Ecology, epidemiology and evolution of enteric microbes in fragmented populations of the endangered takahe (*Porphyrio hochstetteri*)

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Abstract

Pathogenic diseases are increasingly recognised as a challenge to the conservation of wildlife. Complex host-pathogen relationships and transmission dynamics in wild populations can limit our understanding of how pathogens contribute to the decline and endangerment of wildlife. Endangered wildlife populations maintained in reserves present a unique opportunity to investigate wildlife host-microbe relationships in a controlled semi-natural environment where diversity, abundance and the movement of species are restricted. The aim of this study was to investigate the prevalence and molecular differentiation of enteric bacteria carried by endangered takahe (*Porphyrio hochstetteri*). Through the use of network analysis and molecular epidemiology, the study explored the effects of geographic isolation and translocation on the prevalence, transmission and evolution of *Campylobacter* and *Salmonella* spp. within fragmented populations of takahe.

Translocation and conservation management has created a dynamic network of takahe populations which vary in their likelihood to maintain and transmit pathogens. My study suggests that range expansion following a significant bottleneck and intensive conservation management of takahe has had unforeseen consequences on microbial diversity. The management of takahe in different environmental settings has influenced the carriage of *Campylobacter jejuni* and *Campylobacter coli*. A newly discovered rail-associated *Campylobacter* sp. *nova 1* was prevalent in all populations. However, more discriminatory whole genome analysis of isolates detected a significant biogeographic variation in *C.* sp. *nova 1* genotypes. Possible explanations for the observed pattern include the spatial expansion and isolation of hosts resulting in reduced gene flow of *Campylobacter* spp. and allopatric speciation, and the presence of heterogeneous environmental attributes or cross-species transmission of *Campylobacter* spp. from sympatric reservoir hosts. An assessment of vertebrate reservoirs in an island ecosystem indicated cross-species transmission of *Campylobacter* spp. was not likely to be a factor contributing to the maintenance and phylogeographical distribution of *Campylobacter* spp. in takahe.

This study was the first of its kind to explore microbial dynamics in a large proportion of a well-described but fragmented population of a wild bird. Results suggest historic and current management practices may be having unforeseen influences on enteric microbes, the consequences of which are unknown but could be detrimental to the health of translocated populations of takahe.

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Thesis structure and format

This thesis is presented as a series of seven chapters. Encompassed by a general introduction and discussion, five research chapters have been prepared and are presented as discrete papers for publication in peer reviewed journals.

Chapter one

General introduction introduces the concepts behind the research contained in this thesis by discussing and reviewing current literature on the principles of disease ecology and aspects of the epidemiology of infectious organisms in wildlife. The objectives of the study are summarised at the end of this chapter.

Chapter two

Network analysis of translocated takahe populations to identify disease surveillance targets has been published in the journal Conservation Biology (Grange et al. 2014).

Chapter three

Using a common commensal bacterium in endangered takahe (*Porphyrio hochstetteri*), as a model agent to explore pathogen dynamics in isolated wildlife populations is *in press* in the journal Conservation Biology.

Chapter four

Wildlife translocation and the evolution and population structure of a host associated commensal *Campylobacter* spp. is *under review* in the journal Proceedings of the National Academy of Sciences (PNAS) following publication of chapter 3.

Chapter five

Investigation of vertebrate reservoirs of *Campylobacter* spp. in an island ecosystem will be submitted to the Journal of Animal Ecology pending publication of chapters 3 and 4.

Chapter six

Location specific prevalence of *Salmonella* spp. in endangered takahe (*Porphyrio hochstetteri*) will be submitted to the Journal of Wildlife Disease.

Chapter seven

General discussion summarises the significant findings of this study. The relevance and implications of results are discussed and future research directions are suggested.

Chapter eight

Literature cited has been collated at the end of the thesis to reduce repetition. Literature is referred to in the format consistent with the format used for the journal Conservation Biology.

Chapter nine

Appendix contains all supplementary information organised by chapter

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CHAPTER 1

GENERAL INTRODUCTION

1. General introduction

1.1. Wildlife disease ecology

1.1.1. Disease ecology concepts

The impact of infectious disease on the global biodiversity of wildlife and the emergence of human pathogens of zoonotic origins are becoming increasingly apparent as areas of concern. Although not a common driver of extinction (Heard et al. 2013; Smith et al. 2006), infectious disease is a threatening process which may impact species conservation (Heard et al. 2013; Smith et al. 2009). Catastrophic or chronic host population depression may occur as a result of pathogenic outbreaks due to pathogen induced death, increased susceptibility to predation and reduced reproductive success (Boadella et al. 2011; Cunningham 1996). Disease is defined as an abnormal condition of an organism, which can be caused by infection with a pathogen. The terms “parasite”, “pathogen” and “infectious agent” are often used interchangeably. In terms of transmission, pathogens transmit to new hosts not diseases. A pathogen is any infectious organism that causes disease symptoms in its host. Disease ecology is the study of interactions between hosts and pathogens, including variation in infection, transmission and impacts of pathogens on host populations (Archie et al. 2009; Kilpatrick & Altizer 2010).

Exposure of a host to a pathogen and establishment of infection is the result of complex ecological and evolutionary interactions between the host, the pathogen and the environment or habitat of the host and pathogen, sometimes termed the epidemiological triangle (Silvy 2012; Wobeser 2006). However, investigations of wildlife disease are often more complex than the epidemiological triangle suggests, with underlying hierarchical structure and heterogeneous interactions in natural ecosystems adding an additional level of complexity to the transmission and maintenance of infectious organisms (Caillaud et al. 2013). Management and mitigation of wildlife disease is enhanced by an understanding of the ecological factors influencing the transmission and interspecific variation of microorganisms at the level of the host through to the population, community and ecosystem (Tompkins et al. 2011).

1.1.2. Host pathogen relationships: from individuals to ecosystems

1.1.2.1. The host and the pathogen

The functional dependency of a pathogen on a host leads to an inevitable close association between host and pathogen dynamics. Transmission to and colonisation of new hosts is required for the persistence of infectious organisms in an ecosystem. Microorganisms are an important component of biodiversity, contributing to ecosystem function and dynamics. A microorganism which is dependent on a host for its survival may enter into one of four types of relationships: (i) commensalism, the microorganism benefits without detrimental effects on the host, (ii) mutualism, both the host and microorganism experience increased fitness, (iii) parasitism, the microorganism negatively impacts its host's fitness and (iv) opportunism which is a subset of parasitism where microbes whom are commensal in healthy hosts may cause disease in compromised hosts (Goering & Mims 2013). Therefore, not all infections result in the expression of disease and the nature of the association between a host and an infectious organism may be dependent on a range of factors relating to the host and / or pathogen. Colonisation of a host is dependent on the likelihood of transmission and host defences such as behaviour and immunity. Multiple forms of pathogen transmission have been described including: direct contact, indirect contact, or by vectors. Many biotic and abiotic factors may contribute to differences in pathogen exposure during a host's lifetime. Such factors include: age, sex, habitat selection, population density, diet, social structure and the behaviour of the host (Johnson et al. 2012).

Different lifestyles of pathogens are expected to influence their genomic composition. For example, local environmental and host associated factors may impose a selective pressure on a pathogen which can determine pathogen genotype and phenotype composition. Attenuation and evolution of myxoma virus (MYXV) occurred when the virus was introduced and persisted in invasive European rabbits (*Oryctolagus cuniculus*) in Australia and Europe, whereby the pathogen's virulence decreased to maximise transmission rates in field situations, alongside an increase in host immunity and survival (Kerr 2012).

For optimal survival, a microbial species may be highly dependent on the availability of hosts and its ability to exploit transmission opportunities between suitable hosts and adapt a generalist or specialist

lifestyle accordingly. This could be reflected in the microbial genome. High host specificity is thought to limit gene flow within and between microbial species, and thus increase the genetic distance between conspecific microbes infecting different hosts. This may explain the high levels of genomic variation observed within some bacterial pathogens. For instance, 928 wild bird isolates of *Campylobacter jejuni* were sequenced and compared to 1366 domestic animal and human isolates (Griekspoor et al. 2013). Although there was a high level of diversity within the species, *C. jejuni* sequence types clustered according to host, whereby *C. jejuni* genotypes from wild bird species were different from each other and those from other sources (Griekspoor et al. 2013). Grouping in this manner implies niche specialisation where there may be environmental and host associated barriers restricting gene exchange (Griekspoor et al. 2013; Sheppard et al. 2011; Sousa & Hey 2013). In contrast, a generalist lifestyle is thought to create more opportunities for gene exchange, and thus in theory pathogens of the same species isolated from different hosts or locations should on average be homogeneous. Supporting this hypothesis, high rates of gene flow were observed between the generalist nematode *Trichostrongylus axei* infecting multiple sympatric ungulates in North America, with most genetic variation structured within individual hosts (Archie & Ezenwa 2011).Four modes of speciation are postulated to occur in nature based on barriers to gene flow from isolation, niche formation and genetic polymorphism. These theories are termed: allopatric speciation, peripatric speciation, parapatric speciation and sympatric speciation (Figure 1-1), and all are biologically plausible within microbes with generalist or specialist lifestyles.

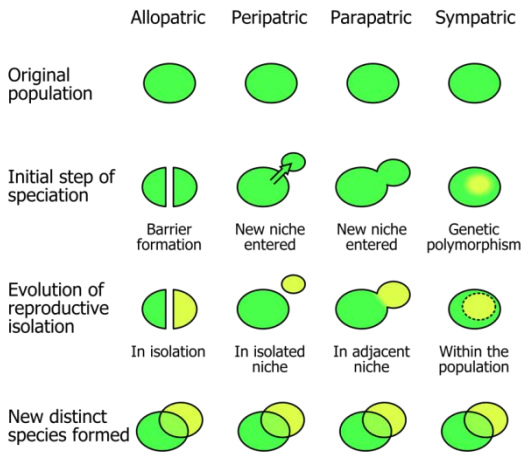

Figure 1-1: Theoretical hypotheses for the modes of speciation (adapted from http://en.wikipedia.org/wiki/Speciation)

1.1.3. The host population

Animal populations are often hierarchical with structural organisation into multi-level societies. On the simplest level, individuals are connected to others through direct or indirect association to form groups. Groups may be formed of familial aggregations or cohorts of solitary individuals sharing a territory. Together conspecific groups form populations. Behaviour and lifestyle choices (Drewe et al. 2011), habitat availability (Almberg et al. 2012) and social structure (Caillaud et al. 2013; Nunn et al. 2014; Nunn et al. 2011) determine the organisation, density and frequency of contact within and between individuals in a population which in turn may determine the likelihood of pathogen exposure and transmission. Habitat influences on pathogen prevalence are also confounded by interactions related to increased host density and connectivity within preferred sites (Almberg et al. 2012). For example, grey wolves (*Canis lupus*) occupying prime habitat in Yellowstone National Park were most susceptible to disease caused by the mite *Sarcoptes scabiei* (Almberg et al. 2012). Heterogeneities in connectivity between susceptible and infected individuals within a population are key factors determining the spread of infectious organisms. Not all infected hosts contribute equally to the transmission of a pathogen in a population. The 20:80 rule is thought to apply in many populations, whereby a few individuals contribute to most infections (Woolhouse et al. 1997; Woolhouse et al. 2005). These "super spreaders" play a central role in dissemination of a pathogen to many individuals or species via high shedding and/or contacts rates (Lloyd-Smith et al. 2005; VanderWaal et al. 2013a). The extent an individual is connected to others can contribute to its propensity to spread infection (Christley et al. 2005) due to increased level of contact and opportunities to become infected. Clustering of highly connected individuals within a population has been shown to contribute to the growth rate of epidemics (Watts & Strogatz 1998).

Transmission dynamics of an infectious organism can vary temporally and geographically because of differences in landscape and host attributes, and the ability of pathogens to persist in the environment (Real & Biek 2007). Sharing of a pathogen may be determined by host behaviour resulting from asynchronous space use, territoriality and shared resources (Nunn & Dokey 2006; Nunn et al. 2014; Nunn et al. 2011). Faecal contamination of soil and water sources with environmentally stable organisms such as *Salmonella* spp., allows indirect transmission of pathogens between independent

groups. Therefore, the spread of infectious agents between animals is not necessarily dependent on coexistence. Vector borne pathogens spend a period of their lifecycle off-host, and thus are less dependent on close proximity between hosts for persistence. For example, given suitable climatic conditions the mosquito *Culex quinquefasciatus* is able to invade a new environment and disseminate avian malaria (such as *Plasmodium relictum*) between multiple populations of susceptible birds, and this has had a profound effect on Hawaiian honeycreeper populations in the Hawaiian archipelago (Warner 1968).

1.1.4. Multi-host pathogens and reservoir dynamics

Many pathogens have complex lifestyles, colonising many species and environments. Multi-host pathogens such as *Salmonella* spp., with over 2500 serotypes identified (Grimont & Weill 2007), are able to colonise and infect a range of taxonomic groups. Commonly termed generalist organisms, multi-host pathogens have significantly more complex transmission networks than that found in single host pathogens. Each host species is effectively a sub-population within a larger framework of susceptible hosts and different hosts and / or subpopulations may vary in their susceptibility and immunity to infection. Heterogeneous contact patterns, behaviour and habitat preferences could all influence colonisation and transmission of a pathogen within and between species sub-populations (Dobson 2004).

Cross species transmission rates can be influenced by extent of genetic relatedness between hosts (Huang et al. 2014). This is thought to be a key determinant of transmission and emergence of rabies virus in different bat species in North America (Faria et al. 2013). Physiological and behavioural similarity between taxonomically related hosts has been attributed to sharing of microbial subtypes of *Escherichia coli* in wild ungulates in Africa (VanderWaal et al. 2014). However, even closely related taxa can vary in their immunity to pathogens, where one host species may be more tolerant of a pathogen than its competitor (Prenter et al. 2004). For example, the introduction of squirrel pox virus to the red squirrel (*Sciurus vulgaris*) in Great Britain has contributed to the decline and ecological replacement of the native squirrel with the closely related non-native North American grey squirrel (*Sciurus carolinensis*), in which the virus is less pathogenic (Rushton et al. 2000; Sainsbury et al. 2008).

However, there does not need to be close taxonomic relatedness between the existing and new host for a pathogen to infect a new host species. Chance transmission, including indirect contact between transient individuals and resident populations can occur along wildlife corridors and might be sufficient to cause a local epidemic (Hess 1994; Simberloff & Cox 1987). Use of corridors may be biased towards one sector of the population, with those having larger ranges being prime vectors for dissemination of disease between populations. Clements et al. (2011) investigated movements of whitetail deer (*Odocoileus virginianus*) in the Midwest of America, in order to predict spread of infectious diseases including chronic wasting disease. They found young males seeking new territory dispersed at high rates along the river corridors, whilst others maintained high fidelity to their territory (Clements et al. 2011). Therefore, young males seeking new territories were the most likely element of the population to contribute to the dispersal of infectious diseases.

Multi-host pathogens are of concern to the conservation of endangered wildlife. Sympatric host species harbouring unselective pathogens pose a disease threat for vulnerable species within transmissible proximity. For example, *Toxoplasma gondii* type X may be contributing to the decline of the threatened southern sea otter (*Enhydra lutris nereis*) in California. *T. gondii* type X has been isolated and may be indirectly transmitted to the sea otters from infected wild and domestic felines in the same region (VanWormer et al. 2014). Epidemiologically connected populations that act as sources of pathogens to vulnerable populations are typically termed reservoirs (Haydon et al. 2002). Reservoir hosts are classified according to their susceptibility to infection by an organism and ability to maintain and transmit that organism without obvious detrimental effects (Johnson et al. 2012). Despite extensive effort, determining the source of a pathogen and the directionality of transmission is hindered by complex interactions and relationships which may be unobservable in wild populations. These difficulties are apparent when trying to decipher the transmission of *Mycobacterium bovis* between Eurasian badgers (*Meles meles*) and domestic cattle (*Bos primigenius*) (Biek et al. 2012). Another example is brucellosis, which is a disease caused by the bacterium *Brucella abortus*. Although it can threaten wild ungulate populations, much interest and conflict is created due to the potential spread of *B. abortus* from reintroduced bison (*Bison bison*) to cohabiting domestic cattle in North America. Although evidence suggests *B. abortus* may have originated from cattle introduced into America, reviewed in Meagher and Meyer (1994), DNA typing of multiple isolates of *B. arbortus*

isolated from ungulates including wild elk (*Cervus canadensis*), bison, and cattle in the Greater Yellowstone area revealed that elk and cattle shared similar bacterial genotypes, but bison isolates were highly divergent from cattle sequence types (Beja-Pereira et al. 2009). This would imply that the main transmission pathways may occur between elk and cattle.

Host switching and establishment of a pathogen in a new reservoir is not an unusual occurrence. When the Australian brushtail possum (*Trichosurus vulpecula*) was introduced and established in New Zealand (Pracy 1974), possum populations, became infected with *Mycobacterium bovis* in the new location (Morris & Pfeiffer 1995). Interestingly, possums are free of *Mycobacterium bovis* in their native range (Corner & Presidente 1981), Introduced possums have become a wildlife reservoir for *M. bovis*, and are thought to be a 'spill back' source of infection to livestock (Morris & Pfeiffer 1995). However, the epidemiology of transmission is not well understood and as with many multi-host pathogens in wildlife ecosystems, questions remain unanswered which hinder control of the disease and the creation of management solutions. Investigations are exploring the intra- and interspecies transmission of *M. bovis* through the use of epidemiological tools, including experimental infection and social networking (Corner et al. 2003; Nugent et al. 2013; Rouco et al. 2013).

1.2. Conservation of wildlife in the face of disease threats

1.2.1. Disease threats posed to and from wildlife

Emerging infectious diseases (EIDs) in wildlife are of increasing concern due to their direct impact on biodiversity and the risk of zoonotic transmission (Daszak et al. 2000; Jones et al. 2008). The epidemiology of emerging infectious diseases (EIDs) in free living wildlife is complex, and our understanding of the drivers of transmission and disease progression in these systems is hampered by the fact that wild animals are difficult to observe and measure (McCallum et al. 2001). Three hypotheses explaining the origins of wildlife EIDs are postulated: (i) spill-over of pathogens from domestic animals, (ii) caused by anthropogenic drivers of infection, or (iii) with no involvement of humans or livestock (Daszak et al. 2000).

Outbreaks of EIDs in nature have two major biological implications. First, wildlife EIDs can have significant impacts on the biodiversity of free living animals, e.g. the role that the chytrid fungus

Batrachochytrium dendrobatidis has had in the decline of amphibian populations in Australia (Berger et al. 1998). Second, the emergence of important infectious diseases in humans and livestock are often reported to originating from wildlife reservoirs. For example, bats are frequently implicated as sources of exotic human pathogens. Fruit bats (suborder *Megachiroptera*, family *Pteropodidae* of the order *Chiroptera*) have been recognised as natural reservoirs of Hendra virus (HeV) in the Australasian region in recent years (Plowright et al. 2011; Plowright et al. 2008). In this system, domestic horses (*Equus ferus caballus*) are thought to be an intermediate host becoming infected through association with fruit bats, with cases of HeV in humans an incidental occurrence after contact with an infected horse (Plowright et al. 2011; Plowright et al. 2008). It is important to note that pathogen transmission threats may not be mutually exclusive in one direction or the other. Bengis et al. (2002) highlight that the interface between wildlife and livestock provides opportunities for bidirectional flow of organisms, creating conflicts not only in domestic animals but also posing threats for co-habiting wildlife.

In an increasingly connected world, humans and animals are frequently travelling between remote locations with the potential to transmit exotic pathogens across barriers which were previously unattainable. The introduced fungus *Pseudogymnoascus destructans* causes a disease termed white nose syndrome in susceptible North American bats, with up to 95% mortality in some hibernacula and killing over one million bats in North America (Frick et al. 2010). The fungus has been detected in some bat species (*Myotis myotis*) in Europe (Pikula et al. 2012). However, it appears less pathogenic in these populations and has not been associated with the mass mortalities observed on the American continent (Cryan et al. 2013). It is thought that suitable environmental conditions, human travel and the availability of susceptible naive hosts has allowed the rapid spread of the fungus upon introduction to the eastern states of North America (Foley et al. 2011).

Stresses imposed by anthropogenic manipulation of the environment, habitat fragmentation, introduction of invasive species, global travel and altered climate, often acting synergistically, have contributed to the emergence of infectious disease and the declines of wildlife populations (Brook et al. 2008; Munns 2006; Olival et al. 2013). Pathogenic outbreaks are rarely a sole driver of extinction but in conjunction with additional selective pressures can pose a significant threat to a population

(Heard et al. 2013; Smith et al. 2006). A review of the IUCN red listed species revealed that less than 4% of extinctions and less than 8% of critically endangered species listed infectious disease as a contributing factor to their decline (Smith et al. 2006). The threat of disease appears to increase as population size decreases when a species, particularly amphibians and birds, moves further towards extinction (Heard et al. 2013). However, these findings may be influenced by discovery and species specific sampling bias whereby as a species heads towards extinction, the knowledge about threats posed to that species increases (Heard et al. 2013). This would be an intuitive finding as wild animals are notoriously difficult to observe in the wild, with diseased individuals often inconspicuous or rapidly scavenged.

Fragmentation of habitats due to human activity has had negative impacts on wildlife populations around the world (Foley et al. 2005; Plowright et al. 2008). For example, deforestation in the Peruvian Amazon has altered breeding of a malarial mosquito (*Anopheles darling*) which has led to an increased biting rate of humans in deforested areas (Vittor et al. 2005). Increased awareness of isolated habitat patches and populations and the impact this has on the ecosystem has driven the need for a solution. Habitat corridors are commonly proposed as a conservation tool to negate the effects of fragmentation by facilitating plant and animal movement between isolated patches and increasing population viability (Hilty et al. 2006). However, these corridors are nonspecific and have the potential to negatively impact the same species that they are meant to benefit by inadvertently facilitating the movement of unsolicited competitors, predators and the novel diseases they may carry (Simberloff & Cox 1987). For example, Sullivan et al. (2011) found corridors had a positive effect on spread of animal mediated transmission of plant parasites across highly connected landscapes. Methods combining mathematical epidemiology and metapopulation modelling found that the spatial arrangement and level of contact between populations within a metapopulation are important in terms of pathogen spread, and that some arrangements can be better for disease control than others (Hess 1996).

Disease control to prevent zoonotic spread between the key players; humans, wildlife and livestock, should be based on a broad understanding of the ecology and epidemiology of the disease agent within its hosts (Woodford 2009). However, a challenge to studying transmission of disease including

wildlife is that the hosts are often inconspicuous and not easily observed, especially those with overt disease.

1.2.2. Anthropogenic management of threatened wildlife populations: translocations and sanctuaries as conservation tools

Translocation is the human mediated process of capture, movement and release of animals or living organisms from one location to another (Soorae 2008). From this definition, translocation has been occurring worldwide for centuries with the introduction of farming and trade of domestic and wild animals. In more recent times, the intentional movement of individuals, populations and species across landscapes is being used as a means to maintain biodiversity in the face of anthropogenic changes to ecosystems (Sainsbury & Vaughan-Higgins 2012; Weeks et al. 2011). Translocation is most often used and referred to in the context of the intensive management of threatened species, where anthropogenic manipulation is required to increase or maintain a population (Weeks et al. 2011). In the United States, approximately 70% of all recovery plans for threatened and endangered species have recommended this approach (Tear et al. 1993). The primary aim of a translocation for conservation is survival and persistence of the species in the new location. Also translocation is being proposed as a major tool for the conservation management of wild species unable to adapt to rapid climate change (Mawdsley et al. 2009). The inadvertent introduction or emergence of infectious disease through translocation of animals into new populations or ecosystems has become a major concern when considering management of wildlife populations (Thompson et al. 2010).

Translocation of individuals between previously isolated ecosystems removes barriers to exchange of pathogens (Power et al. 2013). These animals can potentially transfer exotic pathogens into extant populations with no effective immunity at the release site (Anderson & May 1986; Woodford & Rossiter 1994). Threats posed by management actions are particularly pertinent to endangered species maintained in fragmented isolated populations and are heavily reliant on conservation measures for the persistence of the species. Disease outbreaks can affect population dynamics and management, with the extent of the impact often dependent on the size of the outbreak. Simulation of disease epidemics in endangered Sierra Nevada bighorn sheep (*Ovis canadensis*) indicated severe outbreaks of disease increased adult mortality, thus reducing population size and impeding population

management (Cahn et al. 2011). Although small epidemics did not impact population numbers, they did reduce the number of individuals available for translocation, thus indirectly impacting population management and viability (Cahn et al. 2011). Pathogenic incursions may not necessarily result in mortality. Pathogen associated morbidity can impact hosts in subtle ways. For example animals infected with *Toxoplasma gondii* show no overt symptoms of disease but the protozoan can influence the behaviour of its host, increasing risk and likelihood of transmission between mice and cats (Berdoy et al. 2000).

Although captive breeding and translocation of animals has become an important management tool for threatened species programs worldwide (Fischer & Lindenmayer 2000; Griffith et al. 1989), insufficient research has been conducted regarding the spread of disease associated with these activities. The management of wildlife in artificial environments creates atypical opportunities for exchange of microorganisms between humans, domestic animals and wildlife. During captivity animals may be exposed to new infectious agents from previously unavailable transmission routes and act as carriers and/or vectors when released into a new location (Viggers et al. 1993). Endemic island animal species held in captivity can be particularly susceptible to infection by exotic pathogens. For example, herpes virus infection was unknowingly transmitted from asymptomatic foster rock doves (*Columba livia*) to captive-bred Mauritian pink pigeon chicks (*Nesoenas mayeri*) (Snyder et al. 1985).

Spill-over of human pathogens to animals (reverse zoonosis) in captivity is not well understood. However, if a pathogen establishes in a wildlife reservoir, there is a potential for spillback to humans (Thompson et al. 2010), or *vice versa*. A concerning example is that of reverse zoonotic pathogen emergence in African apes. Increasing evidence indicates that human respiratory and gastrointestinal pathogens are circulating in African gorillas and chimpanzees which are thought to have contributed to significant morbidity and mortality in these populations (Palacios et al. 2011; Rwego et al. 2008; Williams et al. 2008).

In theory, the population size of many endangered populations is thought to be below the threshold required for establishment of disease (Mathews et al. 2006), particularly in small translocated sub populations. However, reality is more complicated and alternative hosts in the form of sympatric species within a given ecosystem may increase the effective population size and allow persistence of

an epidemic (Mathews et al. 2006), with devastating effects on the species of concern. Endangered bighorn sheep (*Ovis canadensis*) in North America are have been translocated to re-establish or augment existing populations (Boyce et al. 2011). Populations are small and are frequently afflicted with epidemics of *Mycoplasma ovipneumoniae* associated with pneumonia and death (Besser et al. 2012a; Besser et al. 2012b). Pathogen spill-over from closely related domestic sheep (*Ovis aries*), poly-microbial disease and the presence of carrier individuals are thought to contribute to localised disease outbreaks in bighorn sheep, summarised in Besser et al. (2013). The presence of sympatric domestic sheep may not only provide a source of infection, but could also effectively increase the population size and in theory provide the suitable system required for maintenance of pathogens within the threatened bighorn sheep populations.

Depending on the study species and ecosystem dynamics involved, translocated or captive reared animals may be at an advantage in terms of host parasitism compared to their home range. The parasite release hypothesis suggests that animals may escape native pathogens when moved to a new location (Torchin et al. 2003). Many pathogens have complex life cycles requiring more than one host, and thus if this host is not present in the new location, the pathogen may not be maintained. The North American starling (*Sturnus vulgaris*) descended from a small population of introduced European starlings (*Sturnus vulgaris*) in the 1800's (Baker et al. 1986). Torchin et al (2003) report a lower diversity of parasite species in the North American starlings compared to those of the source population. The phenomenon may be explained by increased host resilience to parasites at the new site (Torchin & Mitchell 2004), or alternatively host fitness may be higher due to lower levels of parasite incidence and or exposure at the new location (Smith et al. 2009; Torchin & Mitchell 2004). Translocated and reintroduced native species are regularly selected for good health prior to transfer, consequently disease circulating in this select group may be lower than the source population (Almberg et al. 2012). However, these populations are expected to acquire pathogens circulating within the environment (Almberg et al. 2012), but this will often be at a slower rate than what they would be exposed to in their original geographic range (Torchin et al. 2003; Torchin & Mitchell 2004). The dynamics of pathogen spill-over and spill-back have been summarised in a recent review by Tompkins et al. (2011), using the example of the invasion of the North American signal crayfish

(*Pacifastacus leniusculus*) into the United Kingdom to demonstrate potential mechanisms of pathogen transmission between introduced and native species (Figure 1-2).

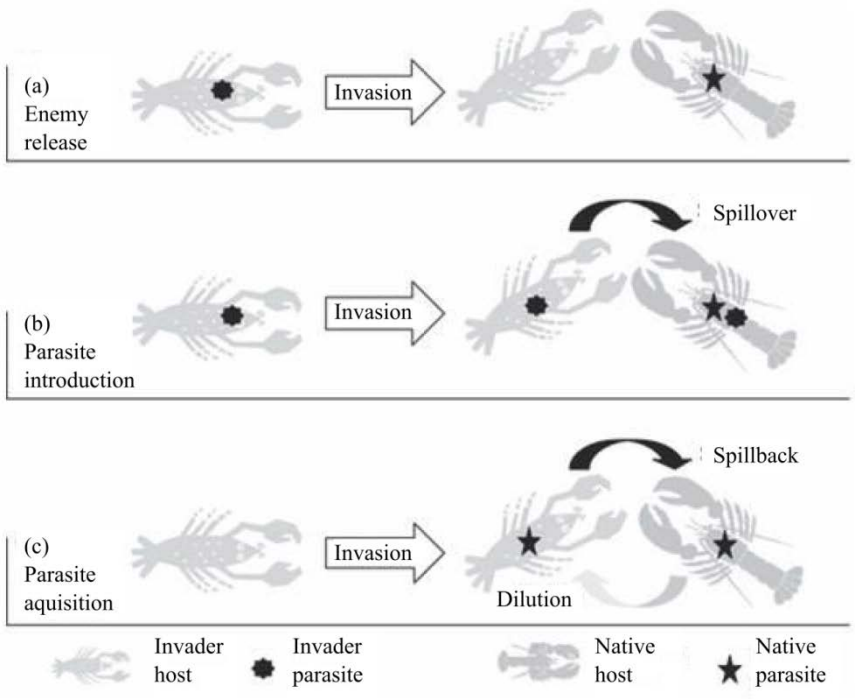

Figure 1-2 Diagrammatic representation of pathogen transmission dynamics between native and introduced populations of crayfish in the United Kingdom (Tompkins et al. 2011)

1.3. Epidemiological tools for species conservation

1.3.1. Risk assessments and disease surveillance

Understanding the epidemiology of an infectious disease in free-ranging wild animals requires an understanding both of the route of natural infection and of the processes underlying development of clinical disease. A lack of knowledge of pathogen dynamics in wild animal populations limits the ability of conservation managers to develop prioritised strategies for disease control and to effectively target disease surveillance. It has been recognised that countries which invest efforts in wildlife disease surveillance are more likely to be prepared for and respond to emerging infectious and zoonotic diseases (Morner et al. 2002). The World Organisation for Animal Health (OIE) was established in 1924 due to increased awareness of animal diseases (www.oie.int/about-us/history). The OIE is an intergovernmental organisation with international policy objectives for the monitoring

and improvement of animal health worldwide (OIE 2011). Active disease surveillance in wildlife is a relatively new concept and is fundamental for prediction and mitigation of disease epidemics in both people and animals. Disease monitoring involves the "systematic recording of epidemiological data, with the specific purpose of detecting spatial and temporal trends as well as presence or absence of the disease" (Boadella et al. 2011). Pathogens selected for screening are often those that would have the greatest impact on human health and the economy, or have a history of causing disease in threatened wildlife populations (Boadella et al. 2011). Selective testing can create bias in detection, with a lean towards exotic zoonotic diseases, especially those which are notifiable (Boadella et al. 2011; Hartley & Gill 2010). Even when screening occurs, we are limited in our interpretation of results due to imperfect tests resulting in false positive and negative results, as well as the intermittent shedding of pathogens from hosts, as is often the case in faecal excretion of *Salmonella* spp. (Ivanek et al. 2012; Van Immerseel et al. 2004). Detecting wildlife disease trends requires: knowledge of diagnostic test sensitivity and specificity, adequate sample size over a significant time period, appreciation of the logistics associated with sample collection, and expertise in data analysis where many parameters are uncertain (e.g. total population size). Increased disease monitoring will provide detailed information of pathogen diversity and relative importance in aetiology of disease in the host (Smith et al. 2009). Improved baseline information with regards to the diversity and abundance of pathogens on a community and ecosystem gradient will increase our grasp on potential transmission of pathogens within and between wildlife and the threats posed to conservation (Thompson et al. 2010).

Globally, wildlife movements carry the risk of transfer of infectious agents and potential impact on the health status of livestock, companion animals and humans (Cunningham 1996). Awareness of this issue has prompted protocols to minimise disease transmission risk associated with wildlife translocations and reintroductions (Cunningham 1996; Viggers et al. 1993; Woodford & Rossiter 1993). The OIE use an assessment model to analyse the disease risk associated with movements of animals (Murray 2004). Several models have attempted to estimate the likelihood that each pathogen would cause disease by assessing the probability and consequences of release and exposure in a new location (Armstrong et al. 2007; Sainsbury & Vaughan-Higgins 2012). Models classified risk ranging from negligible to high and evaluated management options according to outcome. Sainsbury and Vaughan-Higgins (2012) attempted to assess the disease risk of translocating wild animals using the

relocation of the Eurasian crane (*Grus grus*) from Germany to England as a proxy for other species. However, they acknowledge there are limitations in interpreting the outcome of models due to incomplete identification of pathogens affecting the target species. Therefore, animals may be translocated with unknown pathogens which could impact health and population reestablishment (Ewen et al. 2012; Sainsbury & Vaughan-Higgins 2012). For example, three endangered juvenile kakapo (*Strigops habroptilus*) died shortly after translocation to a new location in New Zealand due to an outbreak of the bacteria *Erysipelothrix rhusiopathiae*, which may have been contracted from soil at some stage of transport or arrival, or manifested as a result of the stress of translocation (Gartrell et al. 2005). These animals had been screened for diseases of concern prior to translocation but erysipelas had not been considered as an issue for the parrots prior to this outbreak (Gartrell et al. 2005). This highlights one of the problems in predicting EIDs in small wildlife populations.

