1348.73	2735.46	2739.08	3.10	0.06
g_0~year + sex + age σ~session + sex	14	-1354.64	2737.27	2739.23	3.25	0.06
g_0~session + sex + age σ~session + age	19	-1348.92	2735.83	2739.45	3.47	0.05
g_0~year + sex + age σ~session	13	-1355.93	2737.87	2739.55	3.57	0.05
g_0~session + sex + age σ~session + sex	19	-1349.04	2736.08	2739.70	3.72	0.04
g_0~session + sex + age σ~session + sex + age	20	-1348.72	2737.45	2741.47	5.49	0.02
g_0~year + sex + age σ~session + sex + age	15	-1354.64	2739.27	2741.52	5.54	0.02
g_0~year + sex + age σ~session + age	14	-1355.85	2739.70	2741.65	5.67	0.02
go~1 sigma~1	2	-1391.38	2786.75	2786.80	50.83	0.00

Npar: number of parameters; logLik: log likelihood; AIC(c): Akaike's information criterion (corrected for a small sample size); dAICc: differences in AICc

Table S 6-3 | Hybrid mixture SECR models built including seropositivity as a mixture class. *Only top 4 ranked models with an AICc weight (AICcwt) > 0 are listed describing factors affecting the probability to detect a mouse when home range centre and trap coincide (g_0), the spatial scale over which this probability declines (σ), and a latent mixture class (pmix) representing the proportion of seropositive in the population.*

Hybrid mixture model (pmix = seropositivity)	npar	logLik	AIC	AICc	dAICc	AICcwt
g_0~session + age σ~session + h2 pmix~ts + h2	22	-1443.26	2930.52	2935.40	0.00	0.50
g_0~session + age + h2 σ~session + h2 pmix~ts + h2	23	-1442.75	2931.49	2936.85	1.45	0.24
g_0~year + age σ~session + h2 pmix~ts + h2	17	-1450.37	2934.73	2937.62	2.22	0.16
g_0~year + age + h2 σ~session + h2 pmix~ts + h2	18	-1449.74	2935.49	2938.73	3.32	0.09

Npar: number of parameters; logLik: log likelihood; AIC(c): Akaike's information criterion (corrected for a small sample size); dAICc: differences in AICc. h2: mixture term for pmix; ts: trapping session

Table S 6-4 | Hybrid mixture SECR models built including shedding status as a mixture class. *Only top 8 ranked models with an AICc weight (AICcwt) > 0 are listed describing factors affecting the probability to detect a mouse when home range centre and trap coincide (g_0), the spatial scale over which this probability declines (σ), and a latent mixture class (pmix) representing the proportion of shedders in the population.*

Hybrid mixture model (pmix = shedder)	npar	logLik	AIC	AICc	dAICc	AICcwt
g_0~session + age + h2 σ~session + h2 pmix~ts + h2	**23**	**-1455.39**	**2956.78**	**2962.14**	**0.00**	**0.42**
g_0~year + age + h2 σ~session + h2 pmix~ts + h2	18	-1461.96	2959.91	2963.16	1.01	0.25
g_0~year + age σ~session + h2 pmix~ts + h2	17	-1463.75	2961.49	2964.38	2.24	0.14
g_0~session + age σ~session + h2 pmix~ts + h2	22	-1457.84	2959.69	2964.58	2.43	0.12
g_0~session + age σ~session pmix~ts + h2	21	-1460.54	2963.08	2967.52	5.38	0.03
g_0~year + age σ~session pmix~ts + h2	16	-1466.95	2965.91	2968.46	6.32	0.02
g_0~session + age + h2 σ~session pmix~ts + h2	22	-1460.24	2964.48	2969.37	7.23	0.01
g_0~year + age + h2 σ~session pmix~ts + h2	17	-1466.60	2967.20	2970.09	7.95	0.01

Npar: number of parameters; logLik: log likelihood; AIC(c): Akaike's information criterion (corrected for a small sample size); dAICc: differences in AICc. h2: mixture term for pmix; ts: trapping session

Table S 6-5 | Density estimates for all mice and mice shedding *Leptospira* derived from the top ranked hybrid mixture SECR previously selected. *The associated model appears bolded in Table S 6-4. Lcl: Lower confidence limit, ucl = upper confidence limit*

Season	Grid	group	D	lcl	ucl
Spring 2016	Grid 1	Total	8.92	2.68	29.65
Spring 2016	Grid 2	Total	6.15	2.00	18.92
Fall 2017	Grid 1	Total	55.48	36.68	83.90
Fall 2017	Grid 2	Total	55.85	37.53	83.12
Spring 2017	Grid 1	Total	3.61	0.88	14.80
Spring 2017	Grid 2	Total	6.48	2.94	14.28
Fall 2018	Grid 1	Total	38.88	18.27	82.72
Fall 2018	Grid 2	Total	27.06	13.49	54.29
Spring 2016	Grid 1	Shedders	4.32	1.27	14.68
Spring 2016	Grid 2	Shedders	2.91	1.07	7.92
Fall 2017	Grid 1	Shedders	6.06	3.11	11.81
Fall 2017	Grid 2	Shedders	7.64	3.41	17.14
Spring 2017	Grid 1	Shedders	3.61	0.88	14.80
Spring 2017	Grid 2	Shedders	4.57	1.95	10.70
Fall 2018	Grid 1	Shedders	7.52	2.65	21.30
Fall 2018	Grid 2	Shedders	5.10	1.87	13.90

Appendix 7.
Supplementary data for Chapter 5

Table S 7-1 | List of isolates and [m]EpiLab or NCBI genomes included in the SNP analysis and assembly summary statistics. *Summary statistics were obtained using SeqKit (Shen et al., 2016) and in-house code. The name of isolates described in Chapter 5 includes the Farm and session (e.g. A1), the species (Mm = Mus musculus, Ee = Erinaceus europaeus, Rr = Rattus rattus, Tv = Trichosurus vulpecula, Bt = Bos taurus), the animal ID and the letters K or U when isolates where obtained from both kidney (K) and urine (U). Details on [m]EpiLab and NCBI genomes and details on each Profile are given in Chapter 5, Table 5-3 and Table 5-6. Num_seqs is the number of sequences assembled in contigs, sum_len represents the total length of the assembled genome (sum of contigs lengths), N50 is the minimum contig length to cover 50% of the genome (half of the genome sequence is in contigs larger than or equal the N50 contig size), GC is the GC (guanine-cytosine) content, and %Cov represents the proportion of each genome covered by the SNP analysis.*

Isolate (PubMLST_ID)	Profile	num_seqs	sum_len	N50	GC	%Cov
A1_Mm_MM006 (1309)	Ballum	156	3,893,461	48,168	40.01	95.52%
A1_Mm_MM010 (1310)	Ballum	148	3,892,974	49,331	40.01	95.54%
A1_Mm_MM011K (1311)	Ballum	161	3,894,764	43,982	40.02	95.49%
A1_Mm_MM011U (1312)	Ballum	162	3,893,609	43,982	40.02	95.52%
A1_Mm_MM013K (1313)	Ballum	142	3,895,411	48,156	40.02	95.48%
A1_Mm_MM013U (1314)	Ballum	150	3,895,404	48,385	40.02	95.48%
A1_Mm_MM026K (1315)	Ballum	153	3,893,271	48,183	40.01	95.53%
A1_Mm_MM026U (1316)	Ballum	145	3,896,339	48,276	40.02	95.45%
A1_Mm_MM028 (1317)	Ballum	150	3,894,246	48,523	40.02	95.50%
A1_Mm_MM031K (1318)	Ballum	155	3,894,305	43,520	40.03	95.50%
A1_Mm_MM031U (1319)	Ballum	161	3,892,956	48,178	40.02	95.54%
A1_Mm_MM037 (1320)	Ballum	138	3,896,640	53,752	40.02	95.45%
A1_Mm_MM043 (1321)	Ballum	167	3,893,454	42,724	40.03	95.52%
A2_Ee_MM195 (1332)	Ballum	151	3,897,042	48,168	40.02	95.44%
A2_Ee_MM196 (1333)	Ballum	156	3,894,172	48,168	40.02	95.51%
A2_Mm_MM134 (1325)	Ballum	160	3,892,381	48,189	40.02	95.55%
A2_Mm_MM138 (1326)	Ballum	166	3,893,371	44,417	40.02	95.53%
A2_Mm_MM148 (1327)	Ballum	161	3,894,369	44,725	40.02	95.50%
A2_Mm_MM152 (\)	Ballum	196	3,931,733	44,428	40.23	94.59%
A2_Mm_MM159 (1328)	Ballum	183	3,891,275	41,220	40.03	95.58%
A2_Mm_MM161 (1329)	Ballum	153	3,894,185	48,156	40.02	95.51%
A2_Mm_MM189 (1331)	Ballum	144	3,895,323	52,938	40.02	95.48%
A2_Mm_MM200 (1334)	Ballum	156	3,894,979	48,611	40.02	95.49%
A2_Mm_MM211 (1335)	Ballum	150	3,894,721	53,997	40.02	95.49%
A2_Mm_MM220 (1337)	Ballum	131	3,897,582	52,938	40.02	95.42%
A2_Mm_MM225 (\)	Ballum	143	3,897,290	49,331	40.02	95.43%
A2_Mm_MM242 (1338)	Ballum	151	3,894,510	48,189	40.02	95.50%
A2_Mm_MM244 (1339)	Ballum	152	3,893,698	48,189	40.02	95.52%