Encounters with new pathogens may occur at several stages of the process, including before, during and after translocation (Cunningham 1996). Despite the risk of pathogen emergence and spread, many wildlife relocations occur without disease screening. A report found less than a quarter (24%) of translocations which occurred in Australia, Canada, the United States and New Zealand used a health screening protocol prior to movement of animals (Griffith et al. 1993). The primary aim of disease screening associated with translocations is to prevent introduction of novel pathogens into existing populations at the release site, as well as increasing the likelihood of survival of translocated individuals (Cunningham 1996; Viggers et al. 1993; Woodford & Rossiter 1993). There are few laws and regulations in place to enforce biosecurity and disease screening and as previously mentioned there is substantial variability in uptake of disease assessment associated with translocations (Daszak et al. 2000). Nonetheless, conservation interest in quantifying the likelihood of disease transmission has increased reports of health screening, including a recent health assessments of translocated western ring tailed possums (*Pseudocherirus occidentalis*) (Clarke et al. 2013), water voles (*Arvicola terrestris*) in the United Kingdom and marsupial dibblers (*Parantechinus apicalis*) in Australia (Mathews et al. 2006).

1.3.2. Pathogen ecology and epidemiology

Molecular characterisation of pathogens derived from multiple sources provides insights into the epidemiology of infectious disease ecology. When combined with epidemiological data, genetic tools can be used to identify hosts, investigate pathogen adaptation, infer chains of transmission and link heterogeneities in pathogen prevalence to host and environment associated factors (Archie et al. 2009). An epidemiological investigation of *Mycobacterium tuberculosis* isolates from humans in an outbreak in a community in British Columbia, Canada, nicely demonstrates the recent progression of epidemiological modelling in an applied context (Gardy et al. 2011). In this example, researchers integrated social network contact data derived from questionnaires with whole genome sequencing of *M. tuberculosis* isolates from patients. Sequence analysis revealed two genetically distinct lineages of *M. tuberculosis* circulating in the community, indicating that there were concomitant outbreaks; an observation that was not previously readily intuitive from standard molecular methods (Gardy et al. 2011). Additionally, epidemiological investigation identified that the outbreak coincided with increased crack cocaine use in the community and social connections (Gardy et al. 2011). If conducted early in a disease outbreak, integrative studies of this manner may be used to prevent or reduce transmission routes and thus temper the extent of an epidemic.

Fine scale microbial genetics can be used to infer who infected who in a transmission chain based on sequence profiling. Pathogen subtypes are assigned according to genetic similarity or differences. Therefore, if two individuals share a subtype, transmission may be inferred (Bull et al. 2012; VanderWaal et al. 2013b, 2014). However, limitations are still evident in this technique as direction of transmission cannot be determined without further investigation. Additionally, sharing of a subtype may be indicative of exposure to a common source or similar life history rather than direct transmission between two hosts.

Population genomics has been used in order to attribute niche adaptation of the pathogen to the host or environment. A study of *Campylobacter jejuni* in New Zealand used multi locus sequence typing (MLST) of bacteria isolated from humans and attributed infections to the consumption of chicken from certain poultry suppliers due to the genomic relatedness between human and chicken associated isolates (Mullner et al. 2010). However, comparison of a few genes may not capture the full genomic

diversity present between closely related species. Ribosomal multi locus sequence typing (rMLST) of 53 conserved genes encoding ribosomal proteins on the bacterial genome is an alternate method for more defined sequence type comparisons (Jolley et al. 2012). It has been used for taxonomic exploration of *Neisseria* species, revealing previously unobserved complexity within *Neisseria polysaccharea* suggesting it could comprise more than one taxonomically distinct organism (Bennett et al. 2012).

In nature, some individuals interact more than others and thus the likelihood of transmission and infection with a pathogen is likely to follow suit. Social network analysis attempts to mathematically quantify the variation in connectivity and pathogen transmission pathways in relation to host or population biotic and abiotic factors (Newman 2010). For example, in a transmission study investigating *Mycobacterium bovis* infection in captive brushtail possums (*Trichosurus vulpecula*), infected possums had higher connectivity measures than those which were uninfected (Corner et al. 2003). Network concepts can be applied to identify targets to mitigate pathogen spread and thus are a useful tool to study the demographics of infectious diseases in wildlife (Godfrey 2013; Rushmore et al. 2013; VanderWaal et al. 2013a; VanderWaal et al. 2013b, 2014).

1.4. Research focus

1.4.1. New Zealand conservation management

Worldwide, island ecosystems are particularly vulnerable to extinctions. In New Zealand, 41% of all native bird species have become extinct since human settlement (Tennyson & Martinson 2006). Invasive non-native mammalian predators and anthropogenic activities have a long history of impact on the populations of New Zealand's native flora and fauna. As a result, New Zealand has been innovative with its conservation management of endangered species. To prevent further extinction of land birds, translocation and mammal eradication emerged as conservation solutions and as a result numerous offshore islands were utilised for reasons of isolation and conservation value (Sherley et al. 2010). The process of removing exotic animals from island reserves began in the early 20th Century and since then the islands have provided sanctuary for many indigenous populations threatened by introduced mammals (Bellingham et al. 2010). Conditions created due to human disturbance have

allowed non-native birds to colonise these islands, with starlings (*Sturnus vulgaris*) observed as far as Campbell Island in the Sub-Antarctic (Heather & Robertson 2006). Introduced avifauna may compete with natives who occupy the same ecological niche (Bellingham et al. 2010) and may act as reservoirs of disease (Rushton et al. 2006).

Translocations have become a common conservation management strategy for numerous endangered birds and reptiles in New Zealand and many of these have been catalogued (Sherley et al. 2010). A particularly successful example is the North Island saddleback (*Philesturnus rufusater*) which has been subject to multiple translocations and has successfully increased its range from a single source population to occupying at least 13 islands (Parker 2008). However, not all translocations have been successful. The translocation of hihi (*Notiomystis cincta*) to offshore islands has been attempted but has not been effective with remaining populations heavily reliant on supplementary feeding for maintenance (Armstrong et al. 2007).

Translocations of animals between reserves are actively managed by the New Zealand Department of Conservation (DOC), or by local conservancies and communities under the guidance and control of DOC. All known translocations in New Zealand are performed in accordance with the DOC disease risk assessment tool, a flow diagram that assesses risk associated with infectious disease in the source population as well as the target population (McInnes et al. 2004). With all good intentions, risk assessments may not be able to predict outbreaks of some diseases, with some translocation associated deaths reported (Alley et al. 2008; McLelland et al. 2011).

1.4.2.Takahe (*Porphyrio hochstetteri*)

Figure 1-3 Adult takahe (*Porphyrio hochstetteri*) resident on Maud Island, New Zealand. Photo courtesy of Thomas Burns

The takahe (*Porphyrio hochstetteri*) (Figure 1-3) is an iconic endemic New Zealand flightless rail, considered "nationally critical" under the New Zealand DOC threat classification system (Miskelly et al. 2008) and "endangered" on the IUCN Red list of threatened species (BirdLife International 2013). Takahe are a species of high cultural value and are regarded as a "taonga (treasured) species" to the Maori people of the South Island, Ngai Tahu. As a result, takahe are under strict protection to conserve the species, with consultation required when decisions are made with regards to their welfare. The problems facing takahe are representative of most of New Zealand's endangered bird species. The modern day takahe were thought to have been widespread throughout the South Island, New Zealand, while a closely related extinct species of takahe (*Porphyrio mantelli*) inhabited the North Island (Trewick 1996; Trewick & Worthy 2001). All takahe were thought extinct by the end of the 19th Century until a small population of approximately 250 South Island takahe were rediscovered in Fiordland, South Island in 1948 (Ballance 2001). It is believed that their range was reduced to Fiordland as a consequence of confounding attributes including: hunting by Maori, habitat destruction and the introduction of mammalian predators and competitors (Bunin & Jamieson 1995; Trewick & Worthy 2001).

Over the next 15 years, a 518-km^2 takahe conservation area was created in the Murchison mountains, Fiordland (Ballance 2001), and subsequently takahe from the Fiordland population were introduced to five small predator free offshore islands (Crouchley 1994). Takahe were first translocated to Maud Island in 1984, followed by Mana Island in 1988, Kapiti Island in 1989 and Tiritiri Matangi Island in 1991, with additional introductions in subsequent years (Bunin et al. 1997). Takahe are currently located in 16 reserves or sanctuaries across New Zealand (Figure 1-4). Current management efforts are focused towards predator trapping, nest manipulation, captive rearing and regular relocation of birds between sanctuaries (Hegg et al. 2012).

The takahe population has gone through events of rise and decline in recent times, with a current approximate population of 227 adult birds, all of which are located in New Zealand (Figure 1-4) (Wickes et al. 2009). There still remains a free living population of takahe in the Murchison Mountains, Fiordland, as well as populations in a dedicated breeding facility and on offshore and mainland sanctuaries (Figure 1-4) (Wickes et al. 2009). The conservation aim in establishing these sub-populations was to provide insurance populations and mitigate against both deterministic and stochastic threats (Lee 2001). All the birds external to the Murchison Mountains are descendants of the small original Fiordland population and as such are managed as a single population with multiple translocations to minimise the potential detrimental effects of inbreeding (Jamieson et al. 2006). Even so, island populations originate from a small population and this coupled with a low carrying capacity has led to inbreeding and loss of genetic diversity (Bunin et al. 1997; Jamieson & Ryan 2001). Island populations have been less productive and have shown elevated egg infertility compared with the Fiordland and captive breeding populations (Grueber et al. 2010). In addition, inbreeding depression has been linked to in increased susceptibility to disease, due to reduced variation of gene loci in the major histocompatibilty complex resulting from lack of external genetic input into the population (Agudo et al. 2012; Obrien & Evermann 1988). Impaired ability to elicit an immune response and susceptibility to infection is a distinct possibility in such a small population of takahe. However, disease has rarely been questioned as a contributing factor in the decline of the takahe population. A retrospective investigation of post mortem takahe records revealed that 20% of adult deaths were attributable to infectious or inflammatory disease, with the infectious organisms *Erysipelothrix rhusipathiae* and *Salmonella enterica* serotype Typhimurium cultured from dead takahe (McLelland

et al. 2011). This study noted the lack of cohesive records of disease within this endangered species (McLelland et al. 2011). In the early days of takahe recovery management, cross-fostering was used where a pukeko (*Porphyrio porphyrio)* foster mother was trained to rear foster takahe chicks (Bunin & Jamieson 1996). Although the two species are known to share some habitat and are closely related, this practice may have unwittingly introduced exotic pathogens to the takahe population.

A health assessment of the takahe population highlighted that some infectious and parasitic agents may have been introduced to takahe from activities associated with their management (Rose 2000). However, there were no significant findings and it was recommended that a thorough quarantine protocol should be applied to any movements between takahe populations (Rose 2000). Each takahe movement between locations now requires the completion of DOC regulated disease screening and quarantine/biosecurity protocols (McInnes et al. 2004). Active management by DOC involving translocations, conservation strategies and on-going research investigating the health and welfare of takahe is now required to conserve the species (Wickes et al. 2009).

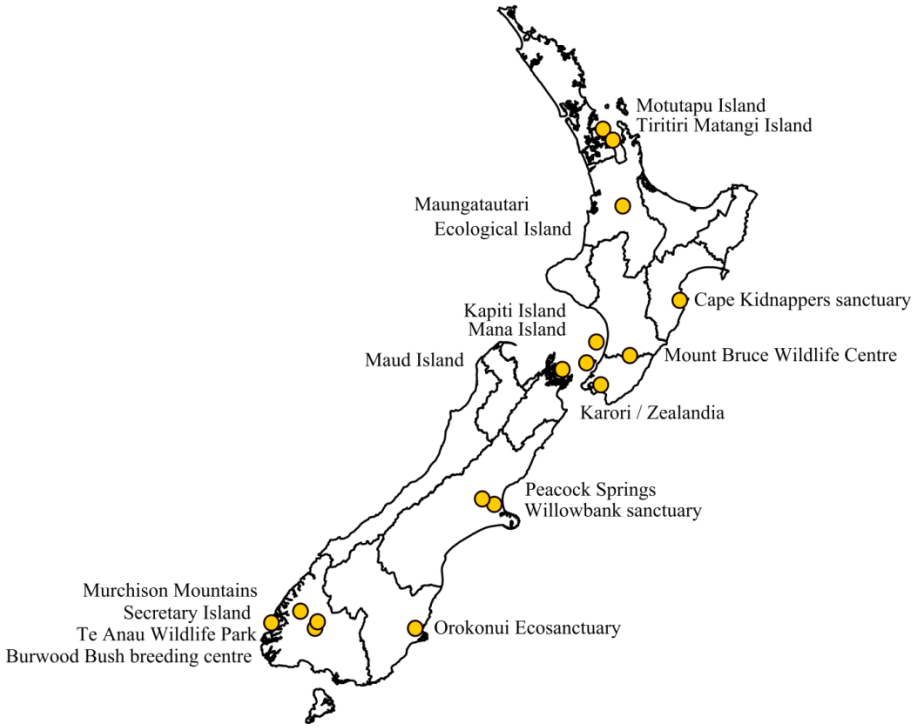

Figure 1-4 Map of the current distribution of takahe in New Zealand. A private island location used as a breeding reserve is undisclosed due to confidentiality restrictions

1.5. Microbes of interest to this study

1.5.1. *Campylobacter* species

Campylobacter spp. have been isolated from humans (Kittl et al. 2013), domestic animals (Colles et al. 2011; Kittl et al. 2013; Mughini Gras et al. 2012) and wildlife, in particular birds (Colles et al. 2011; Colles et al. 2009; French et al. 2009; Hughes et al. 2009; Keller et al. 2011; Sippy et al. 2012) throughout the world. The organisms can survive in host faecal matter and can contaminate the environment including rivers, streams and lakes (Jones 2001). Therefore, *Campylobacter* spp. can transmit non-selectively between different hosts via indirect routes (Kwan et al. 2008). However, *Campylobacter* spp. genotypes demonstrate a degree of host association (Colles et al. 2009; Griekspoor et al. 2013; Mohan et al. 2013). Source attribution studies have extensively investigated the transmission and carriage of the bacteria within and between host species (Colles et al. 2011; French et al. 2009; Sheppard et al. 2010). Observation of the extent of genomic diversity in *Campylobacter* spp. has been made possible by the use of genomic typing primarily by multi-locus sequence typing (MLST) (Maiden et al. 1998). Many studies have used these genomic profiles for sequence comparisons between *Campylobacter* spp. isolates from multiple sources (Colles et al. 2011; French et al. 2009; Hughes et al. 2009; Kittl et al. 2013; Mohan et al. 2013) and have provided insight into the epidemiology and evolution of *Campylobacter* spp. in multiple hosts.

Campylobacter spp. of relevance to animal and human health including *Campylobacter jejuni* and *Campylobacter coli* are frequently isolated from faecal material derived from avian populations (Waldenstrom & Griekspoor 2014) exhibiting no obvious clinical signs of disease (Benskin et al. 2009). Therefore, it is thought that *Campylobacter* spp. are a common commensal component of the avian intestinal microflora. However, there are occasional reports of pathogenic effects in birds (Waldenstrom et al. 2010). The prevalence of *Campylobacter* spp. in birds vary widely between taxa and ecological guilds (Waldenstrom & Griekspoor 2014), ranging from 25% carriage in gulls (Keller et al. 2011) to 78% in coots (Antilles et al. 2013). However, many of these studies have focused on *Campylobacter* spp. of significance to human health and of socio-economic importance, omitting potential incisive information available in unidentified *Campylobacter* spp.

A diverse range of *Campylobacter* spp. genotypes have been reported in domestic and wild birds which are largely host specific and have been associated with ecological attributes relating to domestication, habitat and behaviour (Benskin et al. 2009; Colles et al. 2011; Mohan et al. 2013; Waldenstrom et al. 2002). Host associated factors are thought to be more influential on sequence type carriage than geographical separation, with similar sequence types found in the same avian species on different continents (French et al. 2009; Sheppard et al. 2010). Host behaviour and the ability of birds to fly and migrate between locations may explain the mixing of genotypes between biogeographic regions, with limited gene flow between species due to barriers related to occupation of distinct ecological niches.

1.5.2.*Salmonella* species

Salmonella spp. are ubiquitous opportunistic bacteria and have been isolated from multiple wildlife species around the world (Marin et al. 2013; Marin et al. 2014; Middleton et al. 2014; Vlahović et al. 2004). These organisms can survive outside their hosts for extended periods of time, are prime candidates for contamination of the environment (Winfield & Groisman 2003), and show high potential for cross-infection between susceptible hosts. Avian populations can be asymptomatic carriers of *Salmonella* spp. (Vlahović et al. 2004). Even so, *Salmonella* spp. can cause disease in bird populations and has been a great contributor to passerine mortality in the United States (Saito & Hall 2008). *Salmonella enterica* subspecies *enterica* serotype Typhimurium has been implicated as a cause of death in both native and introduced birds in New Zealand, with a previously undetected strain in a native range restricted passerine (hihi, *Notiomystis cincta*) located on a remote island in New Zealand (Alley et al. 2002; Ewen et al. 2007). Ubiquity of *Salmonella* spp. in the environment and wildlife reservoirs, for example, sparrows (Alley et al. 2002) and reptiles (Middleton et al. 2014), brings cause for concern when considering the management of vulnerable wildlife species. Disease screening protocols associated with translocations in New Zealand include testing for *Salmonella* spp. carriage (McInnes et al. 2004), with the aim of selecting pathogen free individuals and prevent the spread of novel *Salmonella* spp. to naive populations.

1.6. Objectives of the study

The overarching hypothesis of this study is that translocation, population fragmentation and isolation have altered host-microbial dynamics in endangered takahe.

There are four main objectives of this study. The first is to examine population connectivity between takahe subpopulations with respect to pathogen dynamics and identifying targets for surveillance (Chapter 2). The second is to determine the prevalence of a common commensal, *Campylobacter* spp. (Chapter 3) and an opportunistic pathogen, *Salmonella* spp. (Chapter 6) in multiple populations of biogeographically separated takahe. The third is to investigate the influence of geographic isolation and translocation on the molecular differentiation of *Campylobacter* spp. carried by takahe (Chapter 4). The final objective is to determine reservoirs and transmission dynamics of *Campylobacter* spp. in vertebrate communities within an island ecosystem used for conservation of takahe (Chapter 5).

CHAPTER 2

NETWORK ANALYSIS OF TRANSLOCATED TAKAHE POPULATIONS TO IDENTIFY DISEASE SURVEILLANCE TARGETS

Zoë L. Grange, Mary van Andel, Nigel P. French and Brett D. Gartrell

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2. Network analysis of translocated takahe populations to identify disease surveillance targets

2.1. Abstract

Social network analysis is increasingly being used in epidemiology and disease modelling in humans, domestic animals, and wildlife. This study investigated a translocation network (area that allows movement of animals between geographically isolated locations) used for the conservation of an endangered flightless rail, the takahe (*Porphyrio hochstetteri*). Records of takahe translocations within New Zealand were collated, and we used social network principles to describe the connectivity of the translocation network. That is, networks were constructed and analysed using adjacency matrices with values based on the tie weights between nodes. Five annual network matrices were created using the takahe data set, each incremental year included records of previous years. Weights of movements between connected locations were assigned by the number of takahe moved. The number of nodes (i_{total}) and the number of ties (t_{total}) between the nodes were calculated. To quantify the small-world character of the networks, the real networks were compared to random graphs of the equivalent size, weighting, and node strength. Descriptive analysis of cumulative annual takahe movement networks involved determination of node-level characteristics, including centrality descriptors of relevance to disease modelling such as weighted measures of in degree (k_i^{in}), out degree (k_i^{out}), and betweenness (B_i). Key players were assigned according to the highest node measure of k_i^{in}, k_i^{out} and B_i per network. Networks increased in size throughout the time frame considered. The network had some degree of small-world characteristics. Nodes with the highest cumulative tie weights connecting them to other nodes were the captive breeding centre, the Murchison Mountains and two offshore islands. The key player fluctuated between the captive breeding centre and the Murchison Mountains. The cumulative networks identified the captive breeding centre every year as the hub of the network until the final network in 2011. Likewise, the wild Murchison Mountain population was consistently the sink of the network. Other nodes, such as the offshore islands and the wildlife hospital, varied in importance over time. Common network descriptors and measures of centrality identified key locations for targeting disease surveillance. This technique provides a visual representation of animal movements in a population that can aid decision makers when they evaluate translocation proposals or attempt to control a disease outbreak.

2.2. Introduction

The introduction of a an infectious disease into small, highly connected wildlife populations can have severe consequences on survival of the population (Daszak et al. 2000). Understanding how a pathogen may spread from the point of entry throughout a connected community can inform important transmission pathways, and thus aid in planning disease surveillance, outbreak control, and contingency planning (Christley & French 2003). Dissemination of an infectious agent within a population is dependent on many factors, including but not limited to susceptibility, mode of transmission, and social interaction (Wey et al. 2008). Analysis of social relations of individuals and identification of those that are more social within a group might identify individuals that are most influential in pathogen transfer. Social network analysis (SNA) allows the pattern of contacts within a complex system to be described and quantified in terms of a network of links between items. SNA is becoming an important tool in epidemiology of human populations. Thus, its use in wildlife settings is a natural progression.

SNA highlights which objects and relations are most important for maintaining connectivity of the network, leading to its use in epidemiological disease modelling. Studies have been conducted describing contact networks in relation to the transmission of infectious agents in animals (Christley & French 2003). However, there have been no reported applications of network analysis to wildlife translocation databases of endangered species to identify high-risk locations for the prioritization of target sites for disease surveillance.

Infectious diseases are increasingly recognized as a challenge to the conservation of wildlife, particularly in intensively managed species (Smith et al. 2009). A lack of knowledge of pathogen dynamics in wild animal populations limits the ability of conservation managers to develop prioritised strategies for disease control and to effectively target disease surveillance. When animals are translocated their parasites and pathogens can also move with them (Bengis et al. 2002). Therefore there is a need for measures to be in place to reduce disease risk during a translocation program (Viggers et al. 1993). The primary aim of disease screening associated with translocations is to prevent introduction of novel pathogens into existing populations at the release site and to increase likelihood of survival of translocated individuals (Viggers et al. 1993).

Island ecosystems worldwide are particularly vulnerable to extinctions. In New Zealand, 41% of all bird species have become extinct since human settlement and the introduction of invasive species (Tennyson & Martinson 2006). To prevent further extinction of land birds, translocation and mammal eradication emerged as conservation solutions. The process of removing exotic animals from island reserves began in the early 20th century, and the islands have provided sanctuary for many indigenous populations threatened by introduced mammals (Bellingham et al. 2010). Translocations of animals between these sites are commonplace and are actively managed by the Department of Conservation (DOC) or by local conservancies and communities under the guidance and control of DOC (Sherley et al. 2010). However, not all translocations have been successful, for reasons often unknown or unexplored.

The takahe (*Porphyrio hochstetteri*) is an iconic endemic New Zealand flightless rail, considered nationally critically endangered under the New Zealand Department of Conservation threat classification system (Miskelly et al. 2008) and endangered by the International Union for Conservation of Nature (BirdLife International 2013). Takahe are thought to have been widespread throughout New Zealand. Their range was reduced to Fiordland, South Island, as a consequence of hunting by Maori and habitat destruction (Trewick & Worthy 2001). The species was thought to be extinct by the end of the 19th century, but a small population was rediscovered in Fiordland in 1948 (Ballance 2001). Over the next 15 years, takahe were introduced to five small predator free offshore islands to provide insurance populations and mitigate against both deterministic and stochastic threats (Lee 2001). The takahe population has fluctuated in recent times, with a current approximate population of 227 adult birds, all of which are located in New Zealand (Wickes et al. 2009). There remains a free-living population of takahe in the Murchison Mountains, Fiordland, and insurance populations on predator free offshore island reserves, mainland sanctuaries, and a dedicated breeding facility (Wickes et al. 2009) (Appendix 9.2-1). To conserve the species, management strategies have been targeted toward captive breeding and annual translocations of the birds between reserves (Wickes et al. 2009).

This study investigated the use of social network theory and measures of node-level dynamics to describe connectivity patterns and identify surveillance targets in isolated populations of translocated

takahe. Applying comparative analysis to random networks, this study determined whether the takahe networks possess small-world properties. That is, a network in which most nodes are not neighbours of one another but can be reached from every other by a small number of steps. Small-world properties are a measure of particular relevance to population disease spread dynamics. This investigation examined annual cumulative yearly changes in the translocation network, identified key player (structurally important) locations in each network, and determined the importance of these for maintaining the integrity of the system and thus their role in pathogen spread.

2.3. Methods

2.3.1. Data set

A census of all recorded takahe movements within New Zealand from 2007 to 2011 was collated into a database. Prior to 2007, accurate records of takahe movements were not maintained. Records analysed in this investigation include a DOC document of 131 takahe translocations within New Zealand between a breeding centre, island reserves, and mainland reserves (Appendix 9.2-2). In addition, 33 catalogued records (patient origin and release location) of hospitalized takahe from the clinic database at Wildbase Hospital, Massey University were included in the analysis. In the context of this analysis, the term takahe refers to takahe eggs, chicks, and adults. Although life stages may carry variable risk of disease transmission, life stages were not separated due to uncertainty of the data provided with regards to age of individuals. One database (Appendix 9.2-2) was analysed in two formats for subsequent network analysis. The dataset consists of the year of the movement, an origin and destination location, and number of takahe moved between the locations. Due to scarcity of information with regards to precise dates of animal movements, each individual takahe moved was considered as a single event. Therefore, within the same year, two birds translocated at the same time would be considered the same as two birds translocated months apart. Both situations would be regarded as two movement events and weighted accordingly. Number of contacts and direction of movement between locations was known, and in the context of this study, the dataset has been interpreted in both the directed and undirected format. For ease of understanding, each location has been assigned a letter (Figure 2-1), and for subsequent analysis locations are referred to in this format.

For the purposes of this analysis, a network is referred to as the entirety of the interactions for a given data set. A node is the location where takahe have been moved to and from. Ties represent the connection, unidirectional, or bidirectional, between locations due to movement of birds. Tie weights were derived from the number of takahe moved between 2 given nodes. Node strength was calculated as the total tie weights associated with a node.

2.3.2. Network description and topology

Networks were constructed and analysed using adjacency matrices with a range of values based on the tie weights between nodes. Five annual network matrices were created using the takahe data set, each incremental year included records of previous years. For example, matrices were composed for 2007, 2007–2008, 2007–2009, and so on until 2011. Therefore, annual networks account for potential reconnection of previously isolated nodes into the network. Weights of movements between connected locations were assigned by the number of takahe moved within the year or cumulative years included in the network. Takahe network matrices were analysed for weighted network measures using tnet (Opsahl 2009) and igraph (Csardi & Nepusz 2006) packages within R software (R Core Team 2013).

Each network was described in terms of size. The number of nodes (i_{total}) and the number of ties (t_{total}) between the nodes were calculated. These parameters allowed comparison between years and monitoring of growth or reduction in the translocation network over time.

Understanding network topology and the pattern of contacts within a population can help predict disease transmission pathways (Christley & French 2003). Small-world networks, as opposed to regular and random networks, can be described as having clusters of connected nodes that have short paths to connect nearby and distant groups within the network (Watts & Strogatz 1998). Small-world determination is of relevance to disease simulations due to the influence such a connected system could have on the rapid spread of disease from local infections across apparent long distances (Watts & Strogatz 1998). For the purposes of evaluation of small-world characteristics, undirected weighted annual matrices were created from the takahe dataset. Cohesion characteristics including weighted geometric mean clustering coefficients (CC_{av}) (Opsahl & Panzarasa 2009) and weighted average

shortest path lengths (L_{av}) (Opsahl et al. 2010) were calculated for each takahe network using appropriate measures previously described within the tnet package. The clustering coefficient measures the average proportion of connections that exist between locations divided by the number of possible connections that could have existed (Dube et al. 2009). This measure was used to compare the structure of the annual networks.

For all networks, the tnet package was used to create a random graph of the equivalent size, weighting, and node strength; weights were locally reshuffled on each node across its outgoing ties (Opsahl et al. 2008). Mean weighted measures of CC_{av} and L_{av} were calculated from the random network per annual observed network (Opsahl et al. 2010; Opsahl & Panzarasa 2009). Random graph measures were then compared with the mean CC_{av} and L_{av} of the observed annual takahe networks, in order to determine whether each network has small-world properties (Watts & Strogatz 1998). To quantify the small-world character of the networks and thus allow comparison over time, we divided the mean L_{av} by the CC_{av} for both the random and real networks (De Nooy et al. 2011).

2.3.3. Network dynamics

Descriptive analysis of the cumulative annual takahe movement networks involved determination of node-level characteristics, including centrality descriptors of relevance to disease modelling such as weighted measures of in degree (k_i^{in}), out degree (k_i^{out}), and betweenness (B_i) (defined below). Key players were assigned according to the highest node measure of k_i^{in}, k_i^{out}, and B_i per network (Appendix 9.2-3).

Weighted betweenness (B_i) is a measure of centrality that represents the likelihood of a node connecting two random nodes via the shortest path accounting for node strength. A node's strength in a weighted network is given by the sum of the weights of its ties. Locations with the highest weighted betweenness measure are most likely to be involved in funnelling an infectious disease among other nodes through the network via the shortest path.

Weighted degree (k_i) is a complimentary centrality measure to betweenness, values of which are attributed according to a tuning parameter that accounts for the traditional measures of number of direct ties associated with a node, and the average number of tie weights of that node (Opsahl et al.

2010). In other words, weighted degree measures the number and strength of direct transfers between a location and its neighbour. In a disease simulation network, weighted degree measures are positively correlated with probability of becoming infected or transmitting infection and therefore could identify locations for disease surveillance. The ability to calculate and compare in degree (k_i^{in}) and out degree (k_i^{out}) allows identification of nodes as hubs or nodes that are centrally placed due to high numbers of inward or outward connections to their neighbours. Additionally, from a disease surveillance perspective, nodes on the periphery of a network could be just as important as those that are centrally placed. Some nodes could act as end points for an infectious disease (sinks) or can harbour novel pathogens that have not yet infiltrated a network (source). Nodes with high k_i^{in} and low k_i^{out} scores are a good indication of potential sinks and or sources.

2.4. Results

2.4.1. Network description and topology

Networks increased in size throughout the timeframe included in this analysis (Table 2-1). This was demonstrated by the incremental number of nodes and ties included in the network each year. These measures suggest that the annual networks were composed of several clusters of well-connected nodes with high density and weight of ties. Over the years the L_{av} of the observed networks remain consistently short, between three and four nodes, similar to that of the random graphs, a characteristic of small-world networks. However, in the comparison of the L_{av}/CC_{av} of takahe networks to that of the random networks, only the 2007 to 2010 network showed some degree of small-world characteristics, represented by a lower L_{av}/CC_{av} than that of the random networks.

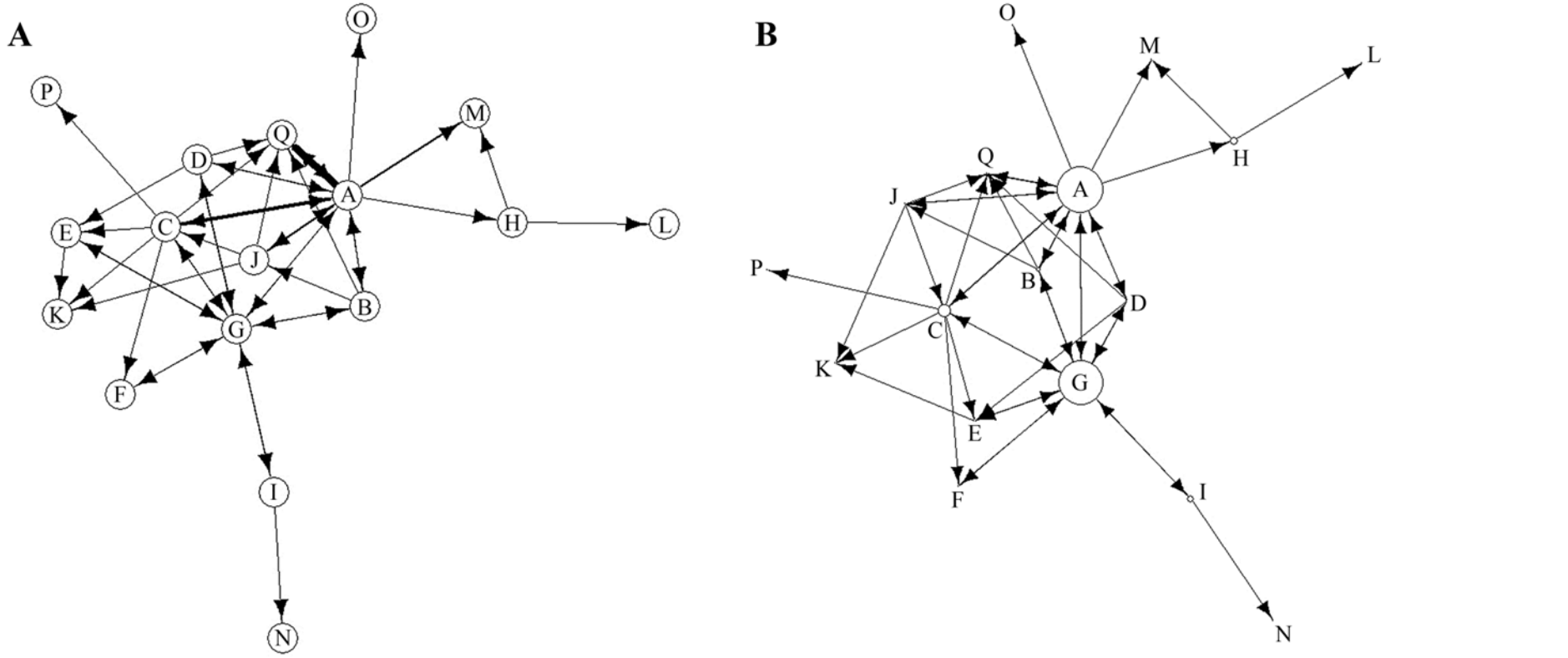
2.4.2. Network dynamics and node-level analysis

Cumulatively nodes with the highest tie weights connecting them were nodes A (captive breeding centre) and Q (wild Murchison Mountain population), A and C (Mana Island), and A and J (Tiritiri Matangi Island) (Figure 2-1).