Isolate (PubMLST_ID)	Profile	num_seqs	sum_len	N50	GC	%Cov
A2_Rr_MM213 (1336)	Ballum	184	3,893,695	43,732	40.03	95.52%
A3_Ee_MM277 (1357)	Ballum	150	3,894,662	52,584	40.02	95.49%
A3_Mm_MM256 (1340)	Ballum	154	3,893,959	48,189	40.02	95.51%
A3_Mm_MM257 (1341)	Ballum	158	3,894,355	48,189	40.02	95.50%
A3_Mm_MM258 (1342)	Ballum	158	3,893,821	48,168	40.02	95.52%
A3_Mm_MM259 (1343)	Ballum	149	3,897,084	48,189	40.02	95.44%
A3_Mm_MM260 (1344)	Ballum	142	3,895,368	49,331	40.02	95.48%
A3_Mm_MM261 (1345)	Ballum	151	3,895,193	48,168	40.02	95.48%
A3_Mm_MM262 (1346)	Ballum	157	3,893,868	44,405	40.02	95.51%
A3_Mm_MM263 (1347)	Ballum	140	3,895,468	49,331	40.02	95.47%
A3_Mm_MM264 (1348)	Ballum	155	3,893,629	48,194	40.02	95.52%
A3_Mm_MM265 (1349)	Ballum	147	3,895,452	49,331	40.02	95.48%
A3_Mm_MM268 (1351)	Ballum	154	3,894,025	43,330	40.02	95.51%
A3_Mm_MM269 (1352)	Ballum	155	3,893,413	52,584	40.02	95.53%
A3_Mm_MM272 (1354)	Ballum	136	3,897,694	52,584	40.02	95.42%
A3_Mm_MM273 (1355)	Ballum	142	3,896,243	52,584	40.02	95.46%
A3_Mm_MM275 (1356)	Ballum	141	3,895,653	57,850	40.02	95.47%
A3_Mm_MM280 (1358)	Ballum	164	3,894,254	43,732	40.02	95.50%
A4_Ee_MM342 (1372)	Ballum	155	3,894,094	48,189	40.01	95.51%
A4_Ee_MM344 (1373)	Ballum	147	3,894,910	48,376	40.02	95.49%
A4_Mm_MM309 (\)	Ballum	137	3,902,365	52,584	40.04	95.31%
A4_Mm_MM310 (1360)	Ballum	143	3,901,359	43,732	40.04	95.33%
A4_Mm_MM313 (1361)	Ballum	140	3,903,421	48,220	40.03	95.28%
A4_Mm_MM317 (1362)	Ballum	134	3,895,585	54,001	40.02	95.47%
A4_Mm_MM320 (1363)	Ballum	149	3,901,375	44,023	40.03	95.33%
A4_Mm_MM324 (1364)	Ballum	120	3,902,099	60,129	40.02	95.31%
A4_Mm_MM325 (1365)	Ballum	131	3,899,138	53,997	40.03	95.39%
A4_Mm_MM327 (1366)	Ballum	142	3,893,679	52,584	40.02	95.52%
A4_Mm_MM328 (1367)	Ballum	170	3,891,793	43,330	40.02	95.57%
A4_Mm_MM330 (1368)	Ballum	130	3,900,803	48,849	40.03	95.34%
A4_Mm_MM332 (1369)	Ballum	159	3,915,734	48,523	40.02	94.98%
A4_Mm_MM336 (1370)	Ballum	145	3,896,664	49,331	40.02	95.45%
A4_Mm_MM340 (1371)	Ballum	140	3,895,857	58,709	40.02	95.47%
A4_Mm_MM350 (1374)	Ballum	139	3,896,680	53,997	40.01	95.45%
A4_Mm_MM351 (1375)	Ballum	117	3,914,439	60,129	40.05	95.01%
A4_Mm_MM354 (1376)	Ballum	143	3,902,360	52,584	40.02	95.31%
A4_Mm_MM361 (1383)	Ballum	159	3,903,123	48,156	40.04	95.29%
A4_Mm_MM370 (1385)	Ballum	140	3,895,732	52,584	40.02	95.47%
A4_Mm_MM372 (1386)	Ballum	150	3,893,888	52,936	40.01	95.51%
A4_Rr_MM366 (1384)	Ballum	136	3,897,427	58,709	40.02	95.43%
B1_Mm_MM050 (1322)	Ballum	156	3,894,116	49,331	40.01	95.51%
B1_Mm_MM082 (\)	Ballum	169	3,916,738	52,584	40.14	94.96%
B1_Mm_MM098 (1323)	Ballum	157	3,894,302	42,012	40.02	95.50%
B1_Mm_MM119 (1324)	Ballum	158	3,894,316	49,331	40.02	95.50%
MU_Rr_LW001	Ballum	114	3,903,118	64,472	40.02	95.29%

Isolate (PubMLST_ID)	Profile	num_seqs	sum_len	N50	GC	%Cov
MU_Rr_LW046	Ballum	152	3,893,231	48,527	40.02	95.53%
H5	Ballum	114	3,912,193	64,472	40.02	95.07%
Arborea	Ballum	122	3,904,949	58,708	40.02	95.24%
Ballum	Ballum	115	3,891,200	60,616	40.01	95.58%
Lb_200801910_Guadeloupe	Ballum	22	3,970,478	315,783	40.02	93.67%
Lb_4E_Brazil	Ballum	2	3,912,599	3,550,837	40.18	95.06%
Lb_56604-Mouse_China	Ballum	229	3,900,819	32,749	40.01	95.34%
Lb_56604-Rat_China	Ballum	4	4,037,579	3,550,837	40.19	92.11%
Lb_56607_China	Ballum	220	3,900,903	34,672	40.01	95.34%
Lb_56648_China	Ballum	222	3,892,822	33,626	40.02	95.54%
Lb_LO-24_Brazil	Ballum	37	3,963,883	174,317	40.02	93.83%
Lb_Muis5_Netherlands	Ballum	212	3,887,643	53,113	40.01	95.67%
A3_Tv_MM270 (1353)	Balcanica	261	3,800,953	24,385	40.34	/
Balcanica_possum	Balcanica	218	3,823,659	31,466	40.34	/
P44	Balcanica	234	3,815,325	26,580	40.34	/
SN_Ce_RL4	Balcanica	262	3,810,748	25,512	40.36	/
SN_Ce_RL16	Balcanica	267	3,800,850	23,233	40.35	/
A_Bt_HO907 (1388)	Hardjo	184	3,686,354	35,321	40.28	95.95%
A_Bt_HO912 (1389)	Hardjo	184	3,687,035	35,847	40.28	95.93%
D12	Hardjo	173	3,694,824	40,933	40.28	95.73%
D25	Hardjo	174	3,694,611	37,315	40.28	95.74%
E44	Hardjo	161	3,705,261	45,893	40.28	95.46%
EVK11	Hardjo	184	3,693,849	35,321	40.29	95.76%
EVK2	Hardjo	178	3,696,872	37,929	40.29	95.68%
EVK25	Hardjo	192	3,691,842	35,321	40.29	95.81%
EVK5	Hardjo	191	3,690,013	34,389	40.29	95.86%
EVK8	Hardjo	187	3,691,973	34,694	40.29	95.81%
G18	Hardjo	154	3,707,674	45,893	40.28	95.40%
I53	Hardjo	157	3,708,831	45,893	40.28	95.37%
I89	Hardjo	174	3,694,795	45,370	40.28	95.73%
K4_12	Hardjo	154	3,708,698	45,893	40.28	95.37%
Lb_203_USA	Hardjo	2	3,907,328	3,589,981	40.22	90.53%
Lb_BK-30_USA	Hardjo	2	3,947,069	3,629,289	39.05	89.61%
Lb_BK-6_USA	Hardjo	2	3,967,801	3,649,879	38.77	89.15%
Lb_BK-9_USA	Hardjo	2	3,949,086	3,631,634	39.01	89.57%
Lb_JB197_USA	Hardjo	2	3,876,235	3,576,473	40.24	91.25%
Lb_L49_Brazil	Hardjo	2	3,935,911	3,620,710	40.17	89.87%
Lb_L550_Australia	Hardjo	2	3,931,782	3,614,446	40.23	89.96%
Lb_Lely_607_Netherlands	Hardjo	198	3,774,172	33,297	40.26	93.72%
Lb_NVSL_S_1343_USA	Hardjo	2	3,932,487	3,613,654	39	89.95%
Lb_NVSL_S_818_USA	Hardjo	2	3,884,697	3,584,464	39.34	91.05%
Lb_Sponselee_CDC_Netherlands	Hardjo	196	3,776,198	31,392	40.23	93.67%
Lb_Sponselee_Netherlands	Hardjo	187	3,755,643	32,590	40.25	94.18%
A_Bt_HO905 (1387)	Pomona	218	4,513,069	41,222	34.93	91.29%
A2_Ee_MM168 (1330)	Pomona	256	4,495,009	30,955	34.92	91.65%

Isolate (PubMLST_ID)	Profile	num_seqs	sum_len	N50	GC	%Cov
A3_Ee_MM266 (1350)	Pomona	233	4,496,886	34,962	34.92	91.62%
A3_Ee_MM281 (1359)	Pomona	216	4,498,625	38,692	34.91	91.58%
AO25ii	Pomona	113	4,522,329	73,113	34.92	91.10%
B33	Pomona	100	4,501,095	78,366	34.92	91.53%
EMY7780	Pomona	100	4,522,652	78,527	34.92	91.09%
ESR8	Pomona	114	4,524,631	67,070	34.93	91.05%
P5661	Pomona	104	4,526,353	73,114	34.93	91.02%
Pomona_str68	Pomona	183	4,505,313	42,521	34.92	91.44%
Li_2006006962_Egypt	Pomona	204	4,563,168	37,213	34.97	90.28%
Li_2006006968_Egypt	Pomona	224	4,555,706	33,291	34.97	90.43%
Li_56133_Australia	Pomona	300	4,518,153	24,981	34.9	91.18%
Li_56608_China	Pomona	308	4,563,098	27,348	34.98	90.29%
Li_56608-V_China	Pomona	299	4,397,635	26,196	34.92	93.68%
Li_56630_China	Pomona	313	4,564,593	27,113	34.92	90.26%
Li_AK-RFB_Argentina	Pomona	31	4,627,117	3,804,209	34.85	89.04%
Li_Col-P036_Colombia	Pomona	591	4,403,805	12,806	34.94	93.55%
Li_CSL10083_USA	Pomona	182	4,574,387	44,679	34.99	90.06%
Li_CSL4002_USA	Pomona	187	4,591,338	41,059	34.97	89.73%
Li_Fox_32256_USA	Pomona	172	4,583,716	47,406	35	89.88%
Li_GR5_Brazil	Pomona	74	4,594,696	121,968	34.97	89.67%
Li_Kennewicki_LC82-25_USA	Pomona	71	4,608,327	128,153	35	89.40%
Li_Pomona_Australia	Pomona	43	4,581,018	198,881	35	89.93%

/ Not available (SNP analysis not conducted)

Table S 7-2 | Core-genome Single Nucleotide Polymorphism and functional impact on associated protein for 2 Hardjobovis isolates

CHR	POS	REF	A_Bt_HO907	A_Bt_HO912	EFFECT	PRODUCT
NC_008508	3151658	A	A	G	missense_variant c.400A>G p.Asn134Asp	glycerophosphodiester phosphodiesterase

Table S 7-3 | List of core-genome Single Nucleotide Polymorphisms and functional impact on associated proteins for 72 Ballum isolates