Year	Nodes (i_{total})	Ties (t_{total})	Average path length (L_{av})[b]		Clustering coefficient (CC_{av})[c]		Small world measure (L_{av}/CC_{av})[d]	
			Observed	*Random*	*Observed*	*Random*	*Observed*	*Random*
2007	6	9	3	3	0.3	0.4	10	9
2007 to 2008	8	20	3	2	0.7	0.5	4	5
2007 to 2009	13	30	4	4	0.6	0.5	6	7
2007 to 2010	14	34	4	5	0.5	0.4	8	12
2007 to 2011	17	43	4	4	0.5	0.4	8	9

[a]The takahe translocation networks represent the movement of takahe between locations used for their conservation management within New Zealand. Weights of movements between connected locations were assigned by the number of takahe moved within the year. The number of nodes (i_{total}) and the number of ties (t_{total}) between the nodes were calculated for each network. To quantify the small-world measure of the networks, the real networks were compared to random graphs of the equivalent size, weighting, and node strength.
[b]Average path length (L_{av}) is the number of steps required to move between two randomly assigned locations in the network.
[c]Clustering coefficient (CC_{av}) is a measure of degree to which nodes in the network tend to cluster together.
[d]Small-world measure is the degree to which most nodes in the network are not neighbours of one another but can be reached from every other node by a small number of steps.

Table 2-1 Measures of observed weighted takahe translocation networks and corresponding random measures which were then compared with the observed annual takahe networks to determine whether each network has small-world properties[a]



A = Burwood Bush breeding center; B = Kapiti Island; C = Mana Island; D = Maud Island; E = Maungatautari reserve; F = Pukaha Mt Bruce; G = Wildbase Hospital; H = Private Island
I = Te Anau wildlife reserve; J = Tiritiri Matangi Island; K = Motutapu Island; L = Peacock Springs wildlife park; M = Secretary Island: N = Wellington Zoo; O = Willowbank reserve
P = Zealandia / Karori Sanctuary; Q = Murchison Mountains

Figure 2-1 Node (circles) and tie (lines) networks of cumulative takahe translocations from 2007 to 2011: (a) tie weight between locations (the thicker the line, the more translocations between two directly connected locations) and (b) weighted betweenness for individual locations (i.e., measure of centrality determining the likelihood of a node connecting two random nodes via the shortest path while accounting for node strength [sum of node weights] of an individual node). Nodes represent isolated geographic locations used for takahe conservation. Lines with either uni- or bidirectional arrows show the direction of human-mediated takahe translocations between locations. Size of the circle illustrates extent of centrality; the larger the node, the more central the location in the network (A, Burwood Bush breeding centre; B, Kapiti Island; C, Mana Island; D, Maud Island; E, Maungatautari reserve; F, Pukaha Mt Bruce; G, Wildbase Hospital; H, Private island; I, Te Anau wildlife reserve; J, Tiritiri Matangi Island; K, Motutapu Island; L, Peacock Springs wildlife park; M, Secretary Island; N, Wellington Zoo; O, Willowbank reserve; P, Zealandia / Karori Sanctuary; Q, Murchison Mountains).

Identification of key players according to the highest measure of weighted degree (k_i) when accounting for historic connections demonstrated relatively consistent key players over time (Table 2-2). The key player according to in degree (k_i^{in}) fluctuated between a captive breeding centre (node A) and the wild Murchison Mountains population (node Q). Node Q had a large number of takahe inputs in 2008 and 2009. Outside these years, node A was the prominent node according to k_i^{in}. Out degree (k_i^{out}) also identified the key player to be node A, with the exception of 2007 where an offshore island, Tiritiri Matangi (node J) was a source of output into the network.

Year	In degree (k_i^{in})[a]	Out degree (k_i^{out})[b]	Betweenness (B_i)[c]
2007	Burwood Bush breeding centre	Tiritiri Matangi Island	Burwood Bush breeding centre, Mana Island, Maud Island
2007 to 2008	Murchison Mountains	Burwood Bush breeding centre	Burwood Bush breeding centre
2007 to 2009	Murchison Mountains	Burwood Bush breeding centre	Burwood Bush breeding centre
2007 to 2010	Burwood Bush breeding centre	Burwood Bush breeding centre	Burwood Bush breeding centre
2007 to 2011	Burwood Bush breeding centre	Burwood Bush breeding centre	Burwood Bush breeding centre

Table 2-2 Identification of key takahe population location within the annual translocation networks, categorised by the highest node-based measure.

When plotting k_i^{in} against k_i^{out}, apart from 2007 when measures were less defined between nodes in the network, the cumulative networks identified node A every year as the hub of the network (Figure 2-2). Likewise, the wild Murchison Mountains population (node Q) is consistently the sink of the network with regularly high k_i^{in} and low k_i^{out} scores (Figure 2-2). Although Mana Island (node C) has been in the network since 2007, its k_i^{out} steadily increased, whereas its k_i^{in} remained constant. The emergence of node C as a potential source within the network becomes apparent from 2010 onwards, and in the final 2011 network its k_i^{out} is 3 times that of its k_i^{in} score. An additional note is the increasing importance of Wildbase Hospital (node G). Although not the most obvious hub of the network, its k_i^{in} and k_i^{out} scores increased proportionately and steadily.

Identifying key players according to the highest measure of weighted betweenness (B_i) produced a consistent key player (Figure 2-1, Table 2-2). Node A had the highest measures every year from 2007 to 2011 when previous connections are included. In 2007, B_i measures were more conserved among nodes in the network. Therefore, three nodes, A, C, and D (Maud Island) were identified as key players.

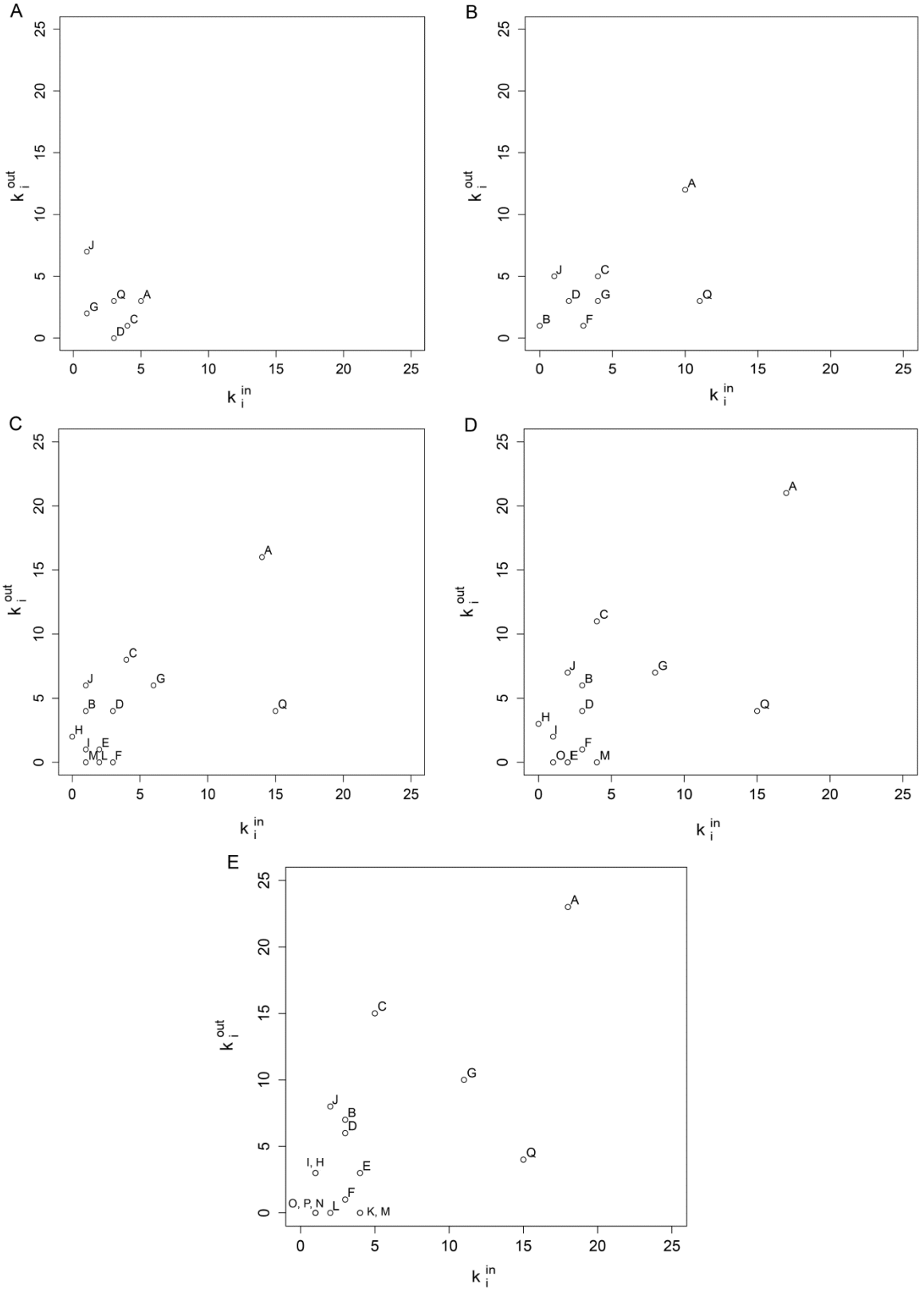


Figure 2-2 Relationship between weighted in degree (k_i^{in}) (animals moving into a location) and weighted out degree (k_i^{out}) (animals moving out of a location) for nodes (i.e., isolated geographic locations used for takahe conservation) of the cumulative (a) 2007, (b) 2007–2008, (c) 2007–2009, (d) 2007–2010, and (e) 2007–2011 final network of takahe translocations. Alphabetical labels are as for Figure 2-1

2.5. Discussion

Conservation of takahe populations has been of significant interest internationally. However, disease has rarely been questioned as a contributing factor in the decline of the population, with only one retrospective investigation highlighting the lack of cohesive disease reports and post-mortem protocols (McLelland et al. 2011). As a consequence of past events, the population has declined to a small number reliant on active management and translocations (Hegg et al. 2012). The translocation database of this flightless endangered species provides an accurate record of locations, individuals, and movements because there is no natural immigration or emigration between the small remnant populations. The ability to model movements of takahe for the last five years has provided the opportunity to construct complete networks and draw valid conclusions for the timespan available. This is often not the case when analysing movements of animals. Data are often missing or unknown, and ascertaining all associations within a country is rarely possible (Ortiz-Pelaez et al. 2006). Therefore, range-restricted endangered animals, such as takahe, are ideal candidates for SNA. This analysis can then be used to determine where to target disease surveillance or to control the spread of disease in an outbreak situation.

2.5.1. Application of network analysis to takahe movements

Approaching the analysis of the takahe database using multiple network measures allowed comparison of network methods and subsequent outcomes. Annual networks were constructed to include translocations from previous years on record because takahe are a long-lived flightless species, and when an individual is moved to a new location, it cannot emigrate to another area without human intervention. Therefore, although the translocation may have occurred several years before the year of analysis, reconnection of the location to the network poses the risk of transmission of pathogens from the extant population back into the network. Annual analysis of the translocation network informs management and identifies shifts in key nodes of contact for the species over time (Figure 2-2, Table 2-2). The final 2007–2011 cumulative network was most important in the assessment of the current state of the network in terms of identifying suitable locations for disease surveillance (Figure 2-1, Figure 2-2).

The number of locations and interactions between these sites increased annually over the time frame of the study (Table 2-1), correlating with the increasing use of translocations as an endangered species management tool worldwide (Fischer & Lindenmayer 2000) and in New Zealand (Sherley et al. 2010). As more locations are added to the network, the likelihood of introduction and exposure to infectious agents may increase due to increased connectivity, and potential clustering between populations. Infectious diseases spread more easily in small-world networks than in regular and random networks. Disease spread is unpredictable in small-world networks due to the high degree of local clustering encountered (Jeger et al. 2007). Limitations of the takahe data, in terms of sample size and lack of complexity in the contact networks, restrict the ability to confirm the presence of small-world attributes in the takahe networks. However, there appears to be a trend for the annual networks to fluctuate and display some attributes of small-world characteristics, with the most obvious being in the final 2007–2010 network where the L_{av}/CC_{av} was substantially smaller than that of the random equivalent networks (Table 2-1). From this it could be inferred that as translocations continue over time and with the establishment of new connections, small-world characteristics may develop. The risks associated with small-world characteristics in terms of ease of pathogen spread between locations should be kept in mind when considering the translocation of birds between populations.

2.5.2. Identification of hubs, sinks, and sources

Detecting wildlife disease trends requires optimal diagnostic sensitivity and specificity, appropriate sample sizes and sampling strategies, and analytical tools that can identify anomalies in space and time in order to inform decision making. However, sampling by its nature is only representative of a small proportion of a real system. Surveillance programs require appropriate sampling to provide a good representation of a population network. Interpreting connectivity within a population can provide insights into the dynamics of a system, identifying players that are most influential. This study used the takahe translocation database as a model to predict which locations may act as hubs, sinks, and sources of pathogens.

A notable result of this investigation is the central position of a captive breeding centre (A), wildlife hospital (G), and wild population (Q) within the translocation network. When looking at weighted centrality measures of degree (k_i^{in} and k_i^{out}) and betweenness (B_i), although these measures

occasionally identified different key players within a network, consistently locations A, G, and Q demonstrated important roles in connectivity between locations (Table 2-2).

Rediscovery of a few wild takahe in the Murchison Mountains in 1984 led to the translocation of birds to and from the wild to sanctuaries across New Zealand (Jamieson & Ryan 1999). The wild population soon became a source for genetic input into the captive populations; however, a large proportion of takahe are still resident in the Murchison Mountains (Hegg et al. 2012). Our networks illustrate the importance of island sanctuaries, with island locations being identified as key players according to k_i^{out} and B_i measures in 2007. After 2007, management increased the role of the wild population in the network. In the timespan included in this investigation, the Murchison Mountain site received many takahe from external sources with relatively little emigration of individuals (Figure 2-2). Management decisions made in 2009 and 2010 to treat the wild birds in the Murchison Mountains as a separate population led to the cessation of relocations to and from Fiordland (Glen Greaves, personal communication). In the network analysis, this is represented by stagnation of centrality measures for this node from 2009 onwards (Figure 2-2). A location such as the Murchison Mountains, which has had multiple immigration events with little emigration, could act as a sink for pathogen evolution within that population; the birds could carry unique microflora that could be pathogenic to naïve-introduced animals.

Captive breeding facilities, such as Burwood Bush breeding centre (A), often have a high turnover of animals and intensive production of stock for reintroduction to the wider population. A common difficulty with endangered species, such as takahe, is that poor breeding success leads to the need for mixing of mates to find compatible couplings (Jamieson et al. 2006). Additionally input of new breeding lines from external sources is required to minimize inbreeding (Jamieson & Ryan 2001). As such it is not surprising that Burwood Bush breeding centre has emerged as a substantial hub of the takahe network, irrespective of management decisions with regards to other reserves. Consistently, the location increased its connections to and from multiple locations (Figure 2-2) and played a major role as an intermediary between sites, as shown by the B_i and k_i measures (Table 2-2). With such connected locations containing animals originating from multiple locations, focusing effort and surveillance within a hub provides insight into what is flowing through the entire network.

Wildlife health treatment centres, such as Wildbase Hospital (G), are of a different nature than the mainland and island reserves included in the network. The number of takahe sent to veterinary hospitals is likely to be variable depending on the health status of the population, whereas movements between other locations are controlled by management decisions. The veterinary hospital (G) appeared to gain importance over time where the number of birds transiting through the location increased, as shown by growth in k_i^{in} and k_i^{out} scores (Figure 2-2). The hospital was also an intermediary location, as shown by high measures of B_i, particularly evident in the final 2011 network (Figure 2-1). Animals sent to a hospital are more likely to be ill or injured. Therefore, the animals may be more vulnerable to pathogen invasion and could present a significant disease risk by acting as carriers or vectors of new diseases to wild populations when released (Viggers et al. 1993).

A translocation database is by no means static over time; locations may be included one year and not in subsequent years. Establishment of new sites in the network for advocacy or display purposes, such as Peacock Springs wildlife park (L) and Willowbank reserve (O), have had inputs of birds into them but are not connected to other locations (Figure 2-2). Sites such as these are regarded as peripheral and relatively isolated and could be targeted to determine extent of disease spread. If reconnected to the network by the process of translocation of takahe from these nodes to a hub, such as Burwood Bush breeding centre, this could create a transmission pathway to introduce a novel strain to the hub that could subsequently spread throughout the network. If the networks were to be analysed annually without accounting for historic translocations, peripheral sites would drop out from the networks. If a translocation were to occur either to or from this site in the future, we would not know the risk that the birds already in the location may pose due to previous connections from historic translocations.

2.5.3.Limitations

Like most models of complex biological systems, the SNA produced here has limitations and further refinements are required to reflect real situations. Records used in this analysis span only five years of a 30 plus year history of population management due to the scarcity of recorded data prior to 2007. Limitations in the collation of data for this investigation demonstrated the need for conservation managers to maintain accurate records of individual takahe movements and to file this information in one central database. Seasonal timing of translocations and the numbers of birds translocated at a

given time are components that may influence the importance of a location in the network due to differing influences on the likelihood of transmission and susceptibility to pathogens. Relationships among pathogens, seasonal host infection, and transmission are currently poorly understood. This information would add another level of complexity and increase validity of network associations.

Island sanctuaries, both mainland and offshore, used for takahe conservation are also used as predator free reserves for other threatened New Zealand native fauna and flora (Sherley et al. 2010). The introduction of any animal to a new site may expose an immunologically naive animal to potentially pathogenic organisms residing in other species (Cunningham 1996). Many of the sites have histories of translocations of other species, which could interact with takahe and transmit infective agents to cohabiting takahe. Integration of translocation databases of all introduced species within the takahe reserves would provide a better understanding of the reserve networks and connectivity beyond that of just takahe. Disease surveillance could then be assigned according to complete ecological networks. However, if cross-species transmission from resident and migratory species already present in reserves is considered, then the complexity of the network could rapidly increase beyond our ability to describe it. In addition to takahe, people and field equipment associated with a translocation are moving between locations within the network. This could increase the connectivity between previously isolated locations. To compensate for this possibility, it is recommended that strict quarantine and biosecurity checks continue to be practiced prior to and on arrival at these locations.

2.5.4. Conservation implications and future directions

SNA of historical takahe translocations enabled us to characterize key components of the conservation management network that are potentially important in pathogen transmission. Our findings provide advice for takahe conservation management, where networks are used as component of decisions. It could be applied to help reduce the likelihood of disease spread, identify target locations to monitor for disease emergence and inform mitigation actions should an outbreak occur.

Disease screening can be a very costly component of a translocation, and epidemiologists have been limited in gaining sufficient sample sizes to provide informative meaningful results (Gartrell et al. 2006). Additionally, choosing influential pathogens or those which are relevant to the target species

can be a difficult task. Mathews et al. (2006) suggest that initial screening should be as broad as possible, using existing knowledge of related species. Pooled sampling is often proposed as a low-cost solution (Mathews et al. 2006). However, it is less informative than individual testing. This study identified key locations within the takahe network to impose disease surveillance if funds were limited and management was unable to monitor an entire population. Locations can be selected on the basis of potential threats and / or being representative of the takahe population as a whole. Optimisation of sample collection, sampling a broad range of pathogens from individuals in key locations identified from network analysis, would allow economically viable targeted monitoring of potential threats to a population and inform normal ranges of pathogen prevalence, thus providing epidemiologically sound advice on disease management.

Conservation managers could use a translocation network, like the one used in this study, to inform decisions when moving animals between locations. Takahe movement network analysis has highlighted that historic decisions of introducing intensively reared birds from a breeding centre into the one remaining wild population in the Murchison Mountains may have exposed a wild population to exotic pathogens, which in the face of disease threats may be a risky strategy. Therefore, before commencing a translocation from one location to another, it would be worthwhile noting the current network measures of the existing and new location. If the translocation would reconnect a peripheral location, a sink, to a hub within the network, informed decisions on quarantine procedures and additional disease screening could be made.

A retrospective network analysis of the 2001 foot and mouth disease outbreak in the United Kingdom (Ortiz-Pelaez et al. 2006) uncovered how network analysis could inform mitigation to prevent spread of epidemics. If an infectious disease outbreak were to occur within the takahe population, sites with the highest centrality measures within a network could be isolated and movement restrictions imposed to most effectively disrupt transmission pathways.

Targeted and opportunistic disease surveillance, focusing on common pathogens and their respective strain types, provides detailed information on pathogen diversity and relative importance of aetiology of disease in the host (Smith et al. 2009). Comprehension of the diversity and abundance of pathogens in a community and ecosystem will increase our grasp on potential transmission and evolution of

pathogens within the species of interest (Thompson et al. 2010). In addition, it would allow one to trace and overlay a pathogen network with a translocation network and gain insight into the epidemiology of pathogens within an altered ecosystem.

2.6. Acknowledgments

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2.7. Supporting Information

Map of takahe locations within New Zealand (Appendix 9.2-1), takahe movement data (Appendix 9.2-2), and individual node measures (Appendix 9.2-3) are available in the appendix.

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal 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: Zoe Grange

Name/Title of Principal Supervisor: Associate Professor Brett Gartrell

Name of Published Research Output and full reference:

Grange, Z. L., M. Van Andel, N. P. French, and B. D. Gartrell. 2014. Network analysis of translocated takahe populations to identify disease surveillance targets. Conservation Biology.28(2). 518-528

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

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  The candidate was primarily responsible for all aspects of paper preparation including experimental design, data collection and analysis and led the write up of the paper. The co-authors assisted in all aspects of the paper preparation, and their inclusion on the paper recognises this role.

Zoe Grange — Digitally signed by Zoe Grange DN: cn=Zoe Grange, o=Massey University, ou=IVABS, email=z.grange@massey.a.nz, c=NZ Date: 2014.10.19 10:15:24 +13'00'

Candidate's Signature

19/10/2014

Date

Brett Gartrell — Digitally signed by Brett Gartrell DN: cn=Brett Gartrell, o=Massey University, ou=Wildbase, IVABS, email=B.Gartrell@masseyac.nz, c=NZ Date: 2014.10.20 11:39:36 +13'00'

Principal Supervisor's signature

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CHAPTER 3

USING A COMMON COMMENSAL BACTERIUM IN ENDANGERED TAKAHE (*PORHYRIO HOCHSTETTERI*), AS A MODEL TO EXPLORE PATHOGEN DYNAMICS IN ISOLATED WILDLIFE POPULATIONS

3. Using a common commensal bacterium in endangered takahe (*Porphyrio hochstetteri*), as a model to explore pathogen dynamics in isolated wildlife populations

3.1. Abstract

Predicting and preventing outbreaks of infectious disease in endangered wildlife is problematic without an understanding of the biotic and abiotic factors that influence pathogen transmission, and the genetic variation of microorganisms within and between these highly modified host communities. Using a common commensal bacterium, such as *Campylobacter* spp., in endangered takahe (*Porphyrio hochstetteri*) populations enables the development of a model to study pathogen dynamics within isolated wildlife populations which are connected through ongoing translocations. Takahe are an endangered flightless bird endemic to New Zealand, with a population of approximately 230 individuals. Insurance populations were founded from a single remnant wild population and have been established within multiple reserves. Management has resulted in the formation of several fragmented sub-populations maintained and connected through regular translocations. This investigation tested 118 takahe from eight locations for faecal *Campylobacter* spp. via culture and DNA extraction, with species assignment conducted by PCR. Factors relating to population connectivity and host life history were explored using multivariate analytical methods to determine associations between host variables and bacterial prevalence. The apparent prevalence of *Campylobacter* spp. carriage in takahe was 99%, one of the highest reported in avian populations. Variation in prevalence was evident between *Campylobacter* spp. identified; *C.* sp. *nova 1* (90%) colonised the majority of takahe tested. Prevalence of *C. jejuni* (38%) and *C. coli* (24%) was different between takahe sub-populations, and this may be explained by factors related to population management, captivity, rearing environment and the presence of agricultural practices in the location in which birds were sampled. Modelling of a commensal microorganism within takahe meta-populations suggests that anthropogenic management of endangered species within altered environments may have unforeseen effects on microbial exposure, carriage and disease risk. Translocation of wildlife between locations could have unpredictable consequences including the spread of novel microbes between isolated populations.

3.2. Introduction

The inadvertent introduction or emergence of infectious disease through translocation of animals into new populations or ecosystems is a major concern when considering management of wildlife populations (Thompson et al. 2010). Translocation of individuals between previously isolated ecosystems removes barriers to the exchange of pathogens (Power et al. 2013). These translocations can potentially transfer exotic pathogens into extant populations with no effective immunity at the release site (Anderson & May 1986; Woodford & Rossiter 1993). Threats posed by management actions are particularly pertinent to endangered species that are maintained in fragmented isolated populations and are heavily reliant on conservation measures for population viability. Epidemiological investigations attempt to understand the roles biotic and abiotic factors have upon the transmission of pathogens to inform risk assessments and aid in the prevention and control of outbreak of disease. This study used a common commensal bacterium *Campylobacter* spp. as a model to explore host-microbial dynamics in populations of the endangered flightless takahe (*Porphyrio hochstetteri*) in New Zealand.

Human mediated movement of endangered animals through the process of translocation has developed into a commonly-used conservation tool worldwide (Fischer & Lindenmayer 2000; Griffith et al. 1989). In New Zealand, over 900 known translocation events of native terrestrial animals have occurred in the last 70 years (Sherley et al. 2010). The takahe is an endemic New Zealand flightless rail that was thought to be extinct until a small population was rediscovered in Fiordland, New Zealand in 1948 (Ballance 2001). To date a wild Fiordland population remains, with additional insurance populations located in predator-free offshore and mainland reserves (Wickes et al. 2009). Insurance populations are descendants of the original Fiordland population, and as such are actively managed to minimise the potential detrimental effects of inbreeding (Jamieson et al. 2006) by means of captive breeding and multiple translocations per annum (Grange et al. 2014). Sub-populations are geographically isolated and natural dispersal of takahe is not possible. Microbial carriage is predicted to differ between host populations maintained within a meta-population due to variation in social connectivity, the environment and life history (VanderWaal et al. 2013b).

Social network analysis of a takahe translocation database identified a complex network of sub-populations which were predicted to vary in their likelihood of maintaining and transmitting

pathogens (Grange et al. 2014). Highly connected takahe populations may have an important role in pathogen dispersal, whereas groups with fewer translocations could act as sinks or sources of exotic pathogens due to an increased possibility of allopatric speciation after a period of isolation (Grange et al. 2014). The work by Grange et al. (2014) provided a basis for an empirical investigation into the molecular epidemiology of infectious organisms in the fragmented takahe population. The aim of this study was to investigate the effects of population isolation, management, host biotic factors and environmental variation on the carriage of infectious organisms. The outcomes of epidemiological models could inform disease risk assessments for the management of fragmented endangered wildlife populations.

3.3. Materials and methods

3.3.1. Study population

Approximately half (n = 118) of the total population of takahe were opportunistically tested for faecal carriage of *Campylobacter* spp. from eight locations (Table 3-1) within New Zealand between: March and April 2012 (sampling period 1, n = 71), August and November 2012 (sampling period 2, n = 8), and February and April 2013 (sampling period 3, n = 39). Data from individual takahe, identified by Department of Conservation certified band numbers included: age, sex, nest site location, rearing method (wild, puppet (Eason & Willans 2001) or fostered) and location at time of sampling. The two variables age (9 unknown) and sex (22 unknown) were incomplete due to restricted monitoring of the wild population.

3.3.2. Sample collection

Samples were collected opportunistically during pre-translocation disease screening or annual health checks of takahe. Faecal samples were collected during handling and placed into sterile sealed containers. Sterile swabs were immediately inserted into the fresh faecal sample and stored in Aimes charcoal transport media (Copan, California, USA). All samples were transported refrigerated and stored at 4^{o}C for 1 to 7 days prior to culture and faecal DNA extraction.

3.3.3. Microbiological culture and DNA extraction

Swabs were suspended in 2ml Phosphate Buffered Saline (PBS, pH 7.3). 100µl of inoculated PBS was enriched in 2ml Bolton's broth (Lab M, Bury, England) for 48 hours at 42°C in microaerobic conditions. Bolton's broth was sub-cultured onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Fort Richard, Auckland, New Zealand) and incubated for 48 hours at 42°C in microaerobic conditions. Two colonies from each *Campylobacter* spp. positive mCCDA plate were chosen at random and sub-cultured onto Columbia horse blood agar plates (Fort Richard, Auckland, New Zealand). Plates were incubated for 24 hours at 42°C in microaerobic conditions. DNA extraction from pure cultures was conducted after 24 hours using a 2% Chelex 100 resin (BioRad, Auckland, New Zealand) suspension in sterile MilliQ water (Merck, Palmerston North, New Zealand) and boiling at 100°C for 10 minutes, followed by extraction and storage of the supernatant at -20 °C.

Faecal samples were subject to direct DNA extraction using modified stool pathogen detection protocols, outlined in the QiAmp Stool Minikit (Bio-Strategy, Auckland, New Zealand). Initial incubation was at 95°C and elution was in 100µl sterile milliQ water (Merck, Palmerston North, New Zealand). DNA was stored at -20 °C until required.

3.3.4. Molecular confirmation and speciation

Suspected *Campylobacter* spp. isolates and faecal DNA were subject to polymerase chain reaction (PCR) using previously described primers and protocols for identification of the *mapA* gene in *Campylobacter jejuni* (Mullner et al. 2010; Stucki et al. 1995) and the *ceuE* gene in *Campylobacter coli* (Denis et al. 2001; Gonzalez et al. 1997).

Samples were tested for a newly identified putative species of *Campylobacter*, named *Campylobacter* species *nova 1* (French et al. 2014). Despite extensive examination of multiple hosts, including farmed livestock and wildlife, *C.* sp. *nova 1* has only been identified in New Zealand surface water and members of the Rallidae family (French et al. 2014). Identification of *C.* sp. *nova 1* was conducted using an in house PCR that targets a short section of a putative C4-dicarboxylate trans-membrane transport gene believed to be found only in *C.* sp. *nova 1*. Forward Aot10724 5' GGTGTGTTTGCTGGTCTTGTATTGGC 3 and reverse Aot10724 5 AAATCCACTCCCCGTTT TGCGA 3' primers were designed using Geneious software v6.1 (Drummond et al. 2013), and compared to the GenBank database, where no matches were found. The expected product size was

106 base pairs. PCR conditions for *C.* sp. *nova 1* were as follows: 95°C for 2 minutes for initial denaturation, followed by 40 cycles of 94°C for 20 sec, 55°C for 20 sec and 72°C for 10 sec. The 20 µL PCR reaction mix consisted of 2µL 10x PCR buffer, 0.4µL magnesium chloride (1 mM), 1µL dNTPs (200 µM per dNTP), 2µL of each primer (4pmol), 1 unit Platinum Taq DNA polymerase (Life Technologies, Auckland, New Zealand) and 2µL DNA at 40ng.

Isolates and faecal DNA samples which tested negative for the three *Campylobacter* spp. described above were subject to a PCR targeting a region of the 16S rRNA gene for genus level confirmation of unknown *Campylobacter* spp. (Linton et al. 1997). Each PCR reaction was run with positive control identified by whole genome sequencing and a negative control of water. To confirm successful amplification, all PCR products were run on a 1% gel agarose in Tris-borate-EDTA (TBE) buffer followed by staining with ethidium bromide and visualised by exposure to UV light.

3.3.5. Prevalence of *Campylobacter* spp. in takahe

3.3.5.1. Apparent prevalence

Detection of *Campylobacter* spp. in takahe was dependent on the accuracy of test diagnostics. There was no gold standard method for culture of *Campylobacter* spp. This investigation used two methods of testing, culture and faecal DNA extraction both followed by PCR. By combining the results of the two tests, an individual was classified as positive if it was positive for either test. This method reduced the likelihood of false negatives which may be encountered during culture and selection of *Campylobacter* spp. isolates. Apparent overall prevalence estimates of *Campylobacter* spp. were calculated from the combined test outcomes in R software (R Core Team 2013) using the EpiR package (Stevenson 2014). A Venn diagram was created in R software (R Core Team 2013) using the VennDiagram package (Chen 2013) to show single and multiple carriage of *Campylobacter* spp.

3.3.5.2. Estimates of true prevalence, sensitivity and specificity of tests

Markov Chain Monte Carlo (MCMC) latent class analysis (LCA) was conducted (Appendix 9.3-1) in R software (R Core Team 2013) to generate estimates of the true prevalence, and the sensitivity and specificity of the two tests in three populations, assuming that the tests were conditionally independent. The LCA methods were based on those created for analysis of imperfect diagnostic tests in the absence of a gold standard (Branscum et al. 2005). The routine allows posterior testing for differences in prevalence estimations between populations. The apparent prevalence estimates of the

three *Campylobacter* spp.; *C. jejuni, C. coli* and *C.* sp. *nova 1* for the three management populations (wild, breeding and insurance) (Table 3-1) were used for input into the LCA model. Prior estimates of test sensitivity and specificity and estimates of prevalence were derived from expert opinion and were specified using beta distributions calculated in Betabuster software (Chun-Lung 2014). Stability of each model was tested using uninformative priors (Beta(1,1)) and posterior distributions were compared to models with informative priors. Pairwise tests for differences in prevalence of *C. coli*, *C. jejuni* and *C.* sp. *nova 1* between the three host populations were performed using a Bayesian statistical probability for estimating differences in prevalence (Pr) between populations, where values close to 0 and 1 indicate potential significant differences. Plots of true and apparent prevalence estimates with 95% confidence intervals were created in the ggplot2 package (Wickham 2009).