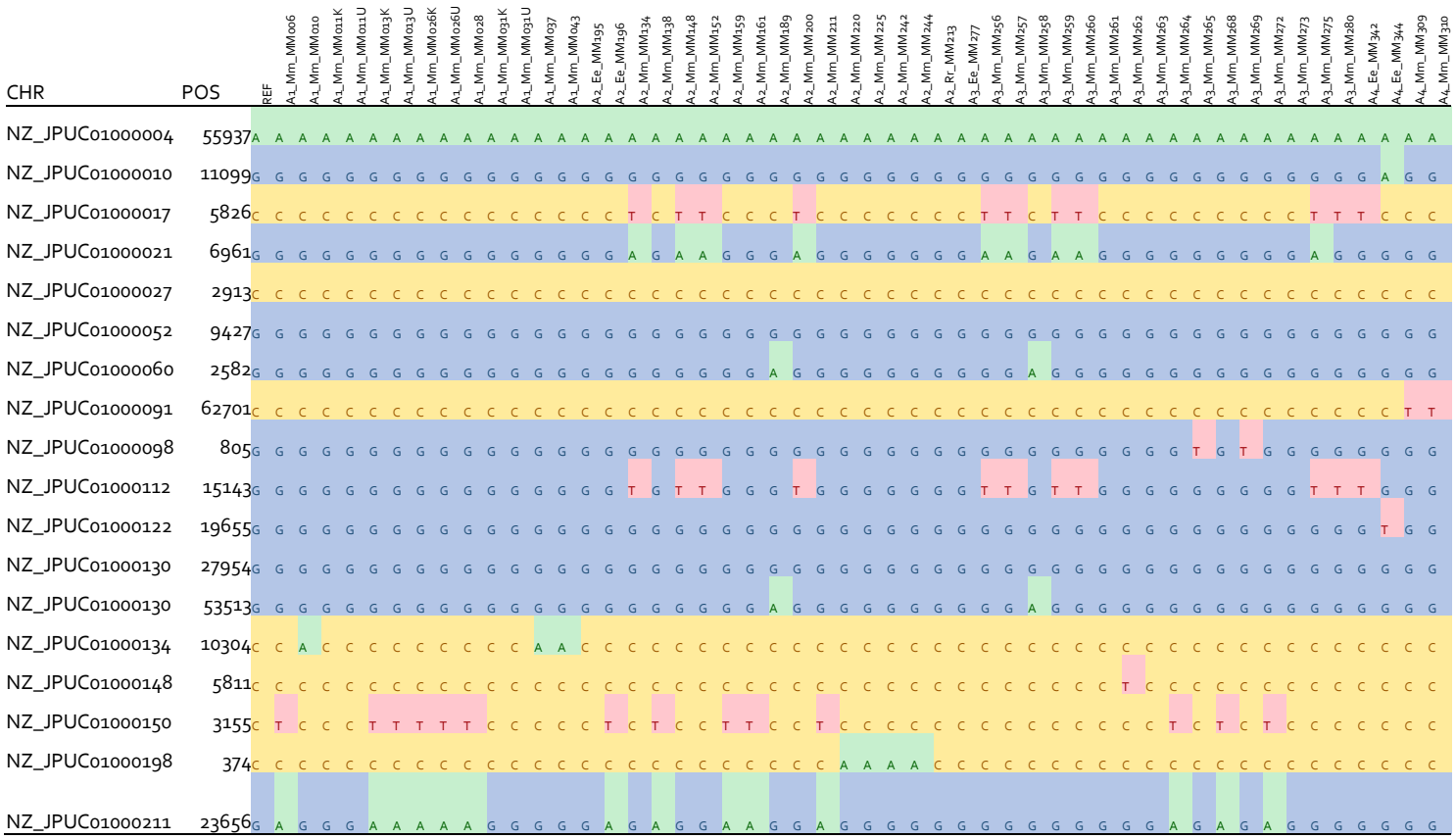


Table S 7-3 | (continued)

A4_Mm_MM313	A4_Mm_MM317	A4_Mm_MM320	A4_Mm_MM324	A4_Mm_MM325	A4_Mm_MM327	A4_Mm_MM328	A4_Mm_MM330	A4_Mm_MM332	A4_Mm_MM336	A4_Mm_MM340	A4_Mm_MM350	A4_Mm_MM351	A4_Mm_MM354	A4_Mm_MM361	A4_Mm_MM370	A4_Mm_MM372	A4_R_MM366	B1_Mm_MM050	B1_Mm_MM082	B1_Mm_MM098	B1_Mm_MM119	EFFECT MV: missense variant SV:synonymous variant SL: Start lost	PRODUCT
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A		MV c.701A>G p.Tyr234Cys	HAD family hydrolase
G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		SV c.264C>T p.Gly88Gly	sulfate adenylyltransferase subunit CysD
C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C		SV c.522G>A p.Lys174Lys	tetraacyldisaccharide 4'-kinase
G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		MV c.1144G>A p.Gly382Arg	hypothetical protein
C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	T	T	T			
G	G	G	G	G	G	G	G	G	G	G	G	G	A	G	G	G	G	G	G	G		MV c.97G>A p.Glu33Lys	two-component system response regulator
G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		MV c.271G>A p.Glu91Lys	FliG protein
C	C	T	T	C	T	C	C	C	C	C	T	T	C	C	C	C	C	C	C	C			
G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G			
G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		MV c.274C>A p.Pro92Thr	pyridoxamine 5'-phosphate oxidase
G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		MV c.277G>T p.Ala93Ser	hypothetical protein
G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	C	G	G	G		SV c.774G>C p.Leu258Leu	hypothetical protein
G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		MV c.1735C>T p.His579Tyr	transcription elongation factor GreA/GreB
C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C		MV c.78G>T p.Met26Ile	PAS domain-containing protein
C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C			
C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C		SL c.3G>A p.Leu1?	hypothetical protein
C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C			
G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		SV c.1197G>A p.Ala399Ala	cellulase (glycosyl hydrolase family 5) domain protein

Appendix 8.
Discrepant results

This appendix is presented as a short communication.

Investigating discrepant results obtained by culture and PCR on animal samples tested for *Leptospira* infection

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Introduction

During the research project described in this thesis, different animal samples were taken and various tests conducted whenever possible. Serum was investigated for the presence of antibodies against *Leptospira* and kidney was cultured and subjected to PCR for direct detection of pathogenic *Leptospira*.