3.3.6. Exploratory analysis of explanatory covariates

3.3.6.1. Allocation of categorical explanatory covariates

All explanatory variables were categorical and assigned as described below and in Table 3-1. Age of the takahe was categorised as juvenile below the age of two, and adult above the age of two. Takahe sex was categorised as male, female or unknown if genetic sex identification had not been performed. Temporal trends were assessed by the comparison of three sampling periods described previously. Location and nest site variables were analysed in multiple formats according to physical location (NZ island, geography, proximity to farming) as well as assignments based on type of location management and connectivity (in degree, out degree, betweenness) (Table 3-1). New Zealand was divided into two categories based on the two major land masses of New Zealand (North Island/South). Geography (mainland/island) was classified according to the location being on the mainland of New Zealand or an offshore island. Conservation management of takahe differs between locations, with three management types being evident (breeding/wild/insurance). The wild population are free ranging in their original location, insurance populations are the translocated populations located in reserves, and the breeding centre acts as a source of birds for the insurance populations. Proximity to farming (farmed/remote) was assigned according to whether the location was directly surrounded by agriculture. An additional variable, translocation status, was measured on an individual level based on whether the animal's location at time of sampling differed from its nest site (location the takahe egg was laid).

Location *	No. of takahe	NZ island	Geography	Management	Proximity to farming	In Degree[#]	Out Degree[#]	Betweenness[#]
Burwood Bush	34	South	Mainland	Breeding	Farmed	High	High	High
Kapiti Island	0	North	Island	Insurance	Remote	NA	NA	NA
Mana Island	6	North	Island	Insurance	Remote	Low	High	Medium
Maud Island	2	South	Island	Insurance	Remote	Low	Low	Low
Maungatautari reserve	6	North	Mainland	Insurance	Farmed	Low	Low	Low
Murchison Mountains	44	South	Mainland	Wild	Remote	High	Low	Low
Private Island	10	South	Island	Insurance	Remote	Low	Low	Medium
Te Anau Reserve	2	South	Mainland	Insurance	Farmed	Low	Low	Medium
Tiritiri Matangi Island	13	North	Island	Insurance	Remote	Low	Low	Low
Willowbank Reserve	1	South	Mainland	Insurance	Farmed	Low	Low	Low

*Location refers to either location at time of sampling or nest site (where egg was laid) [#]Variable only applicable to locations where sampling of takahe occurred, terms and values are in Chapter 2

Table 3-1 Locations, number of takahe included in the analysis and corresponding categorical variables for input into multiple correspondence analysis, latent class analysis and logistic regression modelling

The effect of population connectivity on prevalence of *Campylobacter* spp. was explored using social network measures. Location weighted betweenness, in degree and out degree measures were derived from social network analyses previously conducted on a historic (2007 to 2011) takahe translocation database (Grange et al. 2014). Weighted betweenness (Opsahl et al. 2010) is a measure of centrality that represents the likelihood of a takahe population connecting two random populations via the shortest path, accounting for the sum of translocations in and out of the population. Weighted degree (Opsahl et al. 2010) was calculated from the number of direct connections between a location and its neighbour, and the sum of translocations between those connections. Degree measures can be divided into in and out measures which represent the connections into and out of a population respectively. In and out degree measures were categorised as low below a measure of 10 and high above 10. Betweenness was broken into three categories: low (0), medium (9-19) and high (78).

3.3.7. Multiple correspondence analysis

Multiple correspondence analysis was used to explore interactions between the categorical variables and the apparent prevalence of the three identified *Campylobacter* spp. MCA detects and represents underlying structures in a data set by presenting data as points in a low-dimensional Euclidean space (Greenacre 2007) and was used as an exploratory tool prior to statistical modelling. MCA and hierarchical analysis was performed in R software (R Core Team 2013) using the factoMineR and ggplot2 packages (Husson et al. 2013; Wickham 2009). Values from the first two dimensions, which explain the greatest amount of the variation in the data (Greenacre 2007) were plotted. Graphical overlapping and close proximity between data points indicates a close relationship between variables (words) and individuals (data points). Hierarchical analysis was performed on the outputs of the MCA to determine which variables contributed the most to the clustering of data points.

3.3.8. Multivariate logistic regression modelling

Explanatory variables were analysed using univariate and multivariate logistic regression in R software (R Core Team 2013). Generalised linear models with binomial errors were used to identify variables associated with the prevalence of *C. jejuni*, *C. coli* and *C.* sp. *nova 1*, unidentified *Campylobacter* spp. and multiple *Campylobacter* spp. carriage in takahe. All biologically plausible variables were included in the analysis. However, with limited data available, univariate screening was employed to reduce the number of variables included in the multivariate analyses to those which

had a more direct impact on the outcome. Explanatory variables in univariate analysis with ANOVA test Chi-squared *p*-values of <0.20 when compared to the null model were included in an initial multivariate model (Appendix S6-8). Two-way interactions between univariate explanatory variables were explored, however none met the criteria (*p*-value <0.05) to be included in the final model.

A process of model simplification was used to create a parsimonious final model with variables of significance associated with the carriage of *Campylobacter* spp. Stepwise elimination of variables of least significance was conducted based on Akaike's Information Criterion (AIC) scores. Variables were retained in the model if they showed confounding effects and improved model fit, even if they were not significant. Odds ratios and corresponding 95% confidence intervals were calculated for the categorical variables included in the final model. Significance testing between variable levels was conducted using Wald's tests. The goodness of fit was assessed using the Likelihood ratio and Hosmer-Lemeshow tests.

Two multivariate models were created for each *Campylobacter* spp. Each model contained variables identified as important from univariate analyses. One model was based on a reduced dataset containing age or sex, if they were significant in univariate ANOVA analyses. The second model used the full dataset, excluding age or sex. If the age or sex were not significant in the final model, the multivariate model using the full dataset was accepted. If age or sex were significant in the final multivariate model, the two final models were compared and the best fitting model accepted using AIC.

3.4. Results

3.4.1. Apparent prevalence of *Campylobacter* spp. in takahe

A total of 117 takahe tested positive for *Campylobacter* spp. via either culture or DNA testing of faecal material, giving an overall (based on the results of the two tests) apparent prevalence of 99.2% [95% CI, 95.4 to 100%]. Three *Campylobacter* spp. were identified: *C. jejuni* (apparent prevalence, AP, 38.1% [95% CI, 29.4 to 47.5%]), *C. coli* (AP 23.7% [95% CI, 16.4 to 32.4%]) and *C.* sp. *nova 1* (AP 89.8% [95% CI, 82.9 to 94.6%]) (Figure 3-1). *Campylobacter* spp. belonging to the *Campylobacter* genus were also detected and remain unidentified (AP 5.9% [95% CI, 2.4 to 11.8%]). Multiple carriage of *Campylobacter* spp. was detected, with just over half (50.9%) of takahe testing

positive for two or more *Campylobacter* spp. Most commonly combinations of *C.* sp. *nova 1* / *C. jejuni* (29/118) and *C.* sp. *nova 1* / *C. coli* (18/105) were detected in takahe faeces (Figure 3-2).

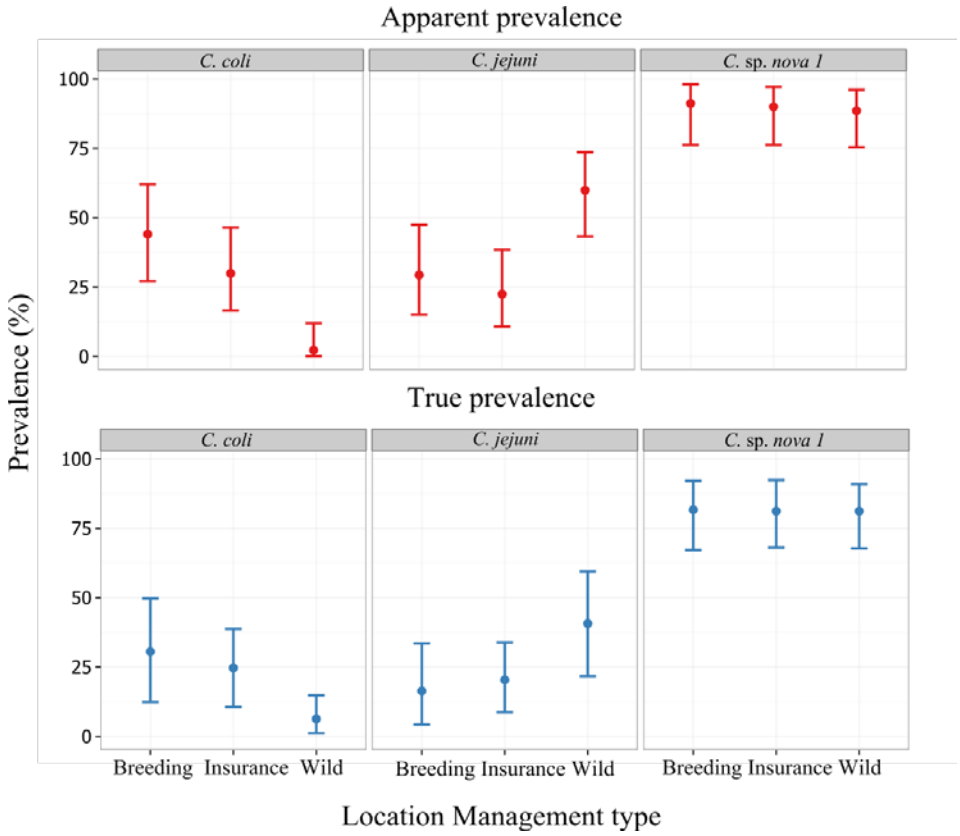

Figure 3-1 Apparent and true prevalence with 95% confidence intervals of *Campylobacter* sp. *nova 1, Campylobacter jejuni and Campylobacter coli* in populations of takahe (*Porphyrio hochstetteri*)

3.4.2. Estimates of true prevalence using imperfect tests

True prevalence estimates for the three identified *Campylobacter* spp., *C. jejuni*, *C. coli* and *C.* sp. *nova 1*, within the three takahe management subpopulations are presented in Figure 3-1. LCA model outputs including estimates of true prevalence, probability of differences in prevalence between population pairs (P), and the true sensitivity / specificity of the tests are detailed in Appendix 9.3-2. There was no significant difference in the prevalence of *C.* sp. *nova 1* in the pairwise comparisons of the breeding, wild and insurance populations. The prevalence of *C. coli* and *C. jejuni* in the wild population was significantly different from the breeding (*C. coli* P = 0.001, *C. jejuni* P = 0.02) and insurance (*C. coli* P = 0.009, *C. jejuni* P = 0.035) populations, with no significant difference observed between the breeding and insurance populations (*C. coli* P = 0.292, *C. jejuni* P = 0.334). Sensitivity and specificity for the two tests did not differ largely across the three species of *Campylobacter*; however there was some variation between the types of tests used. DNA extraction followed by PCR

was more accurate than culture for diagnostic testing, with sensitivity/specificity estimates for *C. jejuni* of 91.4/88.3%, *C. coli* 92.5/90.7% and *C.* sp. *nova 1* 97.8/88.6%. Culture followed by PCR was highly specific but less sensitive than DNA extraction with sensitivity/specificity estimates of *C. jejuni* 62.9/95.7%, *C. coli* 65.3/97.7% and *C.* sp. *nova 1* 69.1/97.8%.

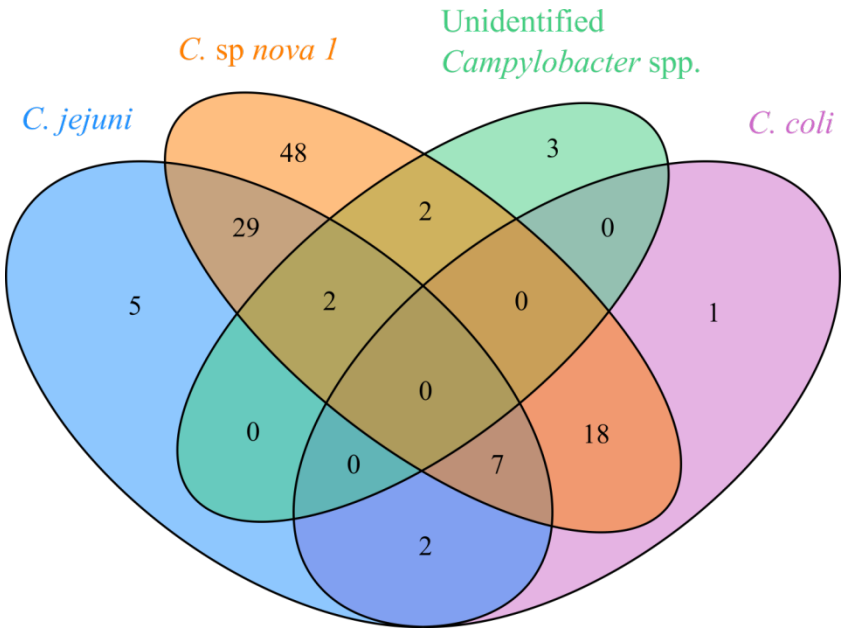

Figure 3-2 Venn diagram of *Campylobacter* spp. carriage in 117/118 takahe (*Porphyrio hochstetteri*). Numbers represent individuals positive for each *Campylobacter* species, overlapping areas represent individuals carrying combinations of *Campylobacter* spp.

3.4.3. Analysis of explanatory covariates associated with the carriage of *Campylobacter* spp.

Takahe showed some variability in covariates associated with carriage, depending on the species of *Campylobacter* concerned. When investigating individual takahe covariate patterns, three groups or clusters are evident, indicating similarities of takahe profiles within but not between the clusters (Appendix 9.3-3A). According to hierarchical analysis, population management (Appendix 9.3-3B) and betweenness (Appendix 9.3-3C) contributed most to the separation of the observations into groups. In addition, whilst carriage of *C.* sp. *nova 1* does not fall within any of these groups due to the very high prevalence, carriage of *C. jejuni* and absence of *C. coli* both fall within the wild population / low betweenness cluster, indicating some correlation between these variables (Appendix 9.3-3A). Aggregation of variables within the MCA plot indicate some co-linearity may be present (Appendix 9.3-3) and could be influential on multivariate analysis outcomes, but confounding variables were retained if they improved model fit.

The high prevalence of *C.* sp. *nova 1* and low prevalence of the unidentified *Campylobacter* spp. in takahe tested limits the analysis of variables which may be associated with the presence or absence of those organisms. Univariate and multivariate analysis found no significant association between the explanatory variables and carriage of *C.* sp. *nova 1* (Appendix 9.3-5, Table 3-2) or unidentified *Campylobacter* spp. (Appendix 9.3-7, Table 3-2).

The final multivariate model for *C. coli* was based on the reduced dataset (96 individuals) due to the significant effect of age on the carriage of *C. coli* in multivariate analyses. Juveniles were more likely (*p*-value = 0.026) to test positive for *C. coli* than adults. Takahe born in the breeding centre were significantly less likely (*p*-value = 0.04) to have *C. coli* than those born in an insurance population (Table 3-2), but there was no significant difference between the breeding and wild populations (*p*-value = 0.3). Being located within close proximity to farming was significantly associated with carriage of *C. coli* (*p*-value = 0.001) (Table 3-2).

Takahe located in the wild founder population were significantly more likely to carry *C. jejuni* (*p*-value = 0.02) than the insurance and breeding populations (*p*-value = 0.003) (Table 3-2). Although no significant differences were observed in the carriage of *C. coli* between the levels of nest site management (Table 3-2), inclusion of the variable improved model fit. Carriage of *C. jejuni* was associated with sampling period, with a significantly lower prevalence observed in the third sampling period (Feb-Apr 2013), compared with the first (Mar-Apr 2012) (*p*-value = 0.003).Multiple carriage (more than 1 species) of *Campylobacter* spp. was apparent in a subsection of the population with some strong associations with nest site. Takahe born in locations within close proximity to farming were significantly more likely to carry multiple species than those born in remote locations (*p*-value = 0.02) (Table 3-2).

Test species	Variable	Level	Coefficient (SE)	Odds ratio (95% CI)	*p*-value
**C. coli*[a]	*Intercept*	Insurance / Remote from farms / Adult	-	-	-
	Nest site	Breeding	-1.57 (0.77)	0.21 (0.04-0.86)	0.041
		Wild	-0.69 (0.72)	0.50 (0.11-1.97)	0.338
	Location	Close to farms	2.42 (0.65)	11.22 (3.41-45.55)	<0.001
	Age	Juvenile	1.39 (0.63)	4.03 (1.23-14.83)	0.026
C. jejuni[b]	*Intercept*	Period 1 (Mar-Apr 2012) / Insurance / Insurance	-	-	-
	Sampling period	Period 2 (Aug-Nov 2012)	0.23 (0.86)	0.43 (0.15-1.11)	0.787
		Period 3 (Feb-Apr 2013)	-1.59 (0.53)	1.26 (0.21-6.77)	0.003
	Nest site	Breeding	1.07 (0.75)	0.20 (0.06-0.55)	0.153
		Wild	-0.32 (0.77)	2.92 (0.69-13.56)	0.683
	Location	Breeding	-0.35 (0.76)	0.73 (0.15-3.32)	0.642
		Wild	1.95 (0.81)	7.02 (1.49-37.33)	0.016
C. sp. *nova 1*[c]	*Intercept*	Remote from farms	-	-	-
	Nest site	Close to farms	-1.42 (1.07)	0.24 (0.01-1.33)	0.183
Unidentified *Campylobacter* spp.[d]	*Intercept*	No / High	-	-	-
	Translocated	Yes	1.17 (0.80)	3.22 (0.66-17.41)	0.145
	In degree	Low	1.09 (0.80)	2.97 (0.61-16.09)	0.175
Multiple carriage[c]	*Intercept*	Remote from farms	-	-	-
	Nest site	Close to farms	0.45 (1.08)	2.93 (1.24-7.41)	0.018

*model based on a smaller dataset due to 22 missing data for age
Likelihood ratio test: [a] p <0.001 d.f = 4, [b] p <0.001 d.f = 6, [c] p=0.113 d.f =1, [d] p=0.148 d.f = 2, [e] p = 0.01 d.f = 1

Table 3-2 Multivariate generalised linear model showing variables of significance for the carriage of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter* species *nova 1* in takahe (*Porphyrio hochstetteri*) faeces.

3.5. Discussion

Exploration of host-microbial relationships and transmission within an intensively managed population is required to understand the epidemiology of infectious organisms in fragmented populations, and to assess the disease risks associated with the fragmentation and translocation of endangered wildlife. Modelling the prevalence of commensal *Campylobacter* spp. within highly managed takahe populations, indicates that anthropogenic management of endangered species within altered environments may have unforeseen effects on microbial exposure and carriage. The results of this study suggest that the transmission dynamics and carriage of bacterial species is heterogeneous between populations. The prevalence of enteric *Campylobacter* spp. in takahe differed according to a number of population and environmental variables. *C.* sp. *nova 1* showed strong host-commensal relationships, whilst the prevalence of *C. jejuni* and *C. coli* were associated with variables relating to age, management and environmental exposures of the host. Whilst modelling of a commensal may not directly simulate the dynamics of a pathogen within fragmented populations due to the lack of pathogenicity and recovery of the host, it has been a good proxy to explore how anthropogenic changes in host population dynamics can influence microbial carriage. The consequences of management-associated influences on host-microbial relationships observed in this study are difficult to predict, but may result in increased exposure to pathogens of significance to wildlife health.

Forced perturbation of populations through frequent translocation of takahe between the geographically isolated locations (Grange et al. 2014), for reasons of conservation and inbreeding (Jamieson et al. 2006) provides a means of population-wide mixing of birds where subpopulation barriers are bypassed. The high prevalence of *C.* sp. *nova 1* in all takahe populations supports the hypothesis of dynamic mixing of infectious organisms irrespective of sub-population biotic and abiotic factors. Despite extensive examination of multiple hosts, including farmed livestock and wildlife, *C.* sp. *nova 1* has only been identified in New Zealand surface water and members of the *Rallidae* family (French et al. 2014). Extant takahe originate from a small population in the Murchison Mountains (Ballance 2001). If *C.* sp. *nova 1* has had a long history of takahe colonisation, niche adaptations to the host may have allowed survival, persistence and transmission of the species through the generations, resulting in the current high prevalence in extant populations. *C.* sp. *nova 1* is newly described, and while the identification methods used in this investigation are unable to observe

differences between isolates, future in depth analyses may identify differences between these isolates (Chapter 4).

Differential carriage of *C. coli* and *C. jejuni* between takahe sub-populations supports the theoretical hypothesis that the takahe sub-populations would vary in their propensity to maintain and transmit infectious organisms (Grange et al. 2014). Depending on the study species and ecosystem dynamics involved, translocated or artificially reared animals may be at an advantage in terms of host parasitism compared to their home range. The parasite release hypothesis suggests that animals may escape native pathogens when moved to a new location (Torchin et al. 2003). Reintroduced native species are selected for good health prior to transfer, consequently disease circulating in this select group may be lower than the source population (Almberg et al. 2012). Many pathogens have complex life cycles requiring more than one host and thus if this host is not present in the new location, the pathogen may not be maintained. Translocated populations of takahe may have escaped from micro-organisms present in the founding population, through random selection of microbe free individuals or the absence of reservoirs in the new environment. Either hypothesis could explain the higher prevalence of *C. jejuni* in the wild Murchison Mountain population.

Translocated populations are expected to acquire pathogens circulating within the environment (Almberg et al. 2012), but it is thought to occur at a slower rate than what they would be exposed to in their original geographic range (Torchin et al. 2003; Torchin & Mitchell 2004). Translocated populations could acquire novel pathogens via spill-over from alternative hosts within the new environment (Tompkins et al. 2011). Contrary to the traditional dogma, domestic animals living in close proximity to wildlife could be a reservoir of exotic pathogens to native species, potentially contributing to their decline (Besser et al. 2013; Besser et al. 2012a; Besser et al. 2012b).

C. coli is frequently isolated from faecal material derived from avian populations whom exhibit no obvious clinical signs of disease (Benskin et al. 2009). Many *C. coli* sequence types are epidemiologically associated with livestock, including pigs and chickens (Kittl et al. 2013). Campylobacter spp. are able to survive in the environment (Bull et al. 2006), thus livestock-associated *C. coli* may have been transmitted from livestock to takahe populations in areas associated with farmland, via faecal contamination of shared resources and/or other wildlife passing between farms and takahe locations. This transmission theory could also explain the higher diversity of

Campylobacter spp. associated with takahe born in populations close to farmland. However, carriage of multiple species is not necessarily an indication of being a persistent host to multiple species, as some infections may be transient due to temporal patterns of shedding (Colles et al. 2009).

Early exposure appears associated with microbial carriage in takahe, with a higher prevalence of *C. coli* reported in juveniles than adults. Similar findings were observed in a study of *C. jejuni* in European starlings (*Sturnus vulgaris*) where shedding of the bacteria was higher in young birds (Colles et al. 2009). Carriage of *C. coli* in juveniles is unlikely to be attributable to differences in behaviour, as juvenile takahe shadow adults for up to two years prior to independence (Maxwell & Jamieson 1997). Juvenile takahe may be more susceptible to infection due to the lack of acquired immunity. However, it has been recognised that further investigations are required to understand the role immunity may play in carriage of *Campylobacter* spp. in wild birds (Waldenstrom & Griekspoor 2014). In accordance with other studies of *Campylobacter* spp. in wild avian populations (Colles et al. 2009; Waldenstrom et al. 2002), sex was not significantly associated with carriage of *Campylobacter* spp. in takahe. A common outcome of multivariate modelling in this study was the involvement of nest environment in explaining the carriage of *Campylobacter* in takahe. Although the mechanisms by which early rearing environment affect the gastrointestinal microbiome of takahe is unclear, there appears to be an association between nest location attributes and the carriage of *Campylobacter* spp. later in life. In the early days of takahe recovery management, cross-fostering was used where a pukeko (*Porphyrio porphyrio)* foster mother was trained to rear foster takahe chicks (Bunin & Jamieson 1996). Although the two species are closely related, this practice could have unwittingly introduced exotic micro-organisms to the takahe population. However, the rearing method of an individual was not significantly associated with the carriage of *Campylobacter* spp.

Although *Campylobacter* spp. are not thought to be pathogenic to takahe, exposure to non-native *Campylobacter* spp. may result in reduction of fitness (Waldenstrom et al. 2010). Several unidentified *Campylobacter* spp. were isolated from takahe, the pathogenicity of which remains unknown. Translocated individuals and isolated populations with few immigrants are potentially more likely to carry unidentified *Campylobacter* spp. Two hypotheses could explain this association; translocated populations may have gained *Campylobacter* spp. from novel reservoirs, and / or *Campylobacter* spp. may have evolved following isolation of takahe.

Limitations in the interpretation of the findings of this study are evident due to the limited sample size, missing information, imperfect tests and potential confounding variables. For example, the breeding and wild subpopulations were comprised of single locations, whereas the insurance group includes many sites. Therefore, populations may be confounded by location specific attributes which were beyond the scope of the study. It is likely that a combination of biotic and abiotic factors explains the observed carriage of different species of *Campylobacter* in takahe in this study. Temporal and seasonal trends in the carriage of *Campylobacter* spp. in wild birds have been reported (Waldenstrom & Griekspoor 2014). Although this study found some associations with the time period samples were collected, the opportunistic design and confounding variables, such as sampling certain locations at a certain time of year, prevents a true assessment of temporal trends in takahe.

Intensive conservation management of endangered species in fragmented populations can influence host-microbial relationships. The disease risk posed to translocated populations of takahe may be determined by the choice of habitat in which they are maintained. Implementing buffer zones around reserves is recommended to reduce the risk of pathogen transmission from reservoir species to endangered wildlife. Additionally, relatively unmanaged populations appear to have different microbial carriage compared to captive populations. Therefore, the movement of animals between these populations may transmit pathogens to naive individuals, and thus would not be advised. This study has provided a good basis for further investigation of microbial dynamics in translocated populations and has the potential to inform risk assessments and aid in our understanding of the epidemiology of infectious disease in wildlife.

3.6. Acknowledgements

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3.7. Supporting information

R script for LCA in diagnostic testing (Appendix 9.3-1), detailed LCA model outputs (Appendix 9.3-2), MCA plots (Appendix 9.3-3), and univariate *Campylobacter* spp. model results (Appendix 9.3-4, 5, 6, 7, 8) are available in the appendix.

CHAPTER 4

WILDLIFE TRANSLOCATION AND THE EVOLUTION AND POPULATION STRUCTURE OF A HOST-ASSOCIATED COMMENSAL *CAMPYLOBACTER* SPP.

4. Wildlife translocation and the evolution and population structure of a host-associated commensal *Campylobacter* spp.

4.1. Abstract

There is an increasing need for the conservation management of threatened wildlife, and yet we have a limited understanding of the effects of tools such as translocation has upon pathogen transmission and disease ecology. Our ability to predict and control outbreaks of infectious disease is hampered by the complex interactions between hosts, pathogens and the environment. The use of commensal bacteria as a proxy for invasive pathogens can help our understanding of some of the epidemiological features of infectious diseases, such as microbial transmission and evolution in natural systems. The genomic structure of a prevalent rail-associated endemic bacterium, *Campylobacter* sp. *nova 1,* was explored in a well-described population of endangered takahe (*Porphyrio hochstetteri*). The distinctive population structure of translocated takahe provides a unique opportunity to investigate the influence of host isolation on enteric microbial diversity. Whole genome sequencing and ribosomal multi-locus sequence typing (rMLST) was carried out on *C.* sp. *nova 1* isolated from one third of the takahe population from multiple locations. *C.* sp. *nova 1* was genomically diverse and multivariate analysis of 52 rMLST alleles revealed location-associated differentiation of *C.* sp. *nova 1* sequence types. While there was evidence of recombination between lineages, bacterial divergence appears to have occurred in the face of host range expansion and isolation. Anthropogenic management of wildlife in highly connected locations such as a breeding centre may create artificial opportunities for exposure and mixing of a broad selection of genotypes. These results support the idea that restricted gene flow in fragmented populations affects the genomic differentiation of microbes. Subtle but important differences in host-microbe relationships as a consequence of management may result in the emergence of pathogens and have important implications when relocating allopatric wildlife populations.

4.2. Introduction

Emerging infectious diseases (EIDs) in wildlife are of increasing concern due to their direct impact on biodiversity and the risk of zoonotic transmission (Daszak et al. 2000; Jones et al. 2008), with over half (54%) of human EID events attributable to bacterial pathogens of zoonotic origin (Jones et al. 2008). Effective management and mitigation of infectious wildlife disease requires an understanding of both biotic and abiotic factors that drive pathogen transmission and persistence within ecosystems. Although infectious disease is rarely the sole driver of extinction (Heard et al. 2013; Smith et al. 2006), it may contribute to local population declines (Berger et al. 1998; Fisher et al. 2012), affect the success of reintroduction programmes (Almberg et al. 2012) and thus the prospect of long term species survival. Our understanding of the epidemiology of infectious diseases in wildlife is often limited by the complex interactions between hosts, pathogens and the environment, and difficulties often encountered when surveying wildlife populations.

Epidemics of invasive pathogens have had significant impacts on wildlife. The spread of chytrid fungus (*Batrachochytrium dendrobatidis*) has been associated with high host mortality or morbidity in amphibians worldwide (Rachowicz et al. 2006; Skerratt et al. 2007). Studies of host-pathogen relationships during an epidemic require an *in situ* outbreak situation. Not only is this a rare occurrence but there are several logistic difficulties associated with studying uncontrolled and unknown pathogens, as exemplified by the 2014 pandemic of Ebola virus in West Africa (Kupferschmidt 2014). Endemic pathogens circulating in a population may regulate host populations but could be more difficult to detect and characterise. An alternative is to study the transmission and population biology of a common host-associated commensal in natural ecosystems as a proxy for an epidemic pathogen (Bull et al. 2012; Chapter 3 ; Chiyo et al. 2014). Such an approach allows key epidemiological and ecological features, such as microbial transmission and evolution to be studied in the absence of disease. Additionally, a commensal in one host may be a pathogen in another (Waldenstrom et al. 2010), which has implications when reconnecting allopatric species through wildlife conservation practices (Cunningham 1996; Daszak et al. 2000).

Heterogeneous social (Bull et al. 2012; Porphyre et al. 2011; VanderWaal et al. 2013b) and meta-population dynamics (Cowled et al. 2012; Guivier et al. 2011) present in natural populations have

been shown to affect microbial diversity and transmission of infectious agents. However, much of our knowledge is based upon theoretical evidence. Robust empirical epidemiological studies of pathogen dynamics in multiple populations require a high prevalence, strong host association and a well-described host population structure. Endangered wild animals are often maintained in relatively small and fragmented sub-populations within highly altered environments. These well-documented populations provide ideal model systems for examining factors contributing to the transmission and population biology of microorganisms including enteric pathogens and commensals.

The model system under investigation in this study captures the carriage, transmission, population structure and evolution of a prevalent, host-associated enteric *Campylobacter* species *nova 1* in takahe (*Porphyrio hochstetteri*). Takahe are an endangered flightless rail endemic to New Zealand (BirdLife International 2013) with a population of approximately 230 individuals (Wickes et al. 2009). The historic translocation of birds from the one remaining wild population in the Murchison Mountains, New Zealand (Ballance 2001) to multiple wildlife sanctuaries has created a network of small sub-populations (Grange et al. 2014). Although fragmented, takahe populations are connected through well-described translocation networks, allowing the effects of within and between microbial population dynamics to be considered (Grange et al. 2014). Social network models have indicated the presence of a dynamic network of sink, source and hub populations with implications for pathogen carriage, transmission and evolution (Grange et al. 2014).

A prior investigation showed that takahe sub-population attributes including management and proximity to agriculture influenced faecal carriage of commensal *Campylobacter jejuni* and *Campylobacter coli* respectively (Chapter 3). The study examined prevalence at the *Campylobacter* species level but lacked the resolution required to explore the microbial population structure of more prevalent *Campylobacter* spp. such as *Campylobacter* species *nova 1*, a proposed novel *Campylobacter* spp. (French et al. 2014) which was isolated from 90% of takahe tested (Chapter 3).

In the first study of its kind, high resolution genotyping was used to model the effects of host population history and spatial characteristics of the environment on the population structure of an enteric microorganism in a well-characterised host population.

4.3. Methods

4.3.1. Sample collection and culture

Cloacal swabs were opportunistically collected from takahe resident in 6 locations in New Zealand (Figure 4-1) during pre-translocation disease screening or annual health checks between November 2011 and April 2013. Samples were stored in Aimes Charcoal transport media (Copan, California, USA) and refrigerated at 4^{o}C for up to 7 days prior to laboratory processing. Previously described protocols were used for microbial culture and extraction of *Campylobacter* spp. DNA for PCR identification of *C.* sp. *nova 1* positive isolates (Chapter 3). This PCR targets a short section of a putative C4-dicarboxylate trans-membrane transport gene believed to be found only in *C.* sp. *nova 1* (Chapter 3).

4.3.2. Selection of *C.* sp. *nova 1* for genomic sequencing

A selection of 70 *C.* sp. *nova 1* isolates were chosen for comparative whole genome sequencing. These isolates originated from 69 (plus 1 repeat) takahe spread between six locations in New Zealand: Burwood Bush breeding centre (n = 22), Murchison Mountains (n = 25), Mana Island (n = 1), Maud Island (n = 4), Maungatautari reserve (n = 4) and a private island reserve which is undisclosed due to confidentiality restrictions (n = 14) (Figure 4-1, Appendix 9.4-1). One takahe moved from Maungatautari reserve to Burwood Bush during the study and isolates from both locations were analysed.

4.3.3. Genomic DNA preparation and processing

Pure cultures were recovered from glycerol broth stored at -80^{o}C and grown in micro-aerobic conditions at 42^{o}C on Columbia Horse Blood agar (Fort Richard, Auckland, New Zealand). DNA was extracted using the Qiagen DNeasy blood and tissue kit (Bio-Strategy, Auckland, New Zealand). Protocols were followed according to the manufacturer's instructions for gram negative bacteria with the final elution step modified to 200µl sterile milliQ water (Merck, Palmerston North, New Zealand). DNA was checked for quality using Qubit dsDNA HS/RNA/Protein assay kits (Life Technologies, Auckland, New Zealand), and stored at -20 ^{o}C prior to sequencing at the New Zealand Genomics Ltd.

Genomes were sequenced using an Illumina MiSeq instrument (Illumina, Victoria, Australia) according to the manufacturer's instructions with paired read lengths each of 250 base pairs. DNA samples were fragmented by nebulisation for 6 minutes at a pressure of 32 psi, purified, then end repaired, A-tailed, adaptor-ligated, fractionated, purified and enriched according to the manufacturer's instructions, using the TruSeq DNA LT Sample Prep Kit v2-Set A and B (Illumina, Victoria, Australia). The prepared libraries were normalized to equal molarity, diluted to 2nM and pooled in libraries of 20 samples. A flow cell was prepared for each pool and sequencing reactions using 9 pmoles of the pooled libraries were performed with the MiSeq Reagent Kit v2 (Illumina, Victoria, Australia) to give approximately 12 to 15 million clusters per run.

4.3.4. Genome assembly, curation and annotation

The algorithm package Velvet (version 1.2.10) (Zerbino & Birney 2008) was used for *de novo* genome assembly and alignment. First, short reads sequences of 250 base pairs were broken into smaller sequences (k-mers). Then, a de Bruijn graph was constructed from those short sequences. The sequences were assembled across a range of k-mer lengths in increments of 10 between 215 and 65 base pairs. The resulting Velvet contiguous sequences (contigs) were stored in a MySQL (version 5.6) database. The best assembly per genome was chosen using a Perl-based in-house ranking system using a score derived from the number of contigs, size of contigs, assembly length and the N_{50} score. Concatenated contigs within an assembly were annotated with the program Prokka (version 1.9) (Seemann 2014). Predicted genes from the annotated genomes were then clustered using OrthoMCL (version 2.09) (Li et al. 2003) and subsequent in-house parsing.

4.3.5. Ribosomal multi locus sequence typing (rMLST) of *C.* sp. *nova 1*

Nucleotide sequences for 52 of the 53 genes (the order *Campylobacterales* does not possess the *rpm*D gene) encoding bacterial ribosomal protein subunits (*rps*) used for rMLST (Jolley et al. 2012), were identified and extracted from the assembled genomes primarily on annotation, but also using a *C. jejuni* reference set of rMLST genes as a database for a BLAST search. Using custom Perl scripts, unique alleles for each gene were defined based on their nucleotide sequence. Each genome was assigned a custom sequence type number defined by the allelic profile of the 52 gene numerical

combination. Each individual gene was aligned using Muscle (Edgar 2004), and these were concatenated to make a single alignment per genome. Both allelic profile data and concatenated rMLST gene sequences were used in subsequent analyses.

Three distance matrices were created from pairwise comparison of 52 gene rMLST profiles of the 70 *C.* sp*, nova 1* genomes (Appendix 9.4-1). A haplotype distance matrix was constructed from the 52 rMLST allelic dataset using the GenAIEx Microsoft Excel add on package (Peakall & Smouse 2012). Two pairwise nucleotide distance matrices were created using uncorrected P measure and general time reversible (GTR) in SplitsTree4 (Huson & Bryant 2006). The uncorrected P measure was obtained by dividing the number of single nucleotide differences by the total number of nucleotides compared between two gene sequences. The GTR model accounts for nucleotide substitution where it assumes a symmetric substitution between bases, for example C change into G at the same rate that C changes into C. Additionally, the GTR model allows for input of variable base frequencies. Values of: A: 35%, C: 15%, G: 15% and T: 35%, were used as this was a good approximation for the GC content of *C.* sp. *nova 1*.

4.3.6. Core genome and rMLST tree construction

A selection of publicly available *Campylobacter* spp. whole genome sequences were downloaded from the NCBI database (Benson et al. 2009; Sayers et al. 2009) (Appendix 9.4-2) and were subject to the methods previously described for the extraction and processing of rMLST sequences. NeighborNet trees of the published *Campylobacter* spp. rMLST sequences and the 70 *C.* sp. *nova 1* isolates were visualised in SplitsTree4 (Huson & Bryant 2006). The tree was constructed from a pairwise comparison of genomic distance using uncorrected P measures (Appendix 9.4-1).

Amino acid sequences of orthologous genes, those which were inherited vertically from a common ancestor, were used for core genome comparison of the 70 *C.* sp. *nova 1* isolates. This was implemented using a custom Perl script and clustered using OrthoMCL version 2.0.9 (Li et al. 2003). A NeighborNet tree was created from a distance matrix of uncorrected P distances based on pairwise comparison of core gene amino acid sequences of the same length, excluding genes of different length. The tree was then visualised in SplitsTree4 (Huson & Bryant 2006).

4.3.7. Multivariate analysis of the relationship between location factors and genetic distance

The three matrices were explored using multidimensional scaling (MDS) followed by permutational multivariate analysis of variance (PERMANOVA) within Primer v6 software (Clarke 1993). Factors used in the PERMANOVA model included sample site and takahe nest site, as well as co-factors relating to location geography (North vs South Island, Island vs Mainland) and management (Wild vs Captive breeding and insurance, Captive breeding vs Insurance). The term insurance is defined as sub-populations maintained in free to roam reserves external to the founding wild population. PERMANOVA models were created with 9,999 permutations using a method which allows unrestricted permutation of raw data. Non-significant factors and interactions were removed in a stepwise manner until the final model containing significant factors and corresponding cofactors remained.

Overall and pairwise genetic distances between *C.* sp. *nova 1* allelic profiles were examined in relation to significant factors identified from the PERMANOVA analysis. Fixation index (F_{ST}) values, a measure of population differentiation due to genetic structure, were calculated using analysis of molecular variance (AMOVA) methods within Arlequin software (Excoffier & Lischer 2010). Pairwise F_{ST} values were visualised in SplitsTree4 (Huson & Bryant 2006).

4.4. Results

4.4.1. *C.* sp. *nova 1* comparison to published *Campylobacter* spp.

All 70 *C.* sp. *nova 1* isolates formed a distinct cluster, separate from other published *Campylobacter* spp. included in this study (Figure 4-2). rMLST gene comparison of the *Campylobacter* spp. genomes correlates with previous investigations (French et al. 2014) where *C.* sp. *nova 1* was closely related to *Campylobacter coli* and *Campylobacter jejuni* but was genomically distinct.

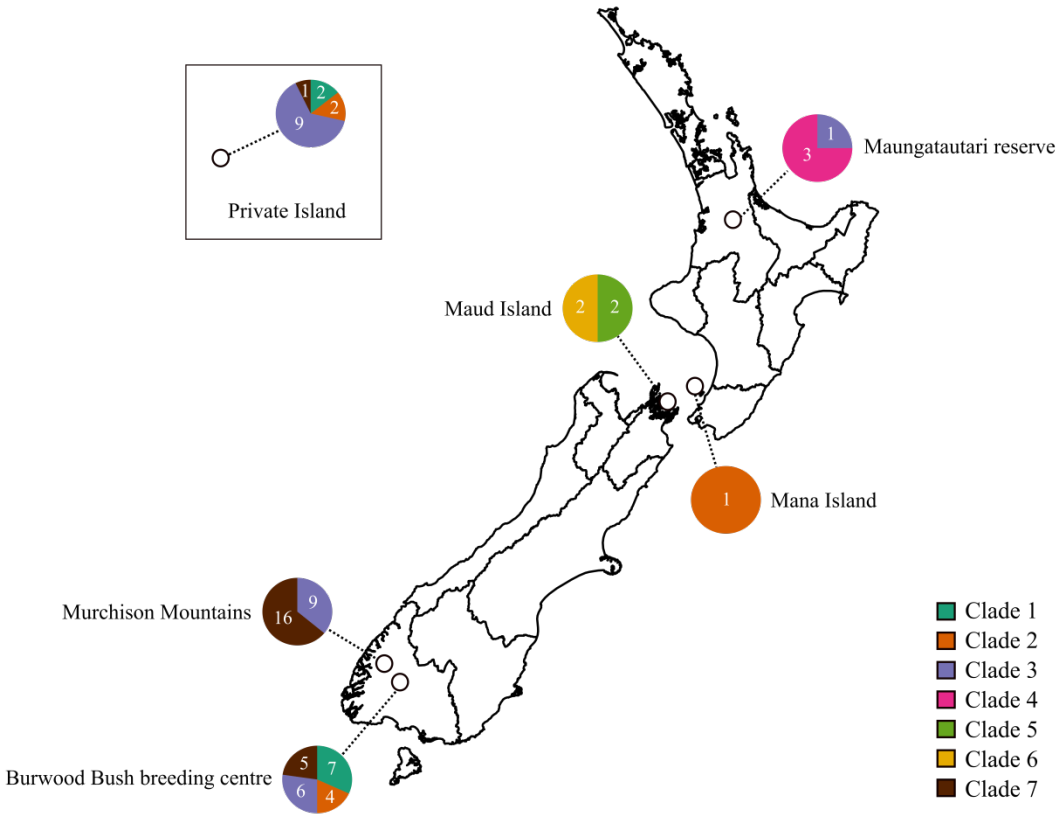

Figure 4-1 Map of takahe (*Porphyrio hochstetteri*) sampling locations in New Zealand. The private island location is undisclosed due to confidentiality restrictions. Pie charts represent the number and clade types, see Figure 4-3, of *Campylobacter* sp. *nova 1* isolated from each location.

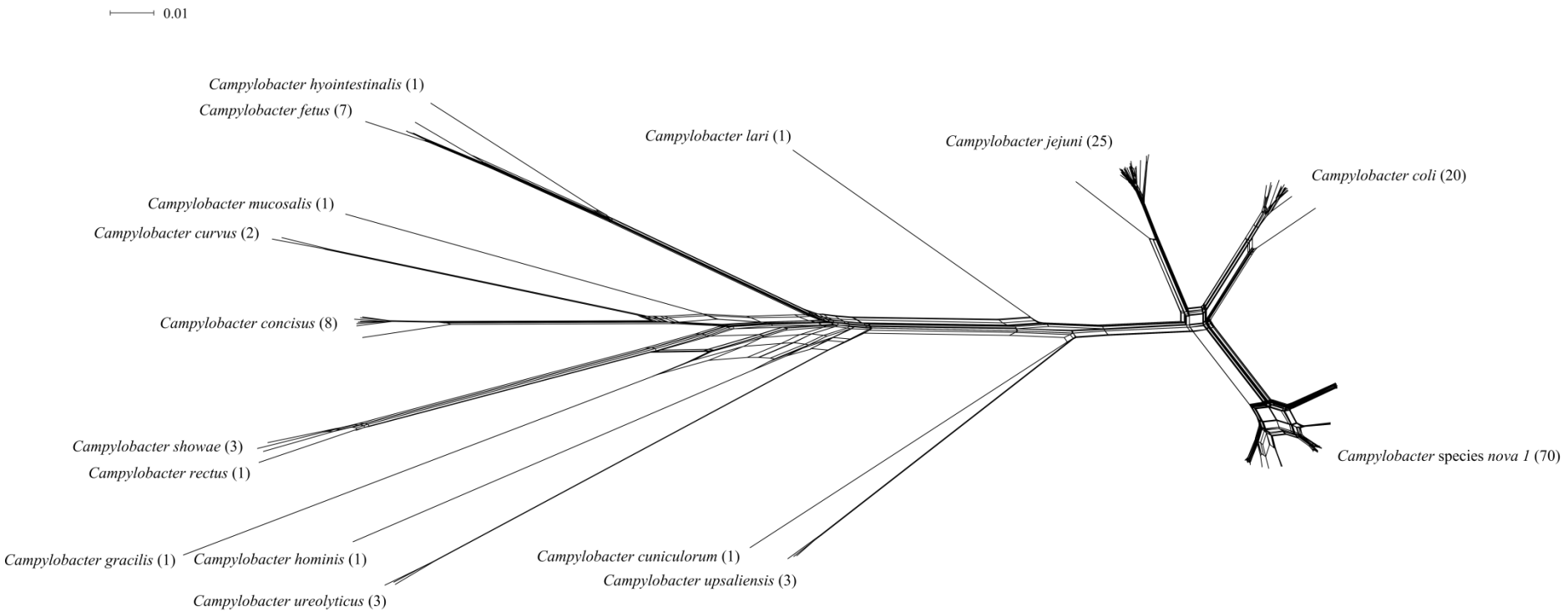

Figure 4-2 NeighborNet tree based on pairwise comparison of rMLST nucleotide sequences of *Campylobacter* sp. *nova 1* isolated from takahe (*Porphyrio hochstetteri*) and a selection of publicly available *Campylobacter* spp.

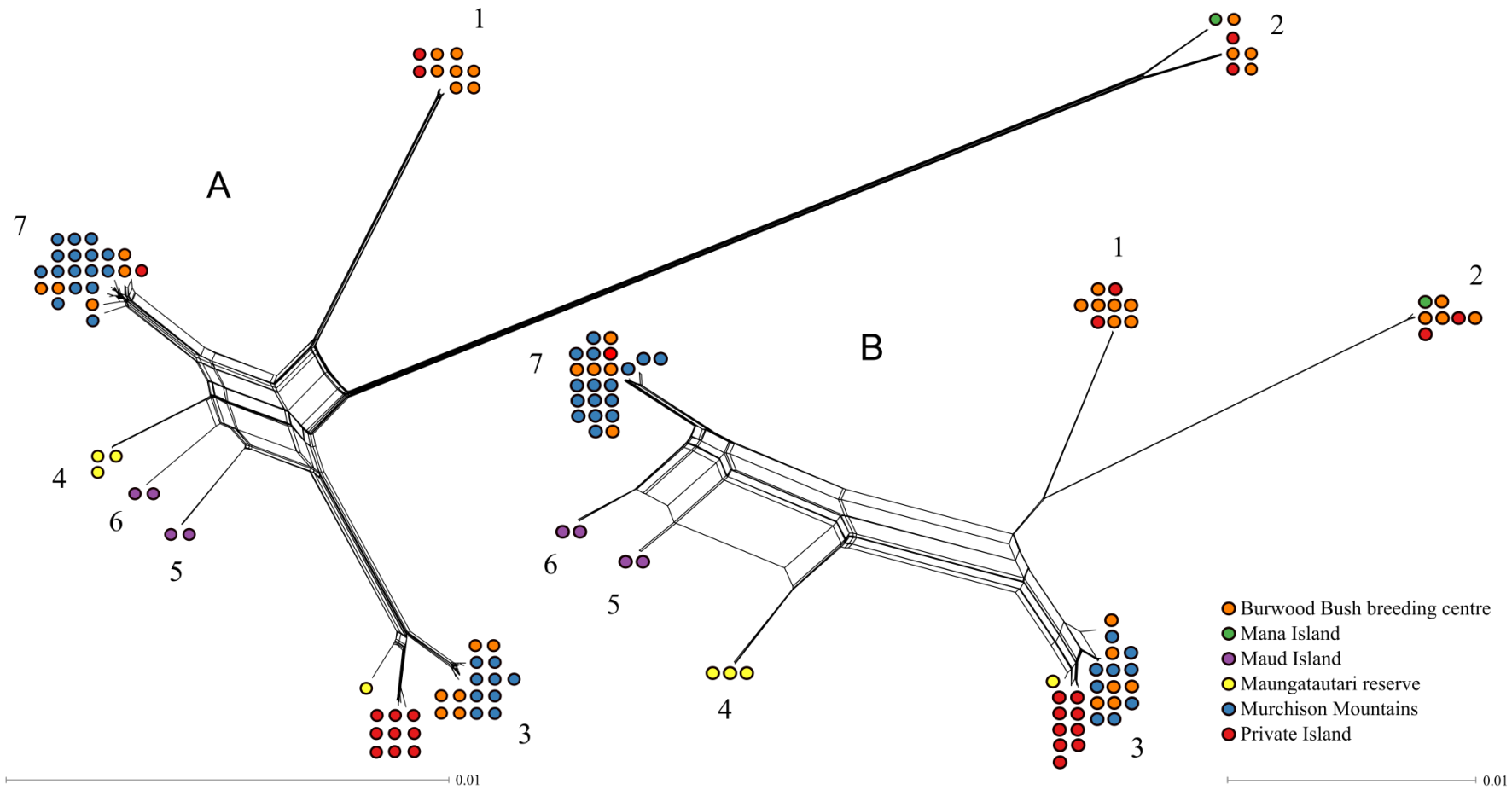

Figure 4-3 NeighborNet trees of 70 *Campylobacter* sp. *nova 1* using unmeasured P distances of a) amino acid core genome comparisons and b) nucleotide comparison of 52 rMLST genes. Dots represent individual *C.* sp. *nova 1* genomes isolated from takahe (*Porphyrio hochstetteri*), coloured by location. Numbers were assigned to each distinct clade on a branch of the tree.

4.4.2. Genomic differentiation of *C.* sp. *nova 1* isolates

Visualisation of nucleotide differences between isolates of *C.* sp. *nova 1* isolates using pairwise uncorrected P distances of the 52 rMLST genes and core genome illustrates fine scale genomic diversity among the isolates (Figure 4-3). Seven clades were observed using both methods, with evidence of genetic recombination between isolates revealed by the net-like structure in the centre of the star shaped tree. These structures represent uncertainty of the rMLST gene ancestry which is characteristic of recombination. The positions of isolates on the tree suggested a correlation between clade and the location of the host from which the bacterium was isolated (Figure 4-3). However, sequence types isolated from Burwood Bush breeding centre are genomically diverse with representatives from four different clades being isolated from takahe within this location.

4.4.3. Multivariate analysis of *C.* sp. *nova 1* rMLST allelic profiles

Initial exploration of *C.* sp. *nova 1* rMLST allelic profiles using multidimensional scaling (MDS) (Appendix 9.4-3) identified three clusters according to similarity in their allelic profile. Large distances were evident between clusters, with some defined grouping of isolates structured within a cluster, particularly within cluster two. Clusters one and three correlated well to clades one and two defined within the NeighborNet tree, whilst MDS cluster number 2 was comprised of all remaining NeighborNet clades (Appendix 9.4-3, Figure 4-3).

Variables relating to sample and host nest location, including co-factors defined by location geography and management, were explored in association with rMLST allelic profiles (Table 4-1), unmeasured P (Appendix 9.4-4) and GTR distance matrices (Appendix 9.4-5) using PERMANOVA, with final models containing only significant factors. All three methods provided similar results with location being a significant factor (P(perm) = 0.0001) explaining the genetic distance observed in pairwise comparisons of rMLST profiles. Genomic differentiation of *C.* sp. *nova 1* according to location may be attributable to co-factors relating to management or historic isolation, with *C.* sp. *nova 1* isolated from the wild being different from those in the breeding and insurance populations (P(perm) = 0.001) (Table 4-1). The sample size did not provide sufficient statistical power to explore interaction terms between sampling location and nest site. Additionally there was no significant

contribution of nest location or its corresponding co-factors on the observed genomic structuring of *C.* sp. *nova 1*.

Factors	**d.f**	**SS**	**Est. of variance**	***Pseudo*-F**	**P(perm) values**	**Perm**
Sampling location	5	9859.9	142.67	3.792	0.0001	9913
Cofactors						
North vs South Island	1	1101.7	52.062	1.7819	0.1211	9149
Wild vs Captive breeding & Insurance	1	3204.4	81.42	5.4559	0.0014	9814
Captive breeding vs Insurance	1	1714.1	45.236	2.46	0.0486	9938
Island vs Mainland	1	1520.1	32.798	2.4835	0.0525	9953
Residuals	64	33283	520.04			
Total	69	43143				

Table 4-1 Reduced PERMANOVA model from 9,999 permutations (perm) containing significant factors and cofactors associated with rMLST allelic profiles of *Campylobacter* sp. *nova 1*. The cofactor insurance is defined as sub-populations maintained in free to roam reserves external to the founding wild population.

Support for strong genetic differentiation of populations according to location was observed with an F_{ST} value of 0.22 ($P < 0.01$), where most variation was within location defined populations (77.8%, d.f. 64, SS 822.75), with the remaining explained among populations (22.2%, d.f. 5, SS 251.37). Pairwise comparison of location F_{ST} values identified some heterogeneity in the genetic distance of isolate populations between locations (Figure 4-4). Branch length and position on the tree correlate with genetic distance. The star-like structure and long branch lengths would suggest location was strongly associated with bacterial divergence. Notably, Burwood Bush breeding centre was placed in the centre of the tree with no apparent branch. Thus isolates derived from this location were less distinct from isolates in other locations included in the analysis, compared to all other pairwise comparisons.

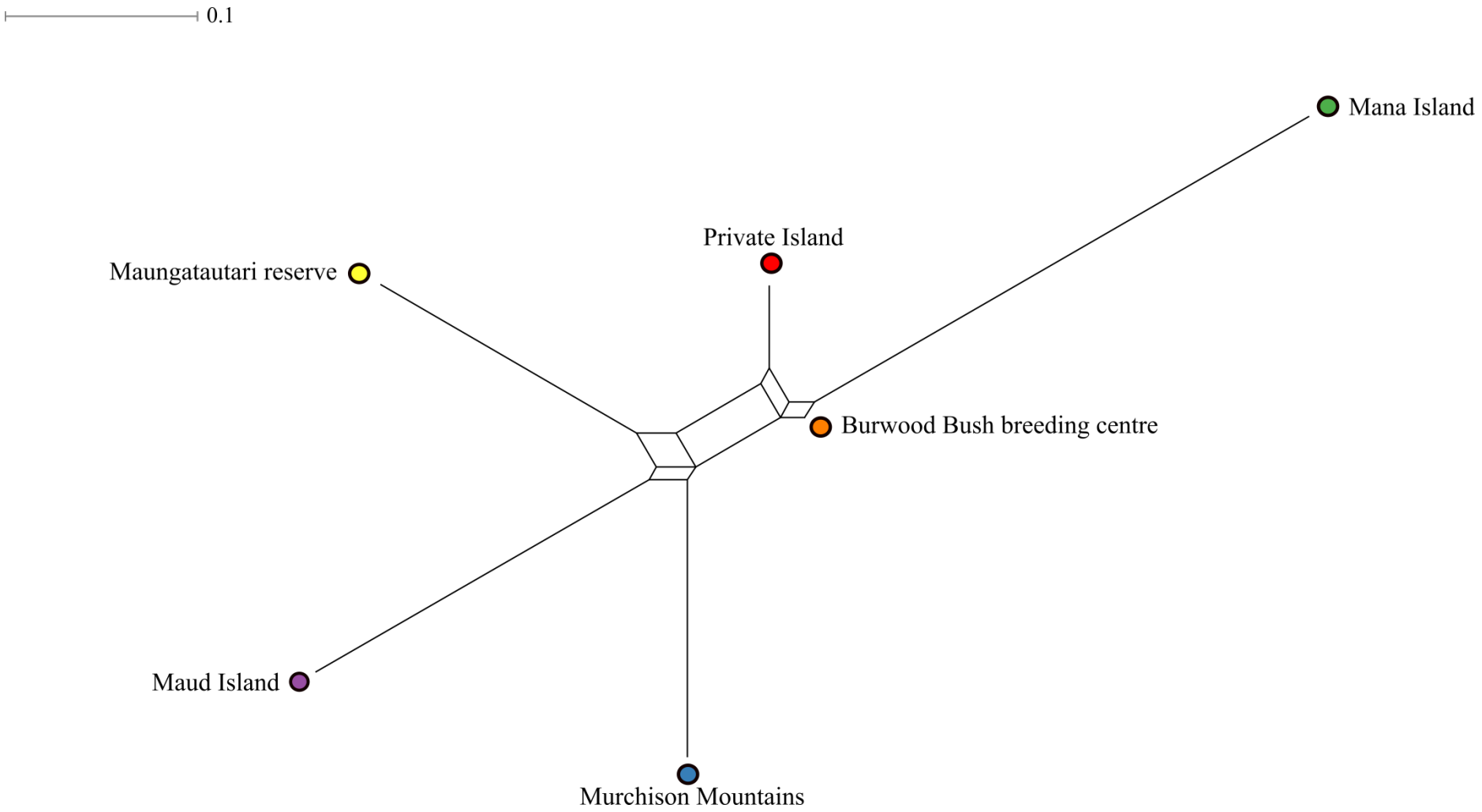

Figure 4-4 F_{ST} tree based on pairwise comparison of 70 rMLST profiles of *Campylobacter* sp. *nova 1* isolated from takahe (*Porphyrio hochstetteri*) populations.

4.5. Discussion

Determining the impacts of host meta-population dynamics on bacterial spread and maintenance in a population has been a key focus of epidemiological studies. The emergence of robust methods integrating survey and genomic approaches allow complex analyses of the relationships between host-associated variables and bacterial genotype (Biek et al. 2012; Girard et al. 2004). This study provides evidence of spatial clustering of bacterial genotypes of a commensal bacterium, *C.* sp. *nova 1*, carried by endangered populations of takahe. Microbial genotypes and rMLST allelic profiles were strongly associated with geographic location, with population isolation and environmental factors potentially contributing to the observed diversity of *C.* sp. *nova 1* sequence types isolated from its host. Our results indicate that fragmentation and anthropogenic manipulation of populations may influence host-microbial relationships, with potential implications on niche adaptation and evolution of microbes in remote environments. Studies of this nature lend insight into the epidemiology of pathogens in natural systems which may threaten wildlife populations or become significant zoonoses.

Environmental attributes can restrict or enhance host population structure. This is particularly pertinent with respect to wildlife whose connectivity between populations has been adversely affected by habitat loss and fragmentation (Hand et al. 2014). Reduced mixing of hosts could have downstream effects on the patterns of disease spread, pathogen population structure and gene-exchange (Gandon et al. 2008). Takahe populations underwent a significant population bottleneck and range contraction to one isolated mountain population due to anthropogenic impacts (Bunin & Jamieson 1995; Trewick & Worthy 2001). *C.* sp. *nova 1* is thought to be endemic to takahe with prevalence estimates of 90% (Chapter 3). A host adapted ancestral lineage of the bacterium may have been present in the founding Murchison Mountain population which subsequently evolved biogeographically as a result of host range expansion, and distribution of populations into multiple new and isolated locations (Ballance 2001). This may explain the observed heterogeneity and spatial clustering of *C.* sp. *nova 1* isolated from takahe in this study, as well as the genomic separation of genotypes isolated from takahe in the Murchison Mountains from those in insurance populations (Figure 4-5A). An alternative hypothesis is that the ancestral population in the Murchison Mountains

consisted of a diverse range of genotypes, bottlenecking then occurred through the translocation of takahe, resulting in a random subset in each population (Figure 4-5B).

A variety of host attributes and behaviour may influence host exposure, prevalence and transmission of microbes. The complexity of such interactions have been explored using social network scientific methods in an epidemiological context (Bull et al. 2012; Chiyo et al. 2014; Hirsch et al. 2013; Porphyre et al. 2011; Rushmore et al. 2013; VanderWaal et al. 2013a). The analysis of population connectivity with respect to the carriage of *C.* sp. *nova 1* genotypes was not able to be conducted in this study. This was due to the fact that each takahe population used in this study possessed unique network measures derived from historic takahe movements (Chapter 2; Grange et al. 2014).

Social interactions between hosts may be more influential on sharing of genotypes than spatial proximity of hosts within an environment (Bull et al. 2012; VanderWaal et al. 2013b). Immigration and emigration of takahe between sub-populations is controlled and mediated by human intervention (Wickes et al. 2009). Therefore, there remain opportunities for continued bacterial gene flow between supposedly allopatric populations. In theory, this would have a homogenising effect on sequence type carriage either through competitive exclusion where dominant sequence types out-compete extant microbial flora, or via genomic recombination and stabilising selection. Carriage of panmictic *Escherichia coli* genotypes isolated from social groups of elephants (*Loxodonta africana*) sharing sources provided support for this concept (Chiyo et al. 2014). However, this study of *C* sp. *nova 1* in takahe provides evidence that bacterial divergence could occur in the face of population isolation with continued gene flow and within relatively short time frames. Initial studies by French et al. (2014) investigated the phylogeny of *C.* sp. *nova 1* isolated from New Zealand water and members of the Rallidae family. Analysis suggested two clades of the species diverged over 1000 years ago (French et al. 2014). If the divergence of *C.* sp. *nova 1* clades isolated from takahe in this study occurred within in similar time frame, it seems unlikely that evolution following translocation from the Murchison Mountain population in the last 30 years (Bunin et al. 1997) explains the biogeographic clustering observed in extant takahe populations.

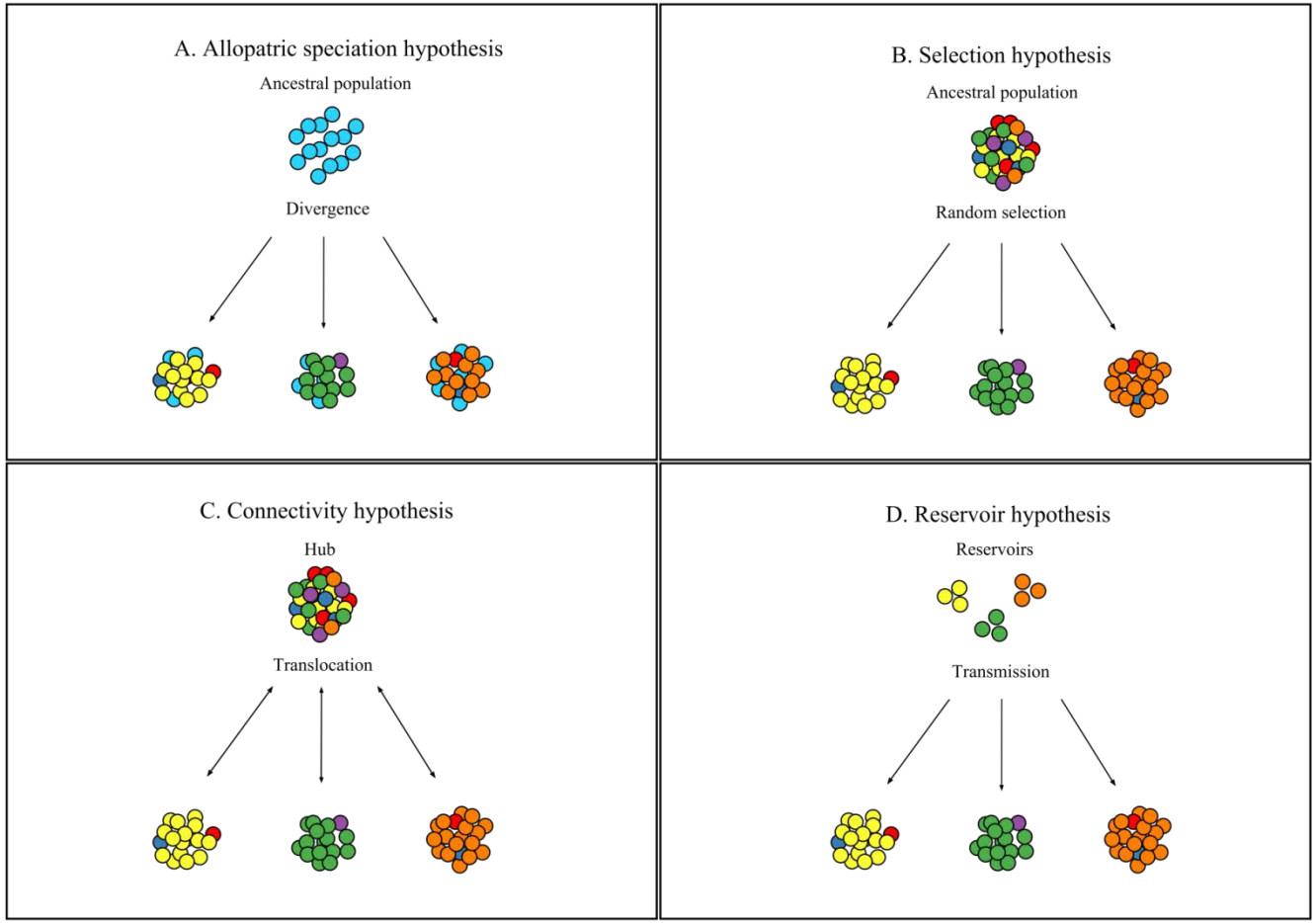

Figure 4-5 Schematic of hypotheses to explain geographic clustering of *Campylobacter* sp. *nova 1* genotypes observed in the takahe (*Porphyrio hochstetteri*) sup-populations. Coloured dots represent *C.* sp. *nova 1* genotypes. (A) By the allopatric speciation hypothesis, there was an ancestral population of *C.* sp. *nova 1* in the Murchison Mountains, and then following translocation and isolation these diverge as a result of rapid genetic drift. (B) By the selection hypothesis, the ancestral population contained a diverse range of genotypes, then bottlenecking occurred through translocation of takahe, with a random subset in each population. (C) By the connectivity hypothesis, the observed biogeographic diversity of genotypes is the result of population connectivity, with 'hub' populations containing multiple genotypes and more isolated populations containing few genotypes due to isolation. (D) By the reservoir hypothesis, the population *C.* sp. *nova 1* genotypes present within a population were determined by the presence and transmission of bacteria from environmental and alternative hosts. Alternatively, the observed diversity is a result of any combination of A to D.

Sequence analysis of *C.* sp. *nova 1* revealed a diverse range of genotypes, with location confounded attributes explaining most of the genetic differentiation. This is suggestive of rapid genomic divergence as a consequence of takahe isolation and is in agreement with previous network and molecular investigations (Chapter 3 ; Grange et al. 2014). Notably, the breeding centre population contained a diverse range of genotypes, with representatives from multiple clusters. This site acts as a hub of the population network with regular immigration from multiple sources (Grange et al. 2014). Unlike most other populations tested where takahe are free to roam and mix socially, takahe within the breeding centre are separated into captive pens according to family groups. This management practice may prevent mixing and allow the maintenance of a diverse range of *C.* sp. *nova 1* genotypes in the breeding centre population which have been derived from other locations, as observed in this study (Figure 4-5C).