Several PCR methods were used across this thesis, with different matrices (kidney, urine), amplification targets (*gyrB*, *lipL32*, *glmU* genes), chemistries (SYTO™ 9, TaqMan™ probe, SybR™ Green) and preparations (QIAamp DNA Mini Kit for kidney and QIAamp RNA Viral Kit or boiled preparations for urine). The *gyrB* quantitative PCR previously validated and in use at ᵐEpiLab (Subharat et al., 2011a) was used on kidney samples from the first capture session. Because results were difficult to interpret, we decided to use another PCR targeting the *lipL32* gene.

The sensitivity of the *lipL32* PCR is considered very high; 100% in the original publication (Stoddard et al., 2009), with a lower limit of detection (LLOD) between 5 and 50 Genome Equivalent (GE)/µℓ for the quantitative PCR (qPCR) used in our study (Galloway and Hoffmaster, 2015). In-house validation of the *lipL32* qPCR and *glmU* PCR gave a LLOD of 2 to 20 GE/µℓ for both (Nisa et al., in prep).

Leptospira are difficult bacteria to grow and isolate, and culture sensitivity is considered low compared to PCR methods (Merien et al., 1995, Slack et al., 2007). However, several samples were PCR negative while leptospires were observed under the dark-field microscope (DFM) or isolated. As some of the observed leptospire-like organisms could not be typed, we investigated the possibility of an issue with the PCR sensitivity.

Because all standard curves in the original articles were determined using either a buffer, blood or urine samples spiked with pure cultures, we investigated the possibility of PCR inhibition due to the presence of the kidney matrix. Firstly, we tested the effect of dilution on samples already extracted as suggested in (Galloway and Hoffmaster, 2015), and secondly, we built a standard curve for the *lipL32* PCR with kidney as a matrix for both *L. interrogans* (serovar Pomona) and *L. borgpetersenii* (serovar Ballum).

Methods

Dilution 1:10

The following samples from animals with conflicting results were investigated:

(a) DNA extracted from six kidney samples from animals for which *Leptospira* were successfully isolated, but *lipL32* PCR was negative (four mice, one ship rat, one beef cattle beast);

(b) DNA extracted from seven kidney samples from animals for which leptospire-shaped organisms were initially observed under the DFM but isolation was unsuccessful, and *lipL32* and *glmU* PCRs were negative (five mice, one hedgehog, one beef cattle beast);

(c) Boiling preparations of four urine samples that were *lipL32* PCR positive with a late amplification (37 < Ct < 42) and culture was negative (dairy cattle)

(d) DNA extracted from two kidney samples with a high Ct in the *lipL32* PCR first run (37 and 35.7) were also included, as well as DNA extracted from two kidney samples with a negative *lipL32* PCR, all culture negative.

All samples were diluted at 1:10 and the *lipL32* PCR re-run.

lipL32 PCR Standard curves with kidney matrix

We tested the effect of the kidney matrix on the sensitivity of the *lipL32* PCR. We used 10-fold serial dilutions of bacterial DNA ranging 200,000 to 0.02 genomic equivalent (GE) and mixed them with renal DNA extracted from *Leptospira* negative animals at a ratio of 1:1. Both renal and bacterial DNA were extracted using the QIAamp DNA Mini Kit as per manufacturer's instructions and quantified using Qubit®. Bacterial DNA was extracted from pure cultures of *Leptospira interrogans* sv Pomona and *L. borgpetersenii* sv Ballum and was previously used to validate the *glmU* PCR (Nisa et al., in prep). Renal DNA was used from three *Leptospira* negative animals (a hedgehog and two mice), that were *lipL32* PCR negative, culture negative and MAT negative at the 48 cut-off (titres < 24 for svs Ballum, Hardjobovis, Pomona, Tarassovi and Copenhageni for mice, and titres < 24 for svs Ballum, Hardjobovis and Pomona and = 24 for Tarassovi and Copenhageni for the hedgehog). The renal DNA concentration before mixing bacterial DNA was 3.8 ng/mℓ (low), 12.9 ng/mℓ (medium), and 74.1 ng/mℓ (high). This provided us with a serial dilution of bacterial DNA ranging 100,000 to 0.01 GE/µℓ with either low, medium or high concentration of renal DNA.

Results

Dilution 1:10

Of all the samples diluted and re-run, three were positive, two from (b) with a Ct = 33.5 and 33.9.and one from (d), with a Ct = 33.5 (the previous Ct before dilution for this sample was 35.7).

lipL32 PCR Standard curves with kidney matrix

The standard curves for Pomona and Ballum are presented in Figure S 8-1(A & B). For a comparison, the standard curve for Ballum using dilutions of *Leptospira* spiked in PBS (Phosphate Buffered Saline) used for the in-house validation of the *lipL32* PCR is presented in Figure S 8-1(C).

When Ballum and Pomona were spiked into a kidney DNA matrix, the lowest dilution at which all three duplicates were positive was 100 GE/µℓ. At lower concentrations, the detection of *Leptospira* DNA was erratic, with no clear effect of renal DNA concentration on the PCR results:

- The PCRs with a low renal DNA concentration were positive down to 100 GE/µℓ for Ballum and 10 GE/µℓ for Pomona, and then positive again at 0.01 GE/µℓ for Pomona.
- The PCRs with a medium concentration of renal DNA were positive down to 10 GE/µℓ for Ballum and 100 GE/µℓ for Pomona.
- The PCRs with a high renal DNA concentration were positive down to 1 GE/µℓ for Ballum and 100 GE/µℓ for Pomona, and then positive again at 0.1 GE/µℓ for Pomona.