Evolution of a microbe within allopatric populations is a concern when considering the management of endangered species. A commensal in one host may be a pathogen in another, and thus the translocation of an infected individual into a naive population may result in morbidity and mortality in a new host. An infection experiment conducted by Waldenstrom et al. (2010), demonstrated a marked decrease in body mass of European robins (*Erithacus rubecula*) following inoculation with a *C. jejuni* isolate derived from another avian species. Similar spatial clustering of microbial genotypes has been observed in the spread of a pathogen in wild populations. Rapid spread of a single multi-locus *Yersinia pestis* genotype within a prairie dog (*Cynomys ludovicianus*) population was followed by localised microbial differentiation within prairie dog sub-populations, which may be attributable to isolation or local maintenance in cryptic mammalian reservoirs (Girard et al. 2004). Modelling of canine distemper virus outbreaks in the Serengeti, suggests an important role of local reservoir species as a source of in pathogen spill-over to African lions (*Panthera leo*) (Craft et al. 2009). It is reasonable to hypothesise that the observed spatial clustering of *C.* sp. *nova 1* sequence types may be a result of local interactions with reservoir species (Figure 4-5D). *C.* sp. *nova 1* has previously been isolated from New Zealand surface water and members of the Rallidae family (French et al. 2014). Given the evidence of strong host-association (Chapter 3) and genomic complexity of *C.* sp. *nova 1* which was previously unobserved from traditional PCR typing methods (Chapter 3), it seems likely

that a combination of interactions between resident takahe, translocation and co-infections of hosts may contribute more to local genomic differentiation (Figure 4-5).

Epidemiological data garnered from investigations of highly managed takahe highlights the need for risk analysis and pathogen screening when considering movements of individuals between locations. Insight gained from this study not only has implications for the management of takahe; it also applies to the relocation of any animal including domestic livestock. Complex agricultural networks regularly move livestock across natural barriers and thus have a high risk of pathogen transmission resulting in outbreaks, as exemplified in the dissemination of foot and mouth disease virus between farms in the United Kingdom 2001 (Ortiz-Pelaez et al. 2006). Gaining a full understanding of pathogen epidemiology in wildlife systems is a challenging prospect. In this study, the small sample size and selection of a single isolate from an individual host limits our interpretation of location associations. For example, the methods used do not account for carriage of multiple *C.* sp. *nova 1* genotypes within a single host. This is a likely occurrence given the evidence of multiple carriage of *Campylobacter* spp. in takahe (Chapter 3). The use of an endemic commensal as a proxy for invasive pathogens can help our understanding of some of the epidemiological features of infectious disease, such as transmission and evolution, but this approach will always be limited by the lack of pathogenicity or recovery from infection. However, with the advent of whole genome analyses which can be applied across multiple bacterial species (Jolley et al. 2012) and robust phylogenetic methods for the exploration of factors that influence host-microbe relationships, these methods may lead the way in understanding and mitigating disease risks imposed by the anthropogenic management of wildlife.

4.6. Acknowledgements

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4.7. Supporting information

Appendix 9.4-1 is available electronically on a disc at the end of this thesis. The list of publically available *Campylobacter* spp. genomes used (Appendix 9.4-2), the MDS plot (Appendix 9.4-3) and PERMANOVA results using the uncorrected P measure (Appendix 9.4-4) and GTR matrix (Appendix 9.4-5) are available in the appendix.

CHAPTER 5

INVESTIGATION OF VERTEBRATE RESERVOIRS OF *CAMPYLOBACTER* SPP. IN AN ISLAND ECOSYSTEM

5. Investigation of vertebrate reservoirs of *Campylobacter* spp. in an island ecosystem

5.1. Abstract

Prediction and prevention of infectious disease in wildlife populations requires a baseline understanding of pathogen transmission, often involving multiple host species in complex ecosystems. Identifying reservoir populations which maintain and transmit pathogens to endangered species is a challenging but important area of epidemiological investigation. In order to explore the evolution and transmission of bacteria between hosts and reservoirs, this study determined the prevalence and genomic composition of enteric *Campylobacter* spp. in five vertebrate orders occupying an island ecosystem used for conservation of endangered takahe (*Porphyrio hochstetteri*). The genus *Campylobacter* contains a number of important pathogens of humans, domestic animals and wildlife but many are considered harmless commensal bacteria in certain hosts. Culture positive *Campylobacter* spp. isolates were subject to PCR for identification, followed by whole genome analysis. Prevalence of *Campylobacter* spp. was variable among sympatric vertebrate communities with estimates ranging from 0% to 100%. Ribosomal multi-locus sequence typing (rMLST) analysis of nineteen *Campylobacter* samples isolated from passerines and rails provided evidence of host-associated but not exclusively host-specific *Campylobacter* spp. genotypes. Genetic similarities were observed between *Campylobacter* spp. genomes isolated within a host species and between closely related hosts. Evidence of allele sharing suggests a common evolutionary history and potential recombination between species of *Campylobacter* colonising different host species. Exploring host-microbe relationships through the use of genomic sequencing of commensal bacteria in a multi-host system study provides insight into potential epidemiological interactions between hosts and pathogens, including the roles that sympatric communities may play in epidemics of more pathogenic generalist microorganisms.

5.2. Introduction

Predicting the emergence of wildlife disease and developing mitigation strategies requires an understanding of complex heterogeneous ecological interactions and the sharing of pathogens between communities. The recent integration of genomics, bioinformatics, epidemiology and ecology provides a combination of tools in which the ecological and evolutionary linkages between host and pathogen may be deconstructed and inter-host transmission dynamics inferred. Reservoirs are regarded as populations which maintain and transmit a pathogen to epidemiologically connected vulnerable target populations, such as endangered species (Haydon et al. 2002). It is often challenging to identify reservoirs of infectious organisms in susceptible hosts. For instance, evidence indicates interspecific interactions between African lions (*Panthera leo*), spotted hyenas (*Crocuta crocuta*) and jackals (*Canis* spp.) contribute to outbreaks of canine distemper virus within lion populations in the Serengeti (Craft et al. 2008; Craft et al. 2009).

Genomic sequencing of commensal microorganisms derived from multiple hosts provides opportunities to model the dynamics of colonisation and within and between-host transmission in an ecosystem, without the need for an infectious disease outbreak. Additionally, the pathogenic potential of generalist microorganisms capable of infecting more than one host species gains importance when reservoirs act as source of infection for spill-over to naive vulnerable species. A classic example is the emergence of squirrel pox virus (SQPV) contributing to the decline of the native red squirrel (*Sciurus vulgaris*) in the United Kingdom. Although transmission of the virus from the introduced grey squirrel (*Sciurus carolinensis*) has been demonstrated, the origin of squirrel pox virus in grey squirrels still remains unclear (Rushton et al. 2006).

Island ecosystems provide the ideal environment for the investigation of bacterial transmission between host communities. Range restriction imposed by geographical isolation can limit immigration and emigration of hosts, with the notable exception of mobile species such as birds which often have extensive home ranges. Conservation practices within New Zealand use mainland and offshore islands as sites for the protection of multiple endangered species (Sherley et al. 2010), due to ease of biosecurity and predator control, thus increasing diversity and density of potential hosts. Concentration of animals at unnaturally high densities on isolated islands may create hubs for the intra- and inter-specific transmission of microorganisms between wildlife communities. Host density and connectivity have been highlighted as influential factors determining the transmission of *Escherichia coli* between ungulates in Kenya (VanderWaal et al. 2013b, 2014). The translocation of animals between ecologically distinct locations may bring together previously allopatric populations and species into sympatry, providing opportunities for pathogens to cross natural barriers

between host communities. If already vulnerable, any additional selective pressure imposed on extant naive populations may lead to disease outbreaks. Although disease is not a common sole driver of population decline (Heard et al. 2013; Smith et al. 2006), it may contribute to localised extinction. For example, the spread of the chytrid fungus *Batrachochytrium dendrobatidis* along with environmental factors has resulted in the decline and extinction of frog species around the world (Berger et al. 1998; Fisher et al. 2012).

Campylobacter spp. are enteric bacteria able to colonise a diverse range of hosts (Kwan et al. 2008), including multiple avian species with variable prevalence reported depending upon species, location and method used (Waldenstrom & Griekspoor 2014). Although *Campylobacter* spp. are often harmless commensals in wildlife, they can be pathogens when hosts are exposed to isolates from another species. An infection experiment demonstrated a marked decrease in the body mass of European robins (*Erithacus rubecula*) following inoculation with a *Campylobacter jejuni* isolate derived from another wild bird species (Waldenstrom et al. 2010). Previous studies have highlighted the exceptionally high prevalence of *Campylobacter* spp. isolated from takahe (*Porphyrio hochstetteri*) (Chapter 3). Takahe are an endemic endangered New Zealand flightless rail (BirdLife International 2013) whose populations have been heavily manipulated through conservation actions. Preservation of the species relies on the frequent translocation of individuals between remote sanctuaries (Wickes et al. 2009). This study describes the prevalence of *Campylobacter* spp. in five vertebrate orders within an island ecosystem used for the conservation of takahe. To explore bacterial transmission between takahe and potential reservoirs, comparative analyses of 52 of the 53 conserved ribosomal protein genes used in ribosomal multi-locus sequence typing (rMLST) schemes (Jolley et al. 2012) were used for the discrimination and differentiation of *Campylobacter* spp. genomes isolated from hosts on Maud Island, New Zealand. To our knowledge, this is the first study to use genomic comparisons of rMLST genes to investigate the epidemiology of a commensal bacterium within abundant and endangered communities inhabiting the same island ecosystem.

5.3. Methods

5.3.1. Study site

Maud Island, also known as Te Hoiere scientific reserve, is located in the Marlborough Sounds, South Island, New Zealand. With a history of farming until it became a wildlife reserve in 1974, the island was cleared of vegetation but through restorative efforts is now comprised of approximately 310 hectares of predominantly regenerating coastal forest. The mainland is 900 metres away at the closest point. The island

is a predator-free conservation reserve and has a long history of translocations of endangered species, including takahe, to the site.

5.3.2. Study populations

Individuals from five vertebrate orders on Maud Island (Table 5-1) were sampled and tested for *Campylobacter* spp. via faecal or cloacal swabs in either a repeated or simple cross sectional design. Four takahe were caught and sampled opportunistically in March 2013 during New Zealand Department of Conservation (DOC) health checks or for management purposes. During the study period there were sporadic incursions by weka (*Gallirallus australis*) onto the island. Samples were collected opportunistically from 59 weka, by DOC staff between January and May 2013 prior to relocation to the mainland. All takahe and weka were uniquely identified by DOC administered leg rings.

Mist nets were erected for up to 3 days within takahe territories, in three month intervals between April 2012 and March 2013 in order to capture, sample and release fifty passerines per season. Nine birds were recaptured in separate events and sampled. Recaptures were not included in prevalence estimates or subsequent analysis. Birds were either marked with paper correction fluid (n = 26) or given a unique leg ring identifier (n = 165). In April 2012, faecal swabs (n = 50) were collected from passerine holding bags. During all subsequent capture periods, passerines were sampled by cloacal swabs (n = 150).

Due to seasonal fluctuations in presence on the island, 50 little blue penguins (*Eudyptula minor*) were captured over three nights during the breeding season in October 2012. Individuals were caught by hand or hand net, restrained for processing and subsequently released at the point of capture. All penguins were microchipped subcutaneously in the midline dorsal scruff of the neck with a unique identifier in order to prevent recapture. All penguins were sampled by cloacal swab prior to release.

In January 2013, 99 reptiles, comprising 80 common geckos (*Hoplodactylus maculatus*) and 19 brown skinks (*Oligosoma zelandicum*), were caught by hand from under artificial and natural retreats. Reptiles were temporarily marked using paper correction fluid on the dorsal surface to prevent resampling over the three days sampling period and cloacal swabs were collected prior to release at point of capture.

Approximately 50 domestic sheep (*Ovis aries*) were present on the island. Sheep were observed within paddocks and 100 fresh faecal samples were collected, over a period of two days in January 2013, with the aim of sampling most individuals. Faecal samples were collected a minimum of one meter radius apart in order to avoid cross contamination.

5.3.3. Sample collection

Cloacal or faecal swabs were collected from all species captured between April 2012 and May 2013 and immediately placed into Aimes charcoal transport media (Copan, California, USA). Cloacal swabs were collected and stored refrigerated at 4^{o}C in Aimes charcoal transport media for up to 7 days.

5.3.4. Microbiological culture, molecular confirmation and speciation

Microbial culture for *Campylobacter* spp. and DNA extraction were conducted using previously described methods (Chapter 3). Two colonies with typical *Campylobacter* spp. morphology were randomly selected from positive modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plates, followed by DNA extraction using methods previously described (Chapter 3). The selection of 2 colonies was applied due to study constraints; however this method limits the detection of multiple species potentially carried by an individual, and thus the interpretation of subsequent analysis. Suspected *Campylobacter* spp. isolate DNA was subject to confirmatory PCR using published protocols targeting: the *mapA* gene in *Campylobacter jejuni* (Mullner et al. 2010; Stucki et al. 1995), the *ceuE* gene in *Campylobacter coli* (Denis et al. 2001; Gonzalez et al. 1997), the putative C4-dicarboxylate trans-membrane transport gene in *Campylobacter* sp. *nova 1* (Chapter 3) and a specific region of the 16S rRNA gene to identify members of the genus *Campylobacter* (Linton et al. 1997).

Apparent and true prevalence estimates with 95% confidence intervals (95% CI) of *C. jejuni* , *C.* sp. *nova 1* and unidentified *Campylobacter* spp. were calculated in the epiR package (Stevenson 2014) in R software (R Core Team 2013) using Blaker's intervals (Blaker 2000) with estimates of sensitivity (se) and specificity (sp) previously described: *C. jejuni* se = 62.9, sp = 95.7% and *C.* sp. *nova 1* se = 69.1, sp = 97.8% (Chapter 3). Sensitivity and specificity values for *Campylobacter* spp. culture and PCR were based on those for *C.* sp. *nova 1*.

One isolate from each *Campylobacter* spp. positive individual was selected at random for whole genome sequencing, unless the two isolates extracted from an individual were identified as different *Campylobacter* spp. by PCR, in which case both were sequenced. One isolate was unable to be recovered from storage and was not included in further genomic analysis. Genomic DNA preparation, sequencing, assembly, curation and annotation were conducted as per protocols previously described in Chapter 4.

5.3.5.rMLST analysis

Nucleotide sequences for 52 of the 53 genes (the order *Campylobacterales* does not possess the *rpm*D gene) encoding bacterial ribosomal protein subunits used for ribosomal multi locus sequence typing (rMLST) (Jolley et al. 2012), were identified and extracted from assembled draft genomes using a *C. jejuni* reference set of rMLST genes as a database for a BLAST search. Using custom Perl scripts, unique alleles for each gene were called based on their nucleotide sequence. Each individual gene was aligned using Muscle (Edgar 2004) and the aligned genes were concatenated to make a single alignment per genome. A NeighborNet tree of the 52 concatenated rMLST gene nucleotide sequences derived from the 19 *Campylobacter* spp. genomes was visualised in SplitsTree4 (Huson & Bryant 2006). Pairwise comparison of genomic distance between *Campylobacter* spp. was conducted within SplitsTree4 (Huson & Bryant 2006) using uncorrected P measures. This distance is obtained by dividing the number of single nucleotide differences by the total number of nucleotides compared between two gene sequences. The lower triangle distance matrix was visualised using the pheatmap package (Kolde 2013) in R software (R Core Team 2013).

5.3.6.*In silico* PCR of the C4-dicarboxylate trans-membrane transport gene

In silico C. sp. *nova 1* PCR was conducted on all sequenced isolates in order to test for the presence of the C4-dicarboxylate trans-membrane transport gene and nucleotide polymorphisms within the target sequence. This was conducted because some isolates were identified as closely related to *C.* sp. *nova 1* by rMLST but tested negative by *in vitro* PCR. Forward and reverse primer sequences for the C4-dicarboxylate trans-membrane transport gene in *Campylobacter* sp. *nova 1* (Chapter 3) were used to find the PCR target region of one isolate (Appendix 9.5-1). The 106 base pair target region was extracted and used as a reference to BLAST against all other isolates in order to determine the presence of the gene within a given genome and any nucleotide polymorphisms which may be present. Sequences were aligned and visualised within Geneious v7.17 (Drummond et al. 2013).

5.4.Results

5.4.1.Prevalence of *Campylobacter* spp. in vertebrate communities

Five vertebrate orders, including 294 birds, 99 reptiles and approximately 50 sheep, inhabiting Maud Island were tested for *Campylobacter* spp. between April 2012 and March 2013. Sampling details and *Campylobacter* spp. apparent (AP) and true (TP) prevalence estimates are presented in Table 2-1. All four takahe tested positive for *Campylobacter* with two species isolated; *C. jejuni* (TP, = 35.3% [95% CI 0-

100%]) and *C.* sp. *nova 1* (TP = 100% [95% CI 68-100%]). *Campylobacter* spp. were isolated from eleven weka tested during the island incursion. Weka prevalence estimates were as follows; *C.* sp. *nova 1* (TP = 17.0% [95% CI 6-33%]) and unidentified *Campylobacter* spp. belonging to the *Campylobacteraceae* family (TP = 4.3% [95% CI 0-17%]). Season was not associated with *Campylobacter* spp. carriage in the passerines tested. Two species of *Campylobacter* were isolated from passerines, including *C. jejuni* isolated from one bellbird (*Anthornis melanura*) (TP = 0% [95% CI 0-5%]) and two silvereyes (*Zosterops lateralis*) (TP = 0% [95% CI 0-10%]). We identified an unknown species of *Campylobacter* from a Eurasian blackbird (*Turdus merula*) (TP = 13.3% [95% CI 0-63%]). *Campylobacter* spp. was not isolated from any of the reptiles, little blue penguins or sheep tested during the study period.

5.4.2. Comparative genomics of *Campylobacter* spp.

Nineteen *Campylobacter* spp. isolates, encompassing all but one *Campylobacter* spp. positive individual due to the inability to recover a weka derived *C.* sp. *nova 1* isolate from storage, were subject to whole genome sequencing. Four distinct clusters were identified based on pairwise comparison of rMLST nucleotide sequences. In addition, potential recombination within and between *Campylobacter* spp. was evident from the net-like structure in some parts of the NeighborNet phylogeny (Figure 5-1A), indicative of uncertainty in the phylogenetic relationships between isolates. The largest genetic distances were observed between different *Campylobacter* spp., with some evidence of host associated grouping of related sequence types within a *Campylobacter* spp. (Figure 5-1B). Takahe *C.* sp. *nova 1* sequence types were highly related to each other and more distantly to three of the *C.* sp. *nova 1* isolated from weka within cluster one (Figure 5-1A). The remaining four weka *C.* sp. *nova 1* isolates were positioned within cluster two next to unidentified *Campylobacter* spp. isolates also isolated from weka. The distance between the two clusters of *C.* sp. *nova 1* was larger (pairwise uncorrected P measure value, PV = 0.1) than that observed between the unidentified *Campylobacter* spp. and *C.* sp. *nova 1* within cluster 2 (PV = 0.01-0.03) (Figure 5-1B).

C. jejuni isolated from passerines (bellbird and silvereyes) were more closely related to each other (PV = 0.001 - 0.01) than to the one takahe associated *C. jejuni* (PV = 0.02) (Figure 5-1B). All *C. jejuni* isolates were genetically separated from *C.* sp. *nova 1*, as indicated by the long branch lengths in the NeighborNet tree. The unknown *Campylobacter* spp. isolated from a Eurasian blackbird was genomically distinct with no shared allele sequences (Figure 5-1, Table 5-2) and a large genetic distance (PV = 0.14 - 0.15) (Figure 5-1B) from all other *Campylobacter* spp. isolated from vertebrates on Maud Island in this study.

Species	Scientific name	No. tested (No. positive)	*Campylobacter* spp.	*Campylobacter* spp. prevalence (95% CI)	
				Apparent (AP)	True (TP)
Passerines					
Bellbird	*Anthornis melanura*	69 (1)	*C. jejuni*	1.5% (0-8%)	0 % (0-5%)
Chaffinch	*Fringilla coelebs*	3	-	-	-
Dunnock	*Prunella modularis*	3	-	-	-
Eurasian blackbird	*Turdus merula*	11 (1)	*Campylobacter* spp.	11.1% (1-44%)	13.3% (0-63%)
European goldfinch	*Carduelis carduelis*	1	-	-	-
Grey warbler	*Gerygone igata*	2	-	-	-
House sparrow	*Passer domesticus*	22	-	-	-
New Zealand fantail	*Rhipidura fuliginosa*	8	-	-	-
Silvereye	*Zosterops lateralis*	66 (2)	*C. jejuni*	3.0% (1-10%)	0% (0-10%)
Song thrush	*Turdus philomelos*	2	-	-	-
Tui	*Prosthemadera novaeseelandiae*	4	-	-	-
Reptiles					
Brown skink	*Oligosoma zelandicum*	19	-	-	-
Common gecko	*Hoplodactylus maculatus*	80	-	-	-
Rails					
Takahe	*Porphyrio hochstetteri*	4 (4❖)	*C. jejuni*	25.0% (1-75%)	35.3% (0-100%)
			C. sp. *nova 1*	100% (47-100%)	100% (67-100%)
Weka	*Gallirallus australis*	59 (11*)	*Campylobacter* spp.	5.1% (1-14%)	4.3% (0-17%)
			C. sp. *nova 1*	13.6% (6-24%)	17.0 (6-33%)
Seabird					
Little blue penguin	*Eudyptula minor*	50	-	-	-
Ruminant					
Sheep	*Ovis aries*	100 faecals	-	-	-

*One *C.* sp. *nova 1* isolate was unable to be recovered for sequencing ❖One takahe tested positive for both *C. jejuni* and *C.* sp. *nova 1.*

Table 5-1 List of species captured and sampled for *Campylobacter* spp. on Maud Island, New Zealand, including sampling effort, apparent (AP) and true prevalence (TP) of *Campylobacter* spp. identified by in vitro PCR.

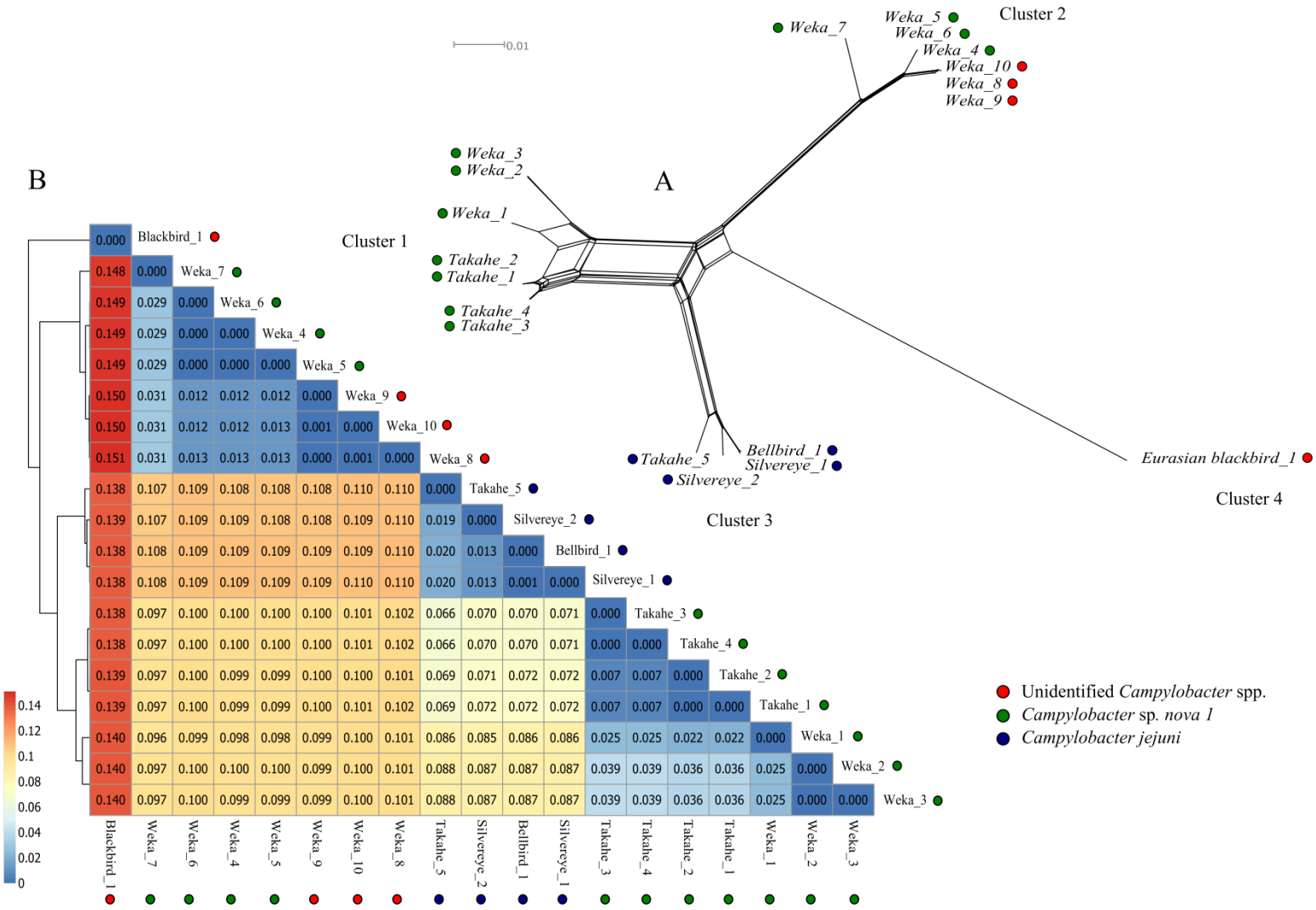

Figure 5-1 NeighborNet tree (A) and pairwise genomic distance matrix (B) based on 52 gene rMLST nucleotide sequences using uncorrected P values (PV) of *Campylobacter* spp. isolated from host species on Maud Island.

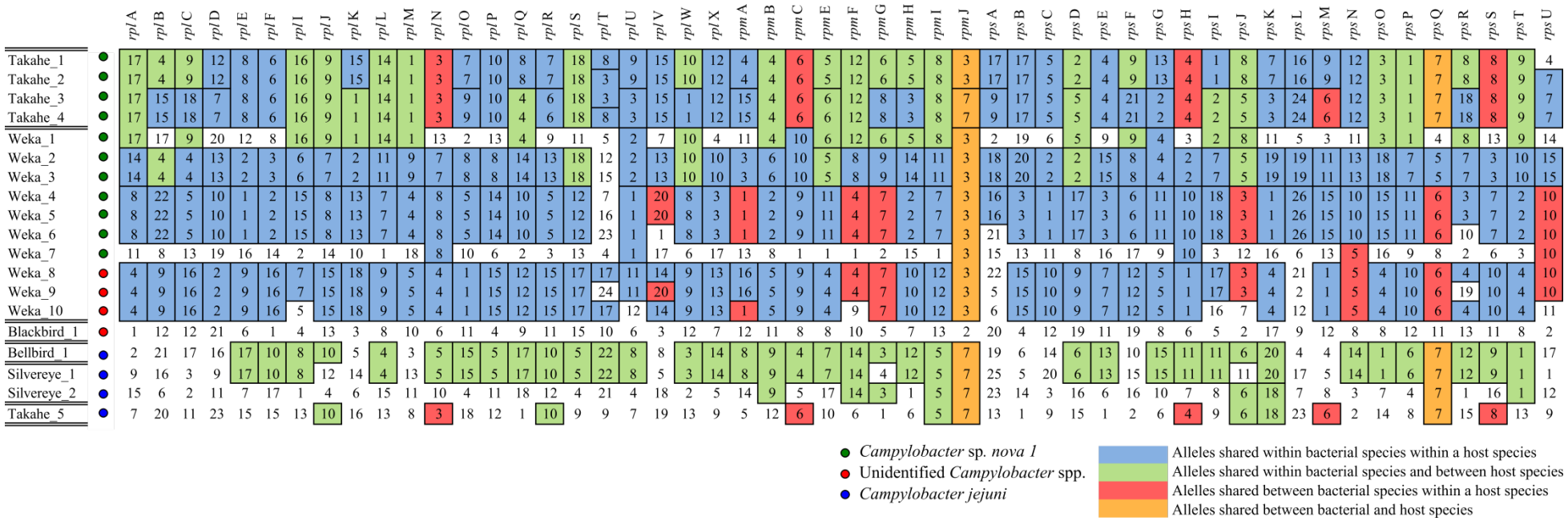

Table 5-2 Allelic profiles of 52 rMLST genes present within *Campylobacter* spp. isolated from host species on Maud Island, New Zealand.

In silico PCR for the putative *Campylobacter* spp., *C.* sp. *nova 1* was conducted on all *Campylobacter* spp. positive isolates and confirmed the presence of the target gene within all *in vitro* PCR positive *C.* sp. *nova 1* (Appendix 9.5-1). One hundred percent sequence homology was observed between 7 out of 11 identified genes. Three isolates originating from weka contained 7 single nucleotide polymorphisms (SNPs) and one weka isolate possessed 4 SNPs within the target region (Appendix 9.5-1). There was no match to the PCR target gene within isolates identified as *C. jejuni* or unidentified *Campylobacter* spp.

Evidence of recombination and sharing of identical alleles was identified on comparison of allelic profiles within and between hosts. This was also observed among *Campylobacter* spp. (Table 5-2). Identical alleles were predominantly nested within a *Campylobacter* spp. Alleles were often conserved within a host with occasional sharing of identical sequences between hosts. Interestingly, five genes (*rpl*N, *rpm*C, *rps*H, *rps*M and *rps*S) showed evidence of homologous sequences within both *C.* sp. *nova 1* and *C. jejuni* isolated from takahe, and 8 alleles were shared between *C.* sp. *nova 1* and unidentified *Campylobacter* spp., indicating potential recombination between *Campylobacter* spp. Some genes were relatively conserved across both bacterial species and host. For example, only three sequence combinations were defined for the *rpm*J gene across the 19 isolate genomes.

5.1. Discussion

Management of existing and emerging wildlife disease threats in endangered or threatened populations requires an understanding of host-pathogen relationships and transmission within natural ecosystems. Previous studies investigating the bacterial dynamics of a common enteric bacterium, *Campylobacter* spp. within fragmented takahe populations discovered a complex relationship between host location and bacterial carriage (Chapter 3 ; Chapter 4). This raised the question of whether location attributes including direct and indirect interactions with sympatric reservoir hosts contributed to the prevalence and bacterial sequence types harboured within takahe. Results from this small-scale cross-sectional prevalence survey provide evidence that avian species, in particular members of the *Rallidae* family, are the predominant host of *Campylobacter* spp. within the study island ecosystem. We observed evidence of allele sharing between different *Campylobacter* spp. isolated from the same and taxonomically distinct hosts suggesting a recent shared evolutionary history of some genes, most likely the result of genetic recombination between these bacteria. Transmission of a bacterium between sympatric hosts may be inferred from the bacterial sequence type they carry (Chiyo et al. 2014; VanderWaal et al. 2013b, 2014). Clonal sequence types were not detected in this study, thus transmission between hosts through direct or indirect means cannot be inferred. However,

conservation of many rMLST genes was found to be within a host species and those that were shared tended to be between species of the same family order, suggesting cross-species transmission would most likely occur between closely related hosts.

Campylobacter spp. are a multi-host enteric organism which has been isolated from a range of species including companion animals (Mughini Gras et al. 2012), wildlife (Colles et al. 2011; French et al. 2009; Keller & Shriver 2013; Mohan et al. 2013; Sippy et al. 2012) and farm animals (Colles et al. 2011; Kwan et al. 2008). Although *Campylobacter* spp. isolated from takahe is thought to be of a commensal nature in takahe with a high prevalence reported in this and previous studies (Chapter 3 ; Chapter 4), exposure to different host associated genotypes may result in reduced fitness and pathogenic effects in the new host (Waldenstrom et al. 2010). We report a low prevalence of *Campylobacter* spp. in sympatric communities sharing an island with takahe. However, prevalence estimates do not necessarily correlate with the likelihood of transmission of a microorganism to new hosts. The ecological concept of "super-spreaders" and "super-shedders" (Lloyd-Smith et al. 2005) based on the 20:80 rule (Woolhouse et al. 1997; Woolhouse et al. 2005), implies that a small proportion of the population infected with a microorganism may be responsible for the dissemination of pathogens to new hosts. Therefore, although the prevalence of *Campylobacter* spp. observed in species co-habiting an environment with takahe was low in comparison to that observed in takahe, it is theoretically possible that the few infected individuals may significantly contribute to the transmission of *Campylobacter* spp. between hosts.

Campylobacter spp. was only detected in terrestrial birds, with a higher prevalence in the flightless takahe and weka. Environmental and behavioural variables may influence transmission between cohabiting vertebrate communities. Faecal contamination of the environment provides opportunities for mixing and chance transmission of enteric bacteria between taxonomically distinct hosts sharing an environment. However, transmission through faecal oral pathways requires a period of survival in the environment (Bull et al. 2006) and given the relative fragility of *Campylobacter* spp. and sub-optimal conditions outside the host (Murphy et al. 2006), it seems likely that mixed species co-infection may be an important means of horizontal gene transfer between *Campylobacter* spp. Similarities in host behaviour, structuring and foraging between closely related hosts may explain the carriage and genetic similarities between *C.* sp. *nova 1* isolated from takahe and weka. This concept is supported by other studies where host exposure to *Campylobacter* spp. has been attributed to ecological guild with a higher incidence reported in ground foraging and scavenging birds (Waldenstrom et al. 2002). In addition, genomic comparisons between *C.* sp. *nova 1* isolated from weka with those from takahe in different locations (unpublished data) identified identical

rMLST profiles between an isolate from a takahe in the Murchison Mountains, New Zealand and a *C.* sp. *nova 1* from a weka in this study.

Bacteria that colonise multiple hosts, with variable pathogenicity in different hosts, are likely to exhibit heterogeneity in carriage between host species due to differences in host immunity, population sizes, social behaviour, spatial distribution and habitat use within an ecosystem (Chiyo et al. 2014; Dobson 2004; Grange et al. 2014). *Campylobacter* spp. were not isolated from sheep on Maud Island during this study, although sheep are known carriers of *Campylobacter* spp. (Mughini Gras et al. 2012; Mullner et al. 2009). Additionally, a recent study found close associations between proximity to agricultural environments and increased likelihood of carriage of *Campylobacter coli* carriage in takahe (Chapter 3). Therefore, co-habitation of livestock with species of conservation concern should only proceed with caution and strict biosecurity including a quarantine period and health testing. Foraging behaviour of marine birds is substantially different from terrestrial species, with penguins spending substantial amounts of time at sea in pursuit of food only returning to shore at night to rest within nests or burrows, thus reducing opportunities for faecal-oral transmission and colonisation of *Campylobacter* spp. *C. jejuni* has been isolated from macaroni penguins (*Eudyptes chrysolophus*) in the Antarctic, however this is a rare report and the incidence was thought to be associated with human effluent contaminating the penguin habitat (Griekspoor et al. 2009). Most reports of *Campylobacter* spp. isolation in reptiles are from captive animals. For example, *Campylobacter fetus* and *Campylobacter hyointestinalis* were isolated from 44.8% of captive reptiles tested in a study in the Netherlands (Gilbert et al. 2014). However, carriage of *Campylobacter* spp. in wild reptilian populations is rare, as supported by this study.

The aim of this study was to investigate potential reservoirs of *Campylobacter* spp. which may be a source of infection in takahe. Interestingly, with such a high incidence unmatched in other animal communities tested, the endangered takahe may themselves be a sustainable source of infection to other vulnerable species. Takahe on Maud Island form part of a complex highly connected network of takahe populations residing within conservation reserves (Grange et al. 2014). Linked through regular translocations, localised and long range transmission through faecal contamination of the environment may be possible. In New Zealand, islands are commonly used as refuges for multiple translocated species of conservation concern (Sherley et al. 2010). Transmission of the apparently rail associated *C.* sp. *nova 1* (Chapter 3 ; Chapter 4 ; French et al. 2014) to naive hosts or humans interacting with takahe may be detrimental, although the pathogenicity of this species in other hosts is unknown.

Detection and identification of *Campylobacter* spp. in biological communities contributes only a small amount to the understanding of the complex interactions involved in the epidemiological triangle of interactions between the host, microbe and the environment. This study was limited by the relatively small populations and sample sizes, and the practical need for opportunistic cross sectional sampling often encountered in investigations of wildlife disease ecology. An inherent risk of ecological studies is there may be a range of unmeasured biotic and abiotic factors influencing the individual through to population and community scales (Tompkins et al. 2011), which may explain the host-microbe relationships observed in this study. For example, survival and establishment of *Campylobacter* spp. within a host may be dependent upon interactions with other members of the gut microbiome, which may in turn be confounded by factors such as host age and ecological interactions (Costello et al. 2012). Additionally, it is likely that this study did not encompass the full diversity of *Campylobacter* spp. present within the study species due to small sample sizes, low prevalence of carriage and the test characteristics of the methods used. Previous comparisons of culture versus faecal DNA detection revealed imperfect sensitivity and specificity of the culture method used for *Campylobacter* spp. detection (Chapter 3). Epidemiological investigations using microbial genomics require bacterial culture of an isolate prior to sequencing and analysis. Therefore, studies of this kind need to take into account the biases resulting from the selective nature of culture which will have downstream effects on the interpretation of results.

Genomic analysis using rMLST profiles seemed to contradict the species definition of *C.* sp. *nova 1* previously described in studies of the bacterium in takahe (Chapter 3 ; Chapter 4), due to the relatively large distances between the isolates within cluster 1 and 2, as well as the close relationship between *C.* sp. *nova 1* and unidentified *Campylobacter* spp. within cluster 2 (Figure 5-1). Although *in silico* PCR confirmed the presence of the *C.* sp. *nova 1* PCR target within all *C.* sp. *nova 1* isolates identified by *in vitro* PCR (Chapter 3), rMLST analysis revealed distinct genomic differences between *C.* sp. *nova 1* isolates (Figure 5-1). A group of *C.* sp. *nova 1* isolates were closely related to unidentified *Campylobacter* spp. isolates which do not possess the PCR target gene. The absence of this gene within the unidentified *Campylobacter* spp. may have been a result of gene deletion or absence of insertion. Alternatively, given the genetic distance between cluster one and two, *C.* sp. *nova 1* within cluster two may be a different species which have acquired or retained the PCR target gene from recombination with an ancestor of the isolates in cluster 1. Either way, this example highlights the restrictive nature of using conventional PCR methods for bacterial species detection and identification, as well as the need to use a large gene pool when comparing bacterial isolates for epidemiological investigations of bacterial transmission or source attribution.

Management of wildlife infectious disease requires the identification, characterisation and quantification of microorganisms in order to understand mechanisms which drive transmission and persistence. This small-scale study demonstrates the use of population genetic tools to explore complex host associated bacterial carriage. High resolution strain typing and determination of host association through genomic sequencing, provides more robust evidence and confidence previously unattainable through conventional molecular methods. Modelling of commensal bacteria in this study provides insight into potential epidemiological interactions between hosts and pathogens, including the roles that ecologically similar and distinct hosts may play in epidemics and the transmission of more pathogenic generalist microorganisms, such as *Salmonella* spp.

5.2. Acknowledgements

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5.3. Animal ethics and permits

Samples were collected under a Massey University animal ethics permit MUAEC Protocol 11/95. Capture, handling and sample collection were in accordance to New Zealand Department of Conservation permits NM-33051-FAU and NM-35424-FAU. Passerines were caught and banded under New Zealand national banding scheme permit number 2012/007.

5.4. Supplementary information

The sequence alignment for the *in silico Campylobacter* sp. *nova 1* target region (Appendix 9.5-1) is available in the appendix.

CHAPTER 6

LOCATION SPECIFIC PREVALENCE OF *SALMONELLA* SPP. IN ENDANGERED TAKAHE (*PORHPYRIO HOCHSTETTERI*)

6. Location specific prevalence of *Salmonella* spp. in endangered takahe (*Porphyrio hochstetteri*)

6.1. Abstract

Generalist opportunistic bacteria such as *Salmonella* spp. are able to cross transmission boundaries between host species. The process of translocation of endangered species to new locations may create novel routes of transmission of potentially pathogenic *Salmonella* spp. between previously isolated hosts and reservoirs, with the potential for negative impacts on vulnerable populations. We investigated the prevalence of *Salmonella* spp. in endangered takahe (*Porphyrio hochstetteri*) from multiple geographically isolated locations in New Zealand. A low *Salmonella* spp. prevalence, 1-5% (95% CI 0-16%), was detected, with three serotypes isolated; *S. enterica* subsp. *enterica* serotype Mississippi, *S. enterica* subsp. *enterica* serotype Saintpaul and *S. enterica* subsp. *houtenae* serotype 40g.t. Takahe from a single island location were significantly (p=0.002) more likely to carry *Salmonella* spp. at the time of sampling than any other location. The serotypes isolated from takahe on this island have been associated with reptiles in other studies, and *S. enterica* subsp. *enterica* serotype Saintpaul is an important zoonotic serotype in New Zealand. The geographic clustering of positive cases suggests the presence of environmental reservoirs and transmission routes on the island which are unavailable elsewhere. The physiological impact of *Salmonella* spp. infection on takahe is unknown; however translocation of *Salmonella* spp. positive individuals to new sites presents a risk of disease expression within the host as well as transmission to other species.

6.2. Introduction

Animals are host to many different organisms which may be commensals, symbiotic, parasitic or pathogenic in nature. *Salmonella* spp. are opportunistic pathogens, able to infect a broad range of hosts, raising concerns about zoonotic transmission through foodborne (EFSA 2013) and direct routes (Cummings et al. 2012) . *Salmonella* spp. infection can have negative impacts on wildlife populations (Hall & Saito 2008; Lawson et al. 2010). Geographical isolation of endangered species, habitat fragmentation and movements of animals to new environments presents opportunities for exposure to novel and potentially pathogenic microorganisms. Therefore, increased understanding of the factors that influence the biological package of parasites and pathogens within hosts and their communities is required to understand the implications of conservation actions such as translocation on the spread of pathogens (Bengis et al. 2002).

Fauna which are translocated to a new environment may pose a significant disease risk to the incumbent wild populations. Animals can act as carriers and / or vectors of exotic pathogens into naive wild populations which have no effective immunity to these pathogens at the release site (Anderson & May 1986; Woodford 1993). Awareness of this issue has prompted protocols to minimise disease transmission risk associated with wildlife translocations and reintroductions (Cunningham 1996; Viggers et al. 1993; Woodford & Rossiter 1993). Due to such concerns, health screening is now required prior to translocations of the endemic endangered New Zealand flightless takahe (*Porphyrio hochstetteri*) (BirdLife International 2013; McInnes et al. 2004). Included in the health test profile is culture for *Salmonella* spp. from cloacal samples. Although useful, interpretation of results remains difficult due to imperfect tests, intermittent shedding of faecal *Salmonella* (Ivanek et al. 2012; Van Immerseel et al. 2004), and the timing of testing. Encounters with new pathogens may occur at several stages of the translocation process (Cunningham 1996). The prevalence of *Salmonella* spp. infection in wild birds is reportedly low, often associated with asymptomatic carriage, however outbreaks of salmonellosis have caused morbidity and mortality in passerines throughout the world, including the USA, United Kingdom and New Zealand (Alley et al. 2002; Hall & Saito 2008; Lawson et al. 2010; Pennycott et al. 2010). Unfortunately there is a lack of baseline information on *Salmonella* spp. prevalence in wild populations of takahe, with anecdotal reports of *Salmonella enterica* subsp. *enterica* serotype Brandenburg (Orr 1997), *Salmonella enterica* subsp. *enterica* serotype Typhimurium (McLelland et al. 2011) and *Salmonella* Mana (ESR 2010) isolated from a few individuals. We investigated the prevalence and geographic distribution of *Salmonella* spp. carriage in takahe from multiple locations in New Zealand.

6.3.Methods

One hundred and forty seven individual takahe, and 15 repeat samples of individuals which had been translocated to a new location in the time frame of this study were tested opportunistically during pre-translocation disease screening or annual health checks. Takahe were tested for cloacal and / or faecal carriage of *Salmonella* spp. from nine locations (Table 6-1) within New Zealand between November 2011 and April 2013. This sample size represents approximately 66% of the total population of this critically endangered species and covers most of its remaining geographic range. For reasons of confidentiality, the location of the private island is undisclosed. All swabs were transported and stored in Aimes Charcoal Transport media (Copan, California, USA) at 4^{o}C for one to seven days prior to culture.

Culture for *Salmonella* spp. was in aerobic conditions at 37^{o}C unless otherwise stated. Each swab was suspended in 2ml Phosphate buffered saline (PBS, pH 7.3). A 500µl aliquot of PBS solution was transferred into 20ml buffered peptone water (BPW) (BioRad, Auckland, New Zealand) and incubated for 24 hours for pre-enrichment. Subsequently, 100µl of PBS was inoculated into two secondary selective enrichment broths, 10ml Rappaport-Vassiliadis *Salmonella* (RVS) at 42^{o}C and 10ml tetrathionate (TET) broth (Fort Richards, Auckland, NZ) enriched with 100µl iodine-iodide solution (0.25g KI, 0.3g I/ml H_2O). Both RVS and TET were incubated for 24 +/- 2 hours. Incubated RVS and TET solutions were subcultured and streaked onto Xylose Lysine Deoxycholate (XLD) and Brilliant Green Modified (BGM) agar (Fort Richards, Auckland, NZ), and were incubated for 18 to 24 hours. The agar plates were examined and two to four suspect *Salmonella* colonies of differing morphology were subcultured onto MacConkey agar (Fort Richards, Auckland, NZ) and incubated for 18 to 24 hours. Cultures presenting as grey on MacConkey plates were inoculated into Triple Sugar Iron (TSI) and Lysine Iron Agar (LIA) slopes (Fort Richards, Auckland, NZ) and incubated for 18 to 20 hours. Isolates with positive reactions on the slopes were further tested for oxidase reactivity and reaction to polyO and polyH antisera (Oxoid, Auckland, NZ). Oxidase negative and polyO and or polyH antisera positive isolates were cultured on to Columbia Horse Blood Agar (Fort Richards, Auckland, NZ) for 24 hours prior to identification using a RapID One test (Oxoid, Auckland, New Zealand) conducted according to manufacturer's instructions. Isolates identified as *Salmonella* spp. were cultured on Dorset Egg agar slopes (Fort Richards, Auckland, NZ) for 24hrs and sent chilled to the Environmental Science and Research (ESR-NCBID) laboratories at Wallaceville, New Zealand for serotyping.

Prevalence calculations were conducted using the EpiTools package (Aragon 2012) within R software (R Core Team 2013). Estimates of the true prevalence, and corresponding 95% confidence intervals, of

Salmonella spp. in the takahe population were made using the test prevalence and assumptions that the culture technique was 50% sensitive and 98% specific based on previous studies (Bager & Petersen 1991). Prevalence estimates were calculated for different sample types, or combinations of sample types (positive for either swab). Influence of location on *Salmonella* spp. presence and absence were performed using the Fisher's exact test in R software (R Core Team 2013).

6.4. Results

Three serotypes of *Salmonella* spp. were isolated from takahe (Table 6-1). Five isolates of *Salmonella enterica* subsp. *enterica* serotype Mississippi and one of *Salmonella enterica* subsp. *houtenae* serotype 40g.t (1) were isolated from takahe located on a private island. A takahe which had previously tested positive for *S.* Mississippi on the private island was later found to be positive for *Salmonella enterica* subsp. *enterica* serotype Saintpaul after translocation to the Burwood Bush breeding centre.

Location	***n (n positive)***	***Salmonella* serotype**
Burwood bush breeding centre	59 (1)	*Salmonella enterica* subsp. *enterica* serotype Saintpaul (1)*
Mana Island	6	-
Maud Island	4	-
Maungatautari reserve	8	-
Murchison Mountains	46	-
Private Island	19 (6)	*Salmonella enterica* subsp. *enterica* serotype Mississippi (5) *Salmonella enterica* subsp. *houtenae* serotype 40 g.t. (1)
Te Anau wildlife reserve	4	-
Tiritiri Matangi Island	15	-
Willowbank reserve	1	-

*One takahe (*Porphyrio hochstetteri*) individual was translocated and previously tested positive for *Salmonella* Mississippi on the private island

Table 6-1 Number (*N*) of takahe (*Porphyrio hochstetteri*) tested by location with corresponding *Salmonella* spp. serotypes isolated from takahe.

Six out of the seven *Salmonella* spp. positive individuals were identified from cloacal swab culture. Of these, four birds had corresponding faecal swabs, two of which were in agreement with cloacal swab results, and two of which were negative for *Salmonella* spp. One *S.* Mississippi isolate was detected in a faecal swab but its corresponding cloacal swab was negative.

Overall true prevalence estimates for *Salmonella* spp. in takahe are relatively low, ranging between 1 and 5% (95% CI 0-16%) (Table 6-2). Significant differences in *Salmonella* spp. prevalence were found according to location (*p*-value 0.002), with *Salmonella* spp. positive individuals only detected in two locations, with true

prevalence estimates of 62% (private island, 95% CI 26-100%), and 0% (Burwood Bush breeding centre 95% CI 0-1%).

Sample type	***n (n positive)***	***Salmonella* true prevalence (95% CI)**
Cloacal swab	147 (6)	4% (0-14%)
Faecal swab	130 (3)	1% (0-9%)
Faecal or cloacal swab	162 (7)	5% (0-14%)

Table 6-2 Estimates of true prevalence of *Salmonella* spp. in takahe (*Porphyrio hochstetteri*) using three different sampling methods.

6.5. Discussion

Salmonella spp. are often regarded as opportunistic pathogens, which may be carried asymptomatically by healthy individuals. However, recrudescence and increased shedding may occur during times of stress, such as that induced by translocation (Verbrugghe et al. 2011). Monitoring two thirds of the takahe population for intestinal carriage of *Salmonella* spp. over a one and half year time period revealed a low prevalence of *Salmonella* spp., with a geographic clustering of asymptomatic *Salmonella* spp. carriage in takahe on a private island.

Takahe populations are highly fragmented, with an extant population of approximately 230 individuals (Wickes et al. 2009). Population management and mitigation against inbreeding effects (Jamieson et al. 2006) has been heavily reliant on translocation of the birds to environments that are substantially different from the founding ecosystem. The isolation of *Salmonella* spp. predominantly from one location, and the serotypes identified in this study, suggests there may be an environmental reservoir and transmission route on the island, not present elsewhere, from which takahe are being exposed to as an incidental host. However the nature of the environmental reservoirs and the epidemiology of infection were not examined in this study.

Herpetofauna are often implicated as sources of *Salmonella* spp., indeed, *S.* Mississippi has been isolated from reptiles in Australia and New Zealand (Ball 1991; Middleton et al. 2014), with some human cases reported in Tasmania correlated to contact with wildlife (Ashbolt & Kirk 2006). Additionally, *S. houtenae* 40g.t (isolated in this study) was recently detected in lizards sampled on New Zealand offshore islands (Baling et al. 2013). Takahe are omnivorous; although grasses comprise the majority of their diet, opportunistic consumption of reptiles and insects has been observed, thus presenting a potential direct transmission route for *Salmonella* spp. infection. Additionally, artificial feeding and water sources provide congregation sites which increase contact rates between multiple hosts and their faeces. For example, aggregations of European starlings (*Sturnus vulgaris*) have been implicated as sources of *Salmonella* spp. in

concentrated animal feeding stations (Carlson et al. 2011), and the role of introduced species such as house sparrows as reservoirs of *Salmonella* spp. is well established (Alley et al. 2002).

Against veterinary advice, a takahe which tested positive for *Salmonella* spp. at the time of translocation was moved from the private island to the breeding centre. One week later when retested in breeding centre, the takahe was positive for *S.* Saintpaul, an unrelated serotype (Table 6-1). Given the short time period between sampling and limitations of *Salmonella* culture, it may be possible the bird was carrying multiple serotypes of *Salmonella* spp. at both time points. *Salmonella* Saintpaul is a common serotype and has been isolated from a range of taxa, including wildlife (Iveson et al. 2009; Middleton et al. 2014; Parsons et al. 2010), and humans (Munnoch et al. 2009). Therefore, an alternative explanation may be that the takahe was exposed to *S.* Saintpaul during translocation or at the new site and the two serotypes isolated represent separate infection events. It is worth noting the translocation of a *Salmonella* spp. positive individual into a breeding centre, a site identified as a dispersal hub and important in terms of connectivity to the takahe population network (Grange et al. 2014), poses a substantial risk of spread of *Salmonella* spp. to takahe within the location, and forward transmission to other populations. With known relative stability in faeces, soil and water for up to and over a year, *Salmonella* spp. may remain viable in the environment for long durations (Winfield & Groisman 2003). Therefore, transmission of *Salmonella* spp. may occur from environmental sources long after an individual has stopped shedding.

Asymptomatic carriage of *Salmonella* spp. in apparently healthy takahe in this study suggests that, in these cases, there was a non-pathogenic host-microbe association. However, the opportunistic pathogenicity of *Salmonella* spp. and its association with reproductive and enteric disease in many species suggests that caution should be used in extrapolating these cases to all serotypes of *Salmonella* in takahe. The process of capture and translocation can have health impacts on the host. For example, the process of translocation could result in significant physiological stress of individuals making them vulnerable to disease outbreaks, as occurred with *Erysipelothrix rhusiopathiae* outbreaks in translocated kakapo (*Strigops habroptilus*) (Gartrell et al. 2005). Additionally, reserves are also home to many other threatened species, many of which are also subject to translocation regimes. Therefore cross-species transmission is a substantial concern where pathogenicity and impact of infection is unknown. Further work is required to understand the ecology and epidemiology of *Salmonella* spp. in the island ecosystem to determine source attribution of *Salmonella* spp. isolated from takahe, which may lead to mitigation and prevention of transmission of *Salmonella* spp. to takahe.

6.6. Acknowledgements

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CHAPTER 7

GENERAL DISCUSSION

7. General Discussion

7.1. Microbial dynamics in translocated takahe (*Porphyrio hochstetteri*)

This study was the first of its kind to explore microbial dynamics in a large proportion of a well-described but fragmented population of a critically endangered bird, the takahe (*Porphyrio hochstetteri*). Sub-populations are connected via a network of movements resulting from historical translocation events. Here I discuss our ability to capture and quantify the epidemiology of infectious organisms resulting from the translocation and isolation of wildlife populations using a case study of microbial dynamics in translocated takahe (*Porphyrio hochstetteri*). The effects of translocation and isolation of takahe were investigated using an array of different techniques and revealed new insights into host-microbe relationships resulting from translocations.

I used a novel approach to explore the history of movements between remote populations of takahe. This study revealed a complex network of sub-populations which were likely to vary in their propensity to spread and maintain pathogens (Chapter 2 ; Grange et al. 2014). After describing the conservation network with descriptive social network principles, I carried out empirical investigations using a commensal enteric bacterium, *Campylobacter* spp., to explore meta-population microbial dynamics by sampling takahe in multiple locations. Traditional multivariate and innovative genomic investigations identified that population isolation and conservation management of the takahe hosts has influenced the carriage and population structure of *Campylobacter* spp. genotypes (Chapter 3 ; Chapter 4).

My study suggests the intensive conservation management of takahe that resulted in a range expansion following a significant bottleneck (Ballance 2001) has had unforeseen impacts on host-microbe relationships and transmission dynamics (Torchin & Mitchell 2004). The management of takahe in different environmental settings has influenced the carriage of *Campylobacter jejuni* and *Campylobacter coli* (Chapter 3). A newly discovered rail-associated *Campylobacter* sp. *nova 1* was prevalent in all populations (Chapter 3). However, more discriminatory whole genome analysis of isolates detected a significant biogeographic variation in *C.* sp. *nova 1* genotypes (Chapter 4). Possible explanations for the observed pattern include spatial expansion and isolation of hosts resulting in reduced gene flow of *Campylobacter* spp. and allopatric speciation, the presence of heterogeneous environmental attributes or cross-species transmission of *Campylobacter* spp. from reservoir hosts in the same location. An assessment of other vertebrate reservoirs in the island ecosystem indicated cross-species transmission of *Campylobacter* spp. may be a factor

contributing to the maintenance and phylogeographical distribution of *Campylobacter* spp. in takahe. Indeed, if spill-over of *Campylobacter* spp. were to occur, it would most likely transmit between takahe and other closely related hosts, such as weka (*Gallirallus australis*) (Chapter 5). Geographical variations in the genotypes of *Campylobacter* spp. carried by the host, suggests isolation and environmental variables are influencing the ongoing evolution of *Campylobacter* spp. Isolation can therefore allow continued evolution of the *Campylobacter* spp. carried by takahe, which may allow a change in state from their current benign nature, to more pathogenic strains that might affect their avian hosts (Waldenstrom et al. 2010) or their human caretakers.

Molecular detection and genomic analysis of *Campylobacter* sp. *nova 1* has revealed new insights into the taxonomy of the genus *Campylobacter*. This study discovered previously undetected diversity within the putative species and identified a relatively close relationship between *C.* sp. *nova 1* and the two human pathogenic species: *C. coli* and *C. jejuni* (Chapter 4). Evidence of recombination between different genotypes within the species (Chapter 4) and between known and unidentified *Campylobacter* species (Chapter 5), suggests a complex ancestry and calls into question our definitions of *Campylobacter* species. Geospatial clustering of *C.* sp. *nova 1* genotypes, combined with the relatively recent translocation events, provides evidence to support a theory that the species is able to adapt and evolve within a relatively short time frame in an isolated host.

Given evidence of host association of *C.* sp. *nova 1* genotypes (Chapter 5 ; French et al. 2014), it is likely that ongoing relocation of takahe provides a route for co-infections and recombination of alleles between genotypes explaining the present day genomic structure of isolates (Chapter 4). Trans-boundary transmission and carriage of *C.* sp. *nova 1* is supported by the isolation of a diverse range of genotypes from the breeding population (Chapter 4). Further study is required to explain the strong association between population connectivity, location and *C.* sp. *nova 1* genotypes. However, I hypothesise historic and current management practices are having unforeseen influences on enteric microbes, the consequences of which are unknown but may be detrimental to the health of translocated populations of takahe. This includes exposure to strains of micro-organisms from food producing animals that may result in increased pathogenicity, antibiotic resistance or novel challenges to host immunity.

The host-pathogen dynamic described for takahe and *Campylobacter* spp. will be different for different species of micro-organisms. In my study, this was examined using another enteric organism, *Salmonella* spp., which has a greater potential for pathogenic effects. The prevalence of *Salmonella* spp. in takahe

(Chapter 6) was significantly lower than that observed for enteric *Campylobacter* spp, with population location being associated with the gastro-intestinal carriage of *Salmonella* spp. in takahe populations. The organism was only isolated from takahe resident on one island, and an individual translocated to the breeding centre (Chapter 6). *Salmonella* spp. have been historically included in disease screening protocols for takahe (McInnes et al. 2004), with individuals barred from translocation if they test positive for *Salmonella* spp. Potentially this may have prevented the spread of *Salmonella* spp. throughout the takahe population, thus explaining the low prevalence observed. However, given the generalist nature of *Salmonella* spp. and ubiquity in the environment, it seems unlikely that this explains the spatial patterns observed in this study. While more work is needed to definitively identify the reservoirs of *Salmonella* spp., the serotypes isolated, *S.* Mississippi and *S. houtenae* 40;gt were suggestive of the presence of local environmental or animal reservoirs. In this case, the *Salmonella* spp. showed no external evidence of causing pathogenic effects on the takahe. However, *Salmonella* spp. have caused mortality, enteritis and reproductive disease in birds in New Zealand (Alley et al. 2002). It is likely that the cases of *Salmonella* spp. in takahe represented a short term host-pathogen dynamic where the takahe were spill-over hosts (Chapter 6), Although little is known about the pathogenicity of *Salmonella* spp. in takahe, it is nevertheless an example of the potential for pathogen transmission between nodes of the takahe network (Chapter 6).

The observational design of these studies of bacterial population genomics in a well-described host have a number of limitations, largely as a result of potential biases introduced by non-random sampling and confounding between variables. However, they did provide insights into the epidemiology of infectious organisms in wild fragmented populations. They also highlight the value of working with previously unidentified host-associated bacterial species and using genomic sequencing. Although it is uncertain whether an investigation into a commensal organism can be used to predict or prevent an epidemic disease emergence or transmission, the finding of management associated genomic differences in a commensal bacterium justifies the need for further investigation of pathogenic organisms within this and other fragmented ecosystems.

7.2. Disease risks associated with translocations

Disease risks associated with translocations of wildlife were addressed in a seminal publication conducted by Cunningham et al. (1996). In the last 14 years, significant advances in our ability to detect and monitor pathogens have improved our epidemiological understanding of pathogen dynamics in natural ecosystems.

Here I evaluate how this study has contributed to new perspectives on disease risk associated with current wildlife management conservation practices.

The role and significance of infectious disease impacts on wildlife remains relatively uncertain. The threat and impact of disease appears to increase as species move closer to extinction (Heard et al. 2013), justifying the need for protection against unnecessary pathogen incursions. The IUCN wildlife health specialist group (IUCN WHSG 2014) and institutes such as the World Organisation for Animal Health (OIE) (OIE 2014) provide guidance upon such matters relating to disease risks associated with the conservation and translocation of wildlife (Jakob-Hoff et al. 2014; OIE & IUCN 2014). However, many translocations occur without sufficient pre- and post-release disease monitoring (Chapter 6 ; Griffith et al. 1993), making it more difficult to assess the role of infectious disease in programme failure.

Correlations between host and pathogen dynamics are inevitable due to dependency of pathogens on their host for survival (Tompkins et al. 2011). Thus any changes in host conditions resulting from translocation may alter this relationship. Although justified for reasons relating to management of small populations, forced perturbation of population dynamics in endangered populations is likely to increase the risk of pathogenic incursions (Torchin & Mitchell 2004). Additionally, translocation mediated stress (Teixeira et al. 2007) may induce immunosuppression and increase susceptibility to disease in the species of concern (Kock et al. 2007; Viggers et al. 1993). Disease can have significant impacts on populations and if an outbreak were to occur within an endangered species it could take decades to recover, particularly if they have low reproductive output (Rushmore et al. 2013). Pathogen incursions may not necessarily result in mortality, instead impacting hosts in subtle ways causing a reduction in fitness, reproduction and changes in behaviour. Pathogen associated morbidity can be difficult to detect, for example animals infected with *Toxoplasma gondii* show no overt symptoms of disease but the protozoan can influence the behaviour of its host, increasing risk and likelihood of transmission between mice and cats (Berdoy et al. 2000).

Co-evolutionary interactions between hosts and their pathogens can be determined by spatial characteristics and resulting gene flow (Archie & Ezenwa 2011; Gandon & Nuismer 2009). If infectious organisms are carried across ecological barriers through the translocation process, allopatric speciation of host-adapted organisms is a distinct possibility within the new environment (Chapter 4). Alternatively, host management and the presence of alternative reservoirs may increase opportunities for genetic recombination between infectious organisms (Chapter 4). For example, antibiotic resistance genes were detected in gram negative bacteria isolated from faecal samples of the captive brush-tail rock wallaby (*Petrogale penicillata*) but were

not present in the bacteria isolated from the wild populations (Power et al. 2013). The class 1 integrons were thought to have been acquired from environmental contamination during captivity (Power et al. 2013). Similarly, the genotype diversity of *C.* sp. *nova 1* carried by takahe populations was associated with host location (Chapter 4). The functional significance of the microbial diversity in my study was not determined, but it does demonstrate that translocation has the potential to cause changes to the host's microbiota. The unforeseen developments described above would not be mitigated by current practices of targeted pathogen screening prior to translocation. Most disease surveillance of wildlife translocations is extremely limited with targeted organisms being identified only to genus or species level. This ignores the possibility of detecting strain variation, or novel organisms such as the *C.* sp. *nova 1* identified in this study.

If the isolation of wildlife into sub-populations is resulting in biogeographic divergence of micro-organisms (Chapter 4), a natural question arises whether conservation practices should be less conservative and increase mixing of sub-populations with the aim of building immunity and resilience for pathogen incursions. A study of plant-fungal relationships found that highly connected host populations experienced lower incidence of pathogens due to increased levels of disease resistance (Jousimo et al. 2014). Many infectious organisms exhibit variation in pathogenicity and can be unpredictable if transmitted to naive hosts; what is a commensal in one species may be a pathogen in another (Waldenstrom et al. 2010). For instance, Hendra virus is carried asymptomatically by bats of the genus *Pteropus* in Australia (Halpin et al. 2011). Incidental viral spill-over into horses or humans in areas where bat colonies overlap with urban centres (Plowright et al. 2011) results in disease symptoms and occasionally death in the new host (Daszak et al. 2006; Playford et al. 2010). Translocation decisions and risk assessments should ideally account for potential reservoirs of pathogens within a location and the possibility of bidirectional transmission of generalist infectious organisms able to colonise and transmit between sympatric wildlife. However, the logistical difficulty of accounting for the increasingly complex interactions of the host and microbiome described in my study make accurate risk assessment of translocations problematic, especially with the limited budgets available to most conservation programmes.

Our ability to inform conservation management is hindered by our inability to capture the influence of complex interactions between hosts and environments and their potential confounding effects on pathogen diversity. *In situ* investigations of wildlife epidemics are insightful, however free living animals are frequently inconspicuous especially if afflicted by illness and the recovery of samples from unhealthy or deceased individuals may be logistically difficult. Although currently underexploited, non-invasive epidemiological investigations of host-commensal relationships in wildlife populations are a good proxy for

the study of infectious disease epidemiology and as a result are gradually increasing in popularity, e.g. (Bull et al. 2012; Chapter 3 ; Chapter 4 ; Chapter 5 ; Chiyo et al. 2014). Recent advances in epidemiological tools, methods and interdisciplinary research are starting to improve our understanding of pathogen dynamics and may in time improve the accuracy of our forecasting of disease risk associated with translocations of wildlife.

7.3. Advancing tools for epidemiological investigations of wildlife

Applied disease ecology is a new and emerging concept which is rapidly evolving into an interdisciplinary field of research. Collaborations between diverse sectors from conservationists through to bioinformaticians are creating new tools for infectious disease research. Here I discuss how this study has informed new approaches to epidemiological investigations of host-microbe and by inference host-pathogen relationships in wildlife.

The development of network models for use on translocation databases is an interesting example of applying social network methods to conservation management (Grange et al. 2014). In chapter 2, I analysed historic translocation records to assess takahe sub-population dynamics and identify targets for disease surveillance. Predictive social network models enable conservation managers to visualise current and historic population connectivity, and geography to inform the risks associated with translocation of individuals between fragmented populations.

Epidemiological frameworks have been developed to incorporate genomic data with multivariate models encompassing both pathogenic and host associated attributes (Chapter 4 ; Kao et al. 2014). Genetic adaptation of infectious organisms within a relatively short time frame provides an ideal system to explore the subtle effects of geographic isolation on the evolution and diversity of microbes in fragmented populations (Chapter 4). Ribosomal multi-locus sequence typing (rMLST) derived from whole genome sequences is a promising tool for the exploration of genomic differences within and between all bacterial genera (Jolley et al. 2012). Investigations have shown it is a good proxy for comparisons of the core genome and requires less computational effort (Chapter 4). rMLST methods provide higher resolution comparisons than traditional molecular typing (Chapter 3) and output can be incorporated into multivariate analysis of biotic and abiotic variables acquired from epidemiological investigations (Chapter 4). However, as with other typing methods reliant on culture of isolates, limitations remain in capturing the full diversity of microbes present within and between hosts. Although currently costly, genomic typing of pathogens is likely to be a major contributor to epidemiological investigations in the future. With the invention of portable

sequencing tools such as the Oxford MinION (Oxford Nanopore Technologies, Oxford, UK) (Eisenstein 2012), rapid testing of wildlife in field situations may be available in the near future. Understanding the functional differences that result from changes in microbial genetic diversity will also be important if we are to reliably predict shifts in important characteristics such as pathogenicity, transmission and antimicrobial resistance.

7.4. Implications for conservation management

Recommended pre-translocation health and pathogen screening has been founded upon theoretical risk assessments (Jakob-Hoff et al. 2014; OIE & IUCN 2014). The lack of resolution in current pathogen testing protocols and empirical research has impeded the formation of robust scientific foundations upon which costly pathogen screening can be justified. Currently, animals may be translocated with identified or unknown parasites which may impact health and population reestablishment due to incomplete identification of pathogens affecting the target species (Sainsbury & Vaughan-Higgins 2012). The development of high resolution analyses afforded by whole genome sequencing of pathogens, as used in this study, provides a more incisive method for the investigation of wildlife disease. As the cost of new technologies decreases, incorporation of these methods into translocation policy is the most beneficial approach to the identification and mitigation of disease.