Discussion/Conclusion

Both dilution at 1:10 and standard curves with a kidney matrix suggested the *lipL32* PCR method used in this thesis suffered from inhibition. The standard curves indicate it had a LLOD 10 times lower than expected on kidney samples, and that above a Ct = 33, the concentration of bacterial DNA was very low and PCRs assays susceptible to inhibition. However, as several samples remained negative, another explanation could be the presence of non-pathogenic or intermediate leptospires. The PCRs employed are

only specific for pathogenic *Leptospira* and intermediate or saprophytic species fail to be detected (Stoddard et al., 2009). Testing samples with a PCR targeting a gene that is not specific to pathogenic leptospires, for instance the *16S rRNA* gene, would be needed to ascertain the absence of non-pathogenic leptospires.

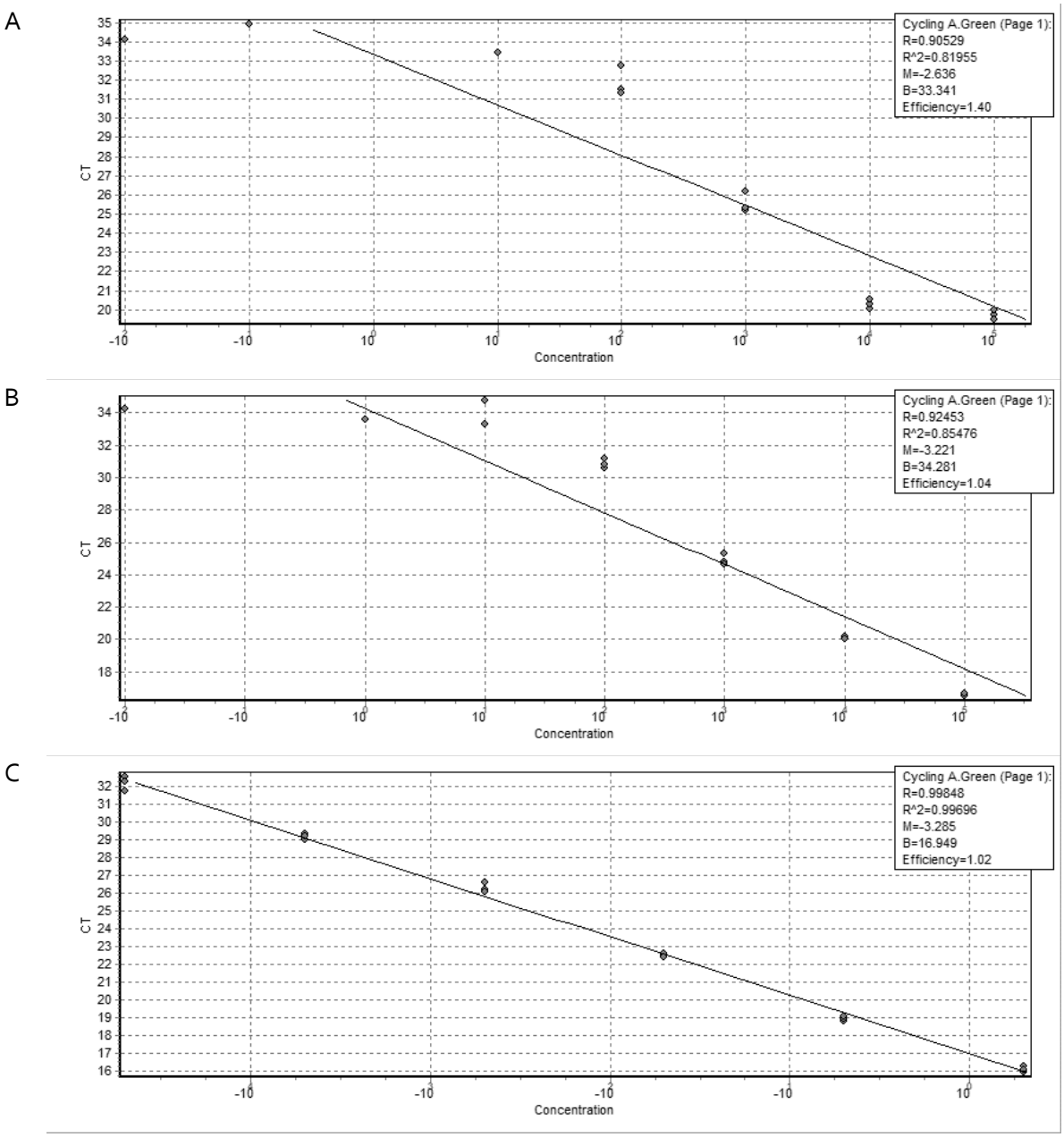

Figure S 8-1 | *lipL32* qPCR Standard curve for the detection of *Leptospira interrogans* serovar Pomona on a kidney matrix (A), and *L. borgpetersenii* serovar Ballum on a kidney (B) or PBS (C) matrix.

Appendix 9. Supplementary data for Chapter 6

Table S 9-1 | Presence of histopathological lesions compatible with leptospirosis among animals tested positive or negative for *Leptospira* spp. on culture, PCR or MAT

Lesion Score†	Culture	PCR	MAT	*Erinaceus europaeus*	*Mus musculus*	*Rattus rattus*	Total
0	negative	negative	negative	5	85	14	104
			positive	9	6	1	16
			NA	0	2	0	2
		positive	negative	1	17	3	21
			positive	0	6	0	6
			NA	0	1	0	1
	positive	negative	negative	0	7	0	7
			positive	1	0	1	2
			NA	0	0	0	0
		positive	negative	0	7	0	7
			positive	2	10	0	12
			NA	0	0	0	0
	NA	negative	negative	0	11	0	11
			positive	0	1	0	1
			NA	0	1	0	1
		positive	negative	0	1	0	1
			positive	0	1	0	1
			NA	0	0	0	0
1	negative	negative	negative	2	3	0	5
			positive	2	0	0	2
			NA	0	0	0	0
		positive	negative	2	2	0	4
			positive	1	2	0	3
			NA	0	0	0	0
	positive	negative	negative	0	1	0	1
			positive	0	1	0	1
			NA	0	0	0	0
		positive	negative	1	0	0	1
			positive	6	39	1	46
			NA	0	1	0	1
	NA	negative	negative	0	0	0	0
			positive	0	0	0	0
			NA	0	0	0	0
		positive	negative	0	0	0	0
			positive	0	2	0	2
			NA	0	0	0	0

† No animals sampled had lesions with a score of 2 or 3; NA = Not available

Table S 9-2 | Apparent prevalence for *Leptospira* spp. per trapping session.

Species	Session	#	Culture and/or PCR positive	Apparent prevalence	95% CI*
Erinaceus europaeus	A1	4	1	25%	
	A2	8	3	38%	[9, 76]
	A3	5	4	80%	[28, 99]
	A4	14	6	43%	[18, 71]
	B1	1	0	0%	
Mus musculus	A1	23	20	87%	[66, 97]
	A2	79	25	32%	[22, 43]
	A3	22	20	91%	[71, 99]
	A4	67	30	45%	[33, 57]
	B1	33	7	21%	[9, 39]
Rattus rattus	A1	1	0	0%	
	A2	3	1	33%	
	A3	2	2	100%	
	A4	5	2	40%	[5, 85]
	B1	11	1	9%	[0, 41]

** 95% confidence interval calculated only when the number of animals tested (#) is ≥ 5*

Appendix 10.
Statements of contribution

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Marie Moinet
Name/title of Primary Supervisor:	Jackie Benschop
Name of Research Output and full reference:	
Of mice, cattle, and men: review of the eco-epidemiology of Leptospira borgpetersenii serovar Ballum in an island setting	
In which Chapter is the Manuscript /Published work:	Chapter 2
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
initial research design, data collection and analysis, manuscript drafting	
For manuscripts intended for publication please indicate target journal:	
Zoonoses and Public Health	
Candidate's Signature:	Marie Moinet Digitally signed by Marie Moinet Date: 2020.01.19 21:26:08 +13'00'
Date:	19/01/2020
Primary Supervisor's Signature:	Jackie Benschop Jackie Benschop (Jan 22, 2020)
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Name of candidate:	Marie Moinet
Name/title of Primary Supervisor:	Jackie Benschop
Name of Research Output and full reference:	
A cross sectional investigation of Leptospira at the wildlife-livestock interface in a beef and sheep farm and a dairy farm in the Manawatū-Whanganui region, New Zealand	
In which Chapter is the Manuscript /Published work:	Chapter 3
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and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
initial research design, data collection and analysis, manuscript drafting	
For manuscripts intended for publication please indicate target journal:	
Plos Negl Trop Dis	
Candidate's Signature:	Marie Moinet Digitally signed by Marie Moinet Date: 2020.01.19 21:30:36 +13'00'
Date:	19/01/2020
Primary Supervisor's Signature:	Jackie Benschop Jackie Benschop (Jan 22, 2020)
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Name of candidate:	Marie Moinet
Name/title of Primary Supervisor:	Jackie Benschop
Name of Research Output and full reference:	
Population dynamics of house mice (Mus musculus) and the epidemiology of Leptospira borgpetersenii serovar Ballum in a dairy farm biotope	
In which Chapter is the Manuscript /Published work:	Chapter 4
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initial research design, data collection and analysis, manuscript drafting	
For manuscripts intended for publication please indicate target journal:	
Journal of Applied Ecology	
Candidate's Signature:	Marie Moinet Digitally signed by Marie Moinet Date: 2020.01.19 21:32:36 +13'00'
Date:	19/01/2020
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Name of candidate:	Marie Moinet
Name/title of Primary Supervisor:	Jackie Benschop
Name of Research Output and full reference:	
Can sequencing methods help us decipher transmission pathways of Leptospira spp. within a community of hosts?	
In which Chapter is the Manuscript /Published work:	Chapter 5
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initial research design, data collection and analysis, manuscript drafting	
For manuscripts intended for publication please indicate target journal:	
Infection, Genetics and Evolution	
Candidate's Signature:	Marie Moinet Digitally signed by Marie Moinet Date: 2020.01.19 21:34:27 +13'00'
Date:	19/01/2020
Primary Supervisor's Signature:	Jackie Benschop Jackie Benschop (Jan 22, 2020)
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Name of candidate:	Marie Moinet
Name/title of Primary Supervisor:	Jackie Benschop
Name of Research Output and full reference:	
Investigating infection in a community of maintenance hosts naturally exposed to Leptospira	
In which Chapter is the Manuscript /Published work:	Chapter 6
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
initial research design, data collection and analysis, manuscript drafting	
For manuscripts intended for publication please indicate target journal:	
EcoHealth	
Candidate's Signature:	Marie Moinet Digitally signed by Marie Moinet Date: 2020.01.19 21:35:57 +13'00'
Date:	19/01/2020
Primary Supervisor's Signature:	Jackie Benschop Jackie Benschop (Jan 22, 2020)
Date:	

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