Historic movement and infection records have important roles in epidemiological studies and have been used for retrospective analysis of pathogenic outbreaks in humans (Gardy et al. 2011), domestic animals (Ortiz-Pelaez et al. 2006) and are now being applied to wildlife. For example, the microbial genomics of *Campylobacter* sp. *nova 1* may have been shaped by the effects of host isolation, the presence of reservoir hosts, and heterogeneous population interactions resulting from historic and current population management (Chapter 4). However, ambiguous movement and health records of translocated individuals limit the ability to assess the importance of disease in translocation failure (Grange et al. 2014). Informative epidemiological investigations require accurate recording of movements and maintenance of wildlife health databases for all government and community controlled population manipulations of wildlife. Similarly, publically archiving full genome information on current micro-organisms, including commensals carried by these animals, will allow future studies to document shifts in the microbial dynamics over time.

Captive management and breeding in artificial environments has been the mainstay of population preservation and augmentation in New Zealand. Intermixing of individuals from sub-populations within a breeding centre can bring together previously isolated individuals into sympatry. These animals may carry

their own endemic infectious organisms which have evolved during a period of isolation into a breeding facility. This creates an opportunity for a diverse range of potential pathogens to mix in the same environment (Chapter 4). In order to prevent co-infection and inter-host transmission of pathogens, a period of quarantine, spelling and decontamination of enclosures is recommended. If a novel pathogen is acquired during captivity, the process of supplementing wild populations with captive bred individuals derived from connected 'hub' populations could facilitate long range dissemination of potentially novel pathogens throughout a population network (Grange et al. 2014). High levels of biosecurity and disease screening of all immigrant and emigrant individuals moving through a breeding facility is highly recommended. However, disease screening currently relies on a pre-existing knowledge of the micro-organisms which are of importance to the species in care, balanced against the costs and availability of testing.

In captivity and in some wildlife sanctuaries, the atypical interface between humans, wildlife and livestock increases interactions and the threat of introduction of exotic pathogens from reservoirs. Although there are obvious benefits to the targeted use of 'advocacy individuals' to promote conservation awareness (e.g. Sirrocco the Kakapo), vulnerable species maintained in open reserves may be at increased risk of exposure to novel pathogens via interaction with humans. In an increasingly connected world, international travel increases the potential for long range pathogen transmission. A notable example is the 2014 reports of people infected with Ebola virus arriving in uninfected countries including the United States (WHO 2014b) and Europe (WHO 2014a) after long distance air travel. Interactions between vulnerable wildlife with humans from around the globe could act as a route for exposure to a complex array of exotic micro-organisms. Contacts of this nature add another level of complexity and threat when assessing risks of wildlife translocation.

When considering the choice of locations used for the introduction and maintenance of endangered species, decisions are rarely based on reducing the threats of pathogen introduction from co-habiting animals. For example, captive reared Arabian oryx (*Oryx leucoryx*) succumbed to capripox virus infection transmitted from domestic sheep grazing along the boundaries of the oryx enclosure (Greth et al. 1992). The installation of buffer zones around reserves and relative isolation from agricultural sources may aid in reducing the likelihood of pathogen transmission from exotic sources (Chapter 3), but will increase the costs of maintaining such reserves. Co-habitation of closely related species within a reserve would not be recommended since research suggests taxonomically related hosts and those whom share behavioural traits are more likely to share pathogens (Chapter 5). An investigation into rabies virus in bat species in North America showed an association between host phylogenetic distance and frequency of cross-species

transmission (Streicker et al. 2010). The likelihood of transmission was lower between bat species that were less closely related (Streicker et al. 2010). Conversely, bringing livestock species directly into contact with endangered wildlife, such as the sheep that shared the takahe sanctuary of Maud Island (Chapter 5), also carries risks of exposing the wildlife to novel micro-organisms that have developed undesirable characteristics such as antibiotic resistance through modern husbandry methods.

Targeting pathways connecting populations through which pathogens can be transmitted has been used as a means of mitigation of disease spread for centuries. The same principles which applied to the quarantine of 'typhoid Mary' in the early 19th Century (Leavitt 1996) are transferable to the mitigation of pathogen spread between susceptible wildlife populations. Quarantine and biosecurity when moving and introducing previously isolated animals into new populations are simple but effective measures to reduce risk. In the hospital setting, education and disinfection measures targeting medical staff that act as bridges between patients, has helped reduce the transmission of multi-drug resistant bacteria between hospitalised patients (Allegranzi & Pittet 2009; Pittet et al. 2006). Transferring the same principles to the management of our endangered wildlife through biosecurity and cleaning of equipment is likely to mitigate the risk of pathogen spread and introduction.

Although there is no current evidence of management actions causing detrimental effects due to infectious disease in takahe conservation, the example of a *Salmonella* spp. positive takahe being moved from a location known to have *Salmonella* spp. positive individuals into quarantine at a breeding centre (Chapter 6) calls into question the efficacy of disease screening recommendations, and more importantly the need for better communication between researchers, veterinarians and conservation managers. Epidemiological investigations into the sources of the generalist pathogens, including obtaining prevalence estimates within each location and testing environmental sources such as food and water sources, can lead to practical mitigation strategies that reduce the risk of infection in the species of concern.

7.5. Future research directions

Threatened wildlife populations are frequently afflicted by a combination of stressors acting synergistically (Brook et al. 2008; Heard et al. 2013; Munns 2006), including habitat loss, predation, invasive species and pathogens. Sampling strategies should account for the mode of transmission of the infectious organism within and between host populations, reservoirs, and the environment in an attempt to determine transfer pathways. Provision of genomic epidemiological data to inform transmission models could translate into a

more comprehensive understanding of threats or risks posed to a species for use in conservation practice decisions.

Family and group dynamics of the host can influence microbial carriage in social species (Caillaud et al. 2013). Overlaying pathogen transmission data with population interactions, and host interaction within populations, was not fully explored in this study but could be approached in a similar manner using proximity contact data and faecal testing for *Campylobacter* spp. In a unique study, researchers in New Zealand are artificially infecting wild brushtail possums (*Trichosurus vulpecula*) with *Mycobacterium bovis*, and are monitoring spread of the organism through social interactions via mark-recapture and proximity collar network analysis (Rouco et al. 2013).

This exploration of microbial evolution and transmission dynamics in takahe was designed as an observational cross-sectional study of microbes in the host species. Repeated sampling over time was not carried out due to the limitations of capturing and sampling wild populations. However, it is recognised that the relationship between a host and a pathogen can be variable over time. One approach to investigate the effects of time would be to conduct a longitudinal investigation into host-pathogen dynamics, but this can be costly in both time and resources. Phylogenetic and coalescent models incorporate a temporal and spatial component and have been used in the analysis of bacterial genetics derived from wildlife (Girard et al. 2004). Applying these methods to bacterial genomics is in its infancy, with most reported studies based on viral genomes (Biek & Real 2010). For example, *post hoc* phylogenetic and coalescent analysis in combination with ecological information suggest the novel avian influenza A H7N9 emerged from multiple gene reassortment events from duck and chicken influenza viruses, suggesting these species may have acted as intermediate hosts (Liu et al. 2013). Bayesian phylogenetic programmes such as BEAST2 (Bouckaert et al. 2014) could be used to retrospectively analyse the influence of historic management practices on the existing diversity of infectious organisms within managed populations. For example, it would be interesting to investigate the temporal component of takahe translocations from the wild to other facilities in the context of gene recombination between *C.* sp. *nova 1* isolates from those populations.

Microbial CRISPR (clustered regularly interspaced short palindromic repeats) typing is an emerging method for genomic resolution between bacterial isolates (Cain & Boinett 2013; Kovanen et al. 2014). Bacterial CRISPRs are DNA loci comprised of short repeat sequences bookmarking non-repetitive spacer DNA insertions of mobile genetic elements derived from interactions with foreign DNA (Jansen et al. 2002). CRISPR-*cas* systems have a role in microbial adaptive immunity and other roles in genetic regulation

(Westra et al. 2014). The identification and characterisation of spacer DNA through tools such as CRISPRTarget (Biswas et al. 2013) and CRISPRDetect (Biswas et al. 2014) can create an effective barcode of historic interactions between bacterial species, and when combined with host data could provide an opportunity to record the subtle effects of translocation has upon the gut microbial community.

This study has only just begun to explore the effects of translocation on a single species of micro-organism. Traditional methods restrict our ability to capture the full diversity of a microbe within a host. Multiple carriage of *Campylobacter* spp. was observed within takahe but limitations in detection were apparent when comparing selective culture and DNA extraction from faecal samples (Chapter 3). Although some randomisation was used in the selection of isolates, more in-depth studies exploring the microbial genomic diversity within takahe would help confirm whether the observed biogeographic patterns of *C.* sp. *nova 1* were real not just an artefact of selective sequencing of a limited number of isolates.

Microbial communities occupying a host are the result of complex interactions and niche specialisation. If this study were to be viewed as an indicator of the effect translocation can have upon a single species, it would be likely that perturbation of host lifestyle would alter the microbial ecosystem within a host. An emerging method is to explore differences in microbiota using metagenomic DNA analysis. Comparison of microbial DNA obtained from skin swabs of co-habiting frogs using metagenomic sequencing of 16S rRNA genes revealed host species as a predictor of microbial community (McKenzie et al. 2012). Studies in New Zealand have used 16S rRNA pyrosequencing techniques to assess the gastrointestinal microbiota of endangered kakapo (*Strigops habroptilus*) and found differences in microbial communities along the gastrointestinal tract (Waite et al. 2012), and an association between age and captive rearing on the faecal microbiota of young birds (Waite et al. 2014). With improvements in faecal extraction methods (Vo & Jedlicka 2014), a broad scale comparison of microbial communities in translocated populations would be an interesting avenue to explore.

7.6. Concluding remarks

The evolutionary responses of pathogens to changes in host management and biodiversity are emerging areas of interest but still require further investigation before they can be applied in a conservation management framework (Joseph et al. 2013). Research into communicable organisms in translocated populations will increase our understanding of threats posed to conserved populations, and be a useful traceable system to inform epidemiological models of infectious disease in natural environments. The case study of the dynamics of microbes within the takahe hosts presented in this thesis is a preliminary example of the insights

fragmented and translocated populations can provide into the epidemiology of epidemics in endangered species. Epidemiological research in endangered species has been limited for reasons of protection and minimal disturbance in vulnerable species. This study has been one of the first to demonstrate the benefits of opportunistic, non-invasive sampling alongside normal management practices. Therefore, I encourage the integration of non-invasive cross-sectional and longitudinal pathogen investigations into pre- and post-translocation policies. The use of whole genome sequencing and bioinformatic tools to quantify the genetic structure of pathogens in wildlife settings is at the leading edge of disease ecology research and is likely to be more frequently reported in the literature in the future.

CHAPTER 8

LITERATURE CITED

8. Literature Cited

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Chapter 4. Wildlife translocation and the evolution and population structure of a host-associated commensal *Campylobacter* spp.

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CHAPTER 9

APPENDIX

9. Appendix

9.2. Chapter 2 supplementary information

Appendix 9.2-1 Map of New Zealand (including region boundaries) and key demonstrating the location of takahe reserves and treatment centres involved in this network analysis. Node H is not shown as it is a private island not under control of the NZ Department of Conservation.

Year	From location	From letter	To location	To letter
2007	Burwood Bush breeding centre	A	Maud Island	D
2007	Burwood Bush breeding centre	A	Tiritiri Matangi Island	J
2007	Mana Island	C	Wildbase Hopsital	G
2007	Wildbase Hopsital	G	Maud Island	D
2007	Tiritiri Matangi Island	J	Burwood Bush breeding centre	A
2007	Tiritiri Matangi Island	J	Burwood Bush breeding centre	A
2007	Tiritiri Matangi Island	J	Burwood Bush breeding centre	A
2007	Tiritiri Matangi Island	J	Mana Island	C
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Burwood Bush breeding centre	A	Murchison Mountains	Q
2007	Tiritiri Matangi Island	J	Murchison Mountains	Q
2007	Tiritiri Matangi Island	J	Murchison Mountains	Q
2007	Tiritiri Matangi Island	J	Murchison Mountains	Q
2007	Murchison Mountains	Q	Burwood Bush breeding centre	A
2007	Murchison Mountains	Q	Burwood Bush breeding centre	A
2007	Murchison Mountains	Q	Burwood Bush breeding centre	A
2007	Murchison Mountains	Q	Burwood Bush breeding centre	A
2007	Murchison Mountains	Q	Burwood Bush breeding centre	A
2007	Murchison Mountains	Q	Burwood Bush breeding centre	A
2007	Murchison Mountains	Q	Burwood Bush breeding centre	A
2007	Murchison Mountains	Q	Burwood Bush breeding centre	A
2007	Murchison Mountains	Q	Burwood Bush breeding centre	A
2008	Burwood Bush breeding centre	A	Mana Island	C
2008	Burwood Bush breeding centre	A	Mana Island	C
2008	Burwood Bush breeding centre	A	Mana Island	C
2008	Burwood Bush breeding centre	A	Wildbase Hopsital	G
2008	Burwood Bush breeding centre	A	Wildbase Hopsital	G
2008	Kapiti Island	B	Burwood Bush breeding centre	A
2008	Mana Island	C	Burwood Bush breeding centre	A
2008	Mana Island	C	Burwood Bush breeding centre	A
2008	Mana Island	C	Pukaha Mt Bruce	F
2008	Mana Island	C	Pukaha Mt Bruce	F
2008	Maud Island	D	Burwood Bush breeding centre	A
2008	Pukaha Mt Bruce	F	Wildbase Hopsital	G
2008	Wildbase Hopsital	G	Mana Island	C
2008	Wildbase Hopsital	G	Pukaha Mt Bruce	F

Year	From	From letter	To	To letter
2008	Tiritiri Matangi Island	J	Burwood Bush breeding centre	A
2008	Burwood Bush breeding centre	A	Murchison Mountains	Q
2008	Burwood Bush breeding centre	A	Murchison Mountains	Q
2008	Burwood Bush breeding centre	A	Murchison Mountains	Q
2008	Burwood Bush breeding centre	A	Murchison Mountains	Q
2008	Burwood Bush breeding centre	A	Murchison Mountains	Q
2008	Burwood Bush breeding centre	A	Murchison Mountains	Q
2008	Burwood Bush breeding centre	A	Murchison Mountains	Q
2008	Burwood Bush breeding centre	A	Murchison Mountains	Q
2008	Mana Island	C	Murchison Mountains	Q
2008	Tiritiri Matangi Island	J	Murchison Mountains	Q
2008	Maud Island	D	Murchison Mountains	Q
2008	Mana Island	C	Murchison Mountains	Q
2008	Maud Island	D	Murchison Mountains	Q
2008	Murchison Mountains	Q	Burwood Bush breeding centre	A
2008	Murchison Mountains	Q	Burwood Bush breeding centre	A
2008	Murchison Mountains	Q	Burwood Bush breeding centre	A
2009	Burwood Bush breeding centre	A	Maud Island	D
2009	Burwood Bush breeding centre	A	Secretary Island	M
2009	Burwood Bush breeding centre	A	Secretary Island	M
2009	Kapiti Island	B	Burwood Bush breeding centre	A
2009	Kapiti Island	B	Wildbase Hopsital	G
2009	Kapiti Island	B	Wildbase Hopsital	G
2009	Mana Island	C	Burwood Bush breeding centre	A
2009	Mana Island	C	Burwood Bush breeding centre	A
2009	Mana Island	C	Burwood Bush breeding centre	A
2009	Mana Island	C	Maungatautari Island reseve	E
2009	Maud Island	D	Maungatautari Island reseve	E
2009	Wildbase Hopsital	G	Burwood Bush breeding centre	A
2009	Wildbase Hopsital	G	Kapiti Island	B
2009	Wildbase Hopsital	G	Te Anau Wildlife Reserve	I
2009	Private island	H	Peacock Springs Wildlife Park	L
2009	Private island	H	Peacock Springs Wildlife Park	L
2009	Private island	H	Peacock Springs Wildlife Park	L
2009	Te Anau Wilidlife Reserve	I	Wildbase Hopsital	G
2009	Tiritiri Matangi Island	J	Burwood Bush breeding centre	A
2009	Tiritiri Matangi Island	J	Burwood Bush breeding centre	A
2009	Burwood Bush breeding centre	A	Murchison Mountains	Q
2009	Burwood Bush breeding centre	A	Murchison Mountains	Q
2009	Burwood Bush breeding centre	A	Murchison Mountains	Q
2009	Burwood Bush breeding centre	A	Murchison Mountains	Q
2009	Burwood Bush breeding centre	A	Murchison Mountains	Q
2009	Burwood Bush breeding centre	A	Murchison Mountains	Q
2009	Burwood Bush breeding centre	A	Murchison Mountains	Q
2009	Burwood Bush breeding centre	A	Murchison Mountains	Q

Year	From	From letter	To	To letter
2009	Burwood Bush breeding centre	A	Murchison Mountains	Q
2009	Mana Island	C	Murchison Mountains	Q
2009	Tiritiri Matangi Island	J	Murchison Mountains	Q
2009	Kapiti Island	B	Murchison Mountains	Q
2009	Tiritiri Matangi Island	J	Murchison Mountains	Q
2009	Mana Island	C	Murchison Mountains	Q
2009	Murchison Mountains	Q	Burwood Bush breeding centre	A
2009	Murchison Mountains	Q	Burwood Bush breeding centre	A
2009	Murchison Mountains	Q	Burwood Bush breeding centre	A
2009	Murchison Mountains	Q	Burwood Bush breeding centre	A
2010	Burwood Bush breeding centre	A	Kapiti Island	B
2010	Burwood Bush breeding centre	A	Kapiti Island	B
2010	Burwood Bush breeding centre	A	Maud Island	D
2010	Burwood Bush breeding centre	A	Wildbase Hopsital	G
2010	Burwood Bush breeding centre	A	Wildbase Hopsital	G
2010	Burwood Bush breeding centre	A	Secretary Island	M
2010	Burwood Bush breeding centre	A	Secretary Island	M
2010	Burwood Bush breeding centre	A	Secretary Island	M
2010	Burwood Bush breeding centre	A	Willowbank Reserve	O
2010	Burwood Bush breeding centre	A	Willowbank Reserve	O
2010	Kapiti Island	B	Burwood Bush breeding centre	A
2010	Kapiti Island	B	Burwood Bush breeding centre	A
2010	Kapiti Island	B	Burwood Bush breeding centre	A
2010	Kapiti Island	B	Burwood Bush breeding centre	A
2010	Kapiti Island	B	Tiritri Matangi	J
2010	Mana Island	C	Burwood Bush breeding centre	A
2010	Mana Island	C	Burwood Bush breeding centre	A
2010	Mana Island	C	Burwood Bush breeding centre	A
2010	Mana Island	C	Burwood Bush breeding centre	A
2010	Mana Island	C	Burwood Bush breeding centre	A
2010	Mana Island	C	Burwood Bush breeding centre	A
2010	Mana Island	C	Burwood Bush breeding centre	A
2010	Mana Island	C	Burwood Bush breeding centre	A
2010	Mana Island	C	Burwood Bush breeding centre	A
2010	Mana Island	C	Burwood Bush breeding centre	A
2010	Mana Island	C	Wildbase Hopsital	G
2010	Wildbase Hopsital	G	Burwood Bush breeding centre	A
2010	Wildbase Hopsital	G	Te Anau Wildlife Reserve	I
2010	Private island	H	Secretary Island	M
2010	Te Anau Wilidlife Reserve	I	Wildbase Hopsital	G
2010	Te Anau Wilidlife Reserve	I	Wildbase Hopsital	G
2010	Tiritiri Matangi Island	J	Burwood Bush breeding centre	A
2010	Tiritiri Matangi Island	J	Burwood Bush breeding centre	A
2011	Burwood Bush breeding centre	A	Private island	H
2011	Burwood Bush breeding centre	A	Secretary Island	M
2011	Burwood Bush breeding centre	A	Secretary Island	M

Year	From	From letter	To	To letter
2011	Burwood Bush breeding centre	A	Secretary Island	M
2011	Kapiti Island	B	Wildbase Hopsital	G
2011	Mana Island	C	Wildbase Hopsital	G
2011	Mana Island	C	Motutapu Island	K
2011	Mana Island	C	Motutapu Island	K
2011	Mana Island	C	Karori Wildlife Sanctuary	P
2011	Mana Island	C	Karori Wildlife Sanctuary	P
2011	Maud Island	D	Burwood Bush breeding centre	A
2011	Maud Island	D	Wildbase Hopsital	G
2011	Maud Island	D	Wildbase Hopsital	G
2011	Maungatautari Reserve	E	Wildbase Hopsital	G
2011	Maungatautari Reserve	E	Wildbase Hopsital	G
2011	Maungatautari Reserve	E	Motutapu Island	K
2011	Wildbase Hopsital	G	Burwood Bush breeding centre	A
2011	Wildbase Hopsital	G	Kapiti Island	B
2011	Wildbase Hopsital	G	Mana Island	C
2011	Wildbase Hopsital	G	Mana Island	C
2011	Wildbase Hopsital	G	Maud Island	D
2011	Wildbase Hopsital	G	Maungatautari Island reseve	E
2011	Wildbase Hopsital	G	Maungatautari Island reseve	E
2011	Te Anau Wilidlife Reserve	I	Wellington Zoo	N
2011	Tiritiri Matangi Island	J	Motutapu Island	K

Appendix 9.2-2 Dataset includes records of individual movements of takahe (*Porphyrio hochstetteri*) between locations in New Zealand from 2007 to 2011.

	2007			2007 to 2008			2007 to 2009			2007 to 2010			2007 to 2011		
Location	k_i^{in}	k_i^{out}	B_i	k_i^{in}	k_i^{out}	B_i	k_i^{in}	k_i^{out}	B_i	k_i^{in}	k_i^{out}	B_i	k_i^{in}	k_i^{out}	B_i
Burwood Bush breeding centre	4	3	4	10	12	21	14	16	42	17	21	52	18	23	78
Kapiti Island	-	-	-	0	1	0	1	4	0	2	6	0	3	7	0
Mana Island	3	1	4	4	5	5	4	8	11	4	11	8	5	14	19
Maud Island	3	0	0	2	3	6	2	4	0	3	4	4	3	6	0
Maungatautari reserve	-	-	-	-	-	-	2	0	0	2	0	0	3	2	0
Pukaha Mt Bruce	-	-	-	2	1	0	2	1	0	2	1	0	2	1	0
Wildbase Hospital	1	2	2	3	3	5	6	6	30	8	7	26	11	10	61
Private Island	-	-	-	-	-	-	0	2	0	0	3	0	1	3	10
Te Anau wildlife reserve	-	-	-	-	-	-	1	1	0	1	2	0	1	3	9
Tiritiri Matangi Island	1	7	4	1	5	0	1	6	0	2	7	0	2	8	0
Motutapu Island	-	-	-	-	-	-	-	-	-	-	-	-	3	0	0
Peacock springs wildlife park	-	-	-	-	-	-	2	0	0	2	0	0	2	0	0
Secretary Island	-	-	-	-	-	-	1	0	0	3	0	0	4	0	0
Wellington Zoo	-	-	-	-	-	-	-	-	-	-	-	-	1	0	0
Willowbank reserve	-	-	-	-	-	-	-	-	-	1	0	0	1	0	0
Zealandia / Karori sanctuary	-	-	-	-	-	-	-	-	-	-	-	-	1	0	0
Murchison Mountains	3	3	1	11	3	0	15	4	0	15	4	0	15	4	0
Key player	**A**	**J**	**ACD**	**Q**	**A**	**A**	**Q**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**

Appendix 9.2-3 Node level measures, in degree (k_i^{in}), out degree (k_i^{out}), and betweenness (B_i), for takahe (*Porphyrio hochstetteri*) networks used for selection of key players according to highest node measure per year.

9.3.Chapter 3 supplementary information

```
# uses Gibbs steps to estimate unknown prevalence, sensitivity and specificity from table data.

# Data
# number of populations
P <- 3
# number of tests
T <- 2
# counts (one per run)
# C. jejuni with Faecal culture and DNA
counts <- c(0,9,1,24,4,15,7,18,5,2,2,31)

#C. coli with Faecal culture and DNA
counts <- c(2,10,3,19,0,1,0,43,5,5,2,28)

#C. nova 1 with Faecal culture and DNA
counts <- c(20,10,1,3,27,12,0,5,22,12,2,4)

# Priors, assuming Beta distribution with parameters alpha, beta.
p_alpha <- rep(2.90, P)
p_beta  <- rep(3.86, P)
s_alpha <- c(99.70, 47.53)
s_beta  <- c(6.20, 20.94)
c_alpha <- c(130.70, 88.28)
c_beta  <- c(15.41, 1.88)

# MCMC control
iters  <- 10000
burnin <- 1000
thin   <- 10

# Now run the MCMC model...

# Simple test for data entered correctly
if (length(counts) != P*(2^T)) {
  stop("counts must be of length (number of populations)*2^(number of tests)")
}

# Setup matrices for y and m
y <- matrix(counts, nrow=P, ncol=2^T, byrow=T)
m <- matrix(0, nrow=T, ncol=2^T)
for (i in 1:T)
{
  for (k in 1:(2^T))
  {
    # does 2^T-k have bit T-i set?
    r <- (2^T-k) %/% (2^(T-i));
    if (r %% 2)
      m[i,k] <- 1
  }
}

# initial values
```

```
p <- rep(0.5, P) # prevalence
s <- rep(0.5, T) # test sensitivity
c <- rep(0.5, T) # test specificity

# latent true positives for each y
x <- y

# posterior
post <- matrix(NA, iters/thin, P+2*T)
post_names <- rep("",P+2*T)
for (i in 1:P)
  post_names[i] <- paste("Pop",i,"Prev",sep="_")
for (i in 1:T)
  post_names[P-i] <- paste("Test",i,"Sens",sep="_")
for (i in 1:T)
  post_names[P-T+i] <- paste("Test",i,"Spec",sep="_")
colnames(post) <- post_names

# run the MCMC
j <- 1
for (l in 1:(iters - burnin))
{
  # Gibbs steps:
  # 1. Sample from x_{iM}
  for (i in 1:P) {
    for (k in 1:(2^T)) {
      p1 <- p[i];
      p2 <- (1-p[i]);
      for (t in 1:T) {
        if (m[t,k]) {
          p1 <- p1*s[t];
          p2 <- p2*(1-c[t]);
        } else {
          p1 <- p1*(1-s[t]);
          p2 <- p2*c[t];
        }
      }
      x[i,k] = rbinom(1, y[i,k], p1/(p1+p2))
    }
  }
  # 2. Sample from p_i
  for (i in 1:P) {
    #               sx <- 0;
    #               sy <- 0;
    #               for (k in 1:(2^T)) {
    #                       sx <- sx + x[i,k];
    #                       sy <- sy + y[i,k];
    #               }
    # optimisation:
    sx <- sum(x[i,])
    sy <- sum(y[i,])
    p[i] <- rbeta(1, p_alpha[i] + sx, p_beta[i] - sy - sx);
```

```
  }
  # 3. Sample from s_t
  for (t in 1:T) {
    alpha <- s_alpha[t];
    beta  <- s_beta[t];
    for (i in 1:P) {
      # optimisation
      alpha <- alpha + sum(m[t,]*x[i,])
      beta  <- beta  + sum((1-m[t,])*x[i,])
      #                   for (k in 1:(2^T)) {
      #                         if (m[t,k]) {
      #                               alpha <- alpha + x[i,k];
      #                         } else {
      #                               beta <- beta + x[i,k];
      #                         }
      #                   }
    }
    s[t] <- rbeta(1, alpha, beta);
  }
  # 4. Sample from c_t
  for (t in 1:T) {
    alpha <- c_alpha[t];
    beta  <- c_beta[t];
    for (i in 1:P) {
      # optimisation
      alpha <- alpha + sum((1-m[t,])*(y[i,]-x[i,]))
      beta  <- beta  + sum(m[t,]*(y[i,]-x[i,]))
      #                   for (k in 1:(2^T)) {
      #                         if (m[t,k]) {
      #                               beta <- beta + y[i,k] - x[i,k];
      #                         } else {
      #                               alpha <- alpha + y[i,k] - x[i,k];
      #                         }
      #                   }
    }
    c[t] <- rbeta(1, alpha, beta);
  }
  # store sample
  if (l > burnin && (l-burnin) %% thin == 0) {
    post[j,] <- c(p, s, c)
    j <- j + 1;
  }
}

# The model likelihood has multiple nodes, particularly in the case where priors are flat.

# In particular, you can swap prev <-> 1-prev and sens -> 1-spec, spec -> 1-sens and get the exact
same likelihood.

# Relabel the output so that sens>spec is greater than (1-sens+1-spec) on average across all tests
for (i in 1:nrow(post)) {
  sens <- post[i,P+1:T]
```

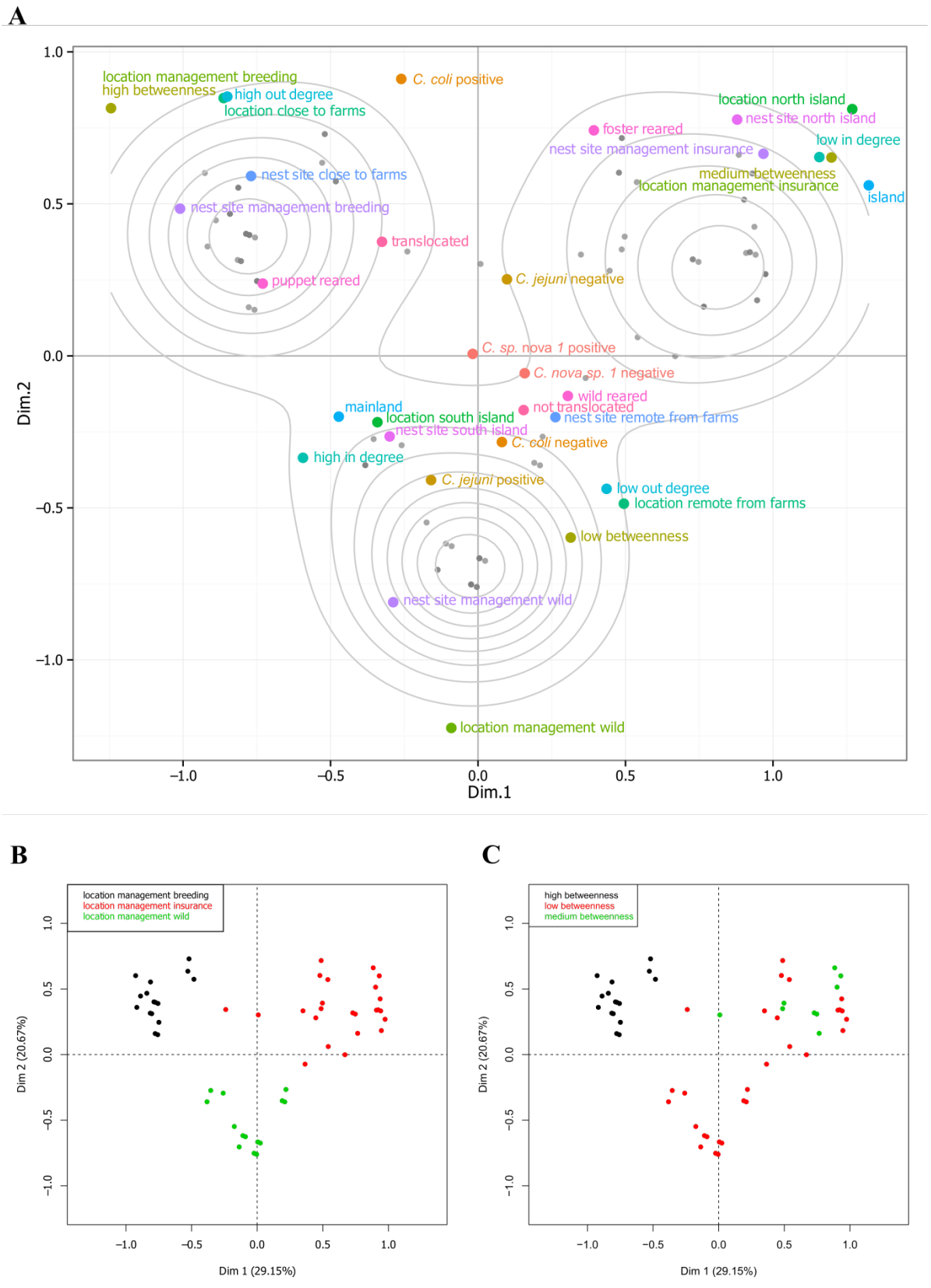
```
  spec <- post[i,P+T+1:T]
  if (sum(sens + spec) < sum(1-sens + 1-spec)) {
    post[i,1:P] <- 1 - post[i,1:P]
    post[i,P+1:T] <- 1 - spec
    post[i,P+T-1:T] <- 1 - sens
  }
}

# Plot posteriors
#pdf("traces.pdf")
par(mfrow=c(3,2))
plot(post[,1], type="l", ylim=c(0,1), main="Prevalence traces")
if (P > 1) {
  for (i in 2:P)
    lines(post[,i], col=i)
}
d <- list(); xlim = NULL; ylim = NULL
for (i in 1:P) {
  d[[i]] <- density(post[,i])
  xlim = range(xlim, d[[i]]$x)
  ylim = range(ylim, d[[i]]$y)
}
plot(d[[1]], ylim=ylim, xlim=xlim, main="Prevalence density")
if (P > 1) {
  for (i in 2:P) {
    lines(d[[i]], col=i)
  }
}

plot(post[,P+1], type="l", ylim=c(0,1), main="Sensitivity traces")
if (T > 1) {
  for (t in 2:T)
    lines(post[,P+t], col=t)
}
d <- list(); xlim = NULL; ylim = NULL
for (t in 1:T) {
  d[[t]] <- density(post[,P+t])
  xlim = range(xlim, d[[t]]$x)
  ylim = range(ylim, d[[t]]$y)
}
plot(d[[1]], ylim=ylim, xlim=xlim, main="Sensitivity density")
if (T > 1) {
  for (t in 2:T) {
    lines(d[[t]], col=t)
  }
}

plot(post[,P+T+1], type="l", ylim=c(0,1), main="Specificity traces")
if (T > 1) {
  for (t in 2:T)
    lines(post[,P+T+t], col=t)
}
```

```
d <- list(); xlim = NULL; ylim = NULL
for (t in 1:T) {
  d[[t]] <- density(post[,P+T+t])
  xlim = range(xlim, d[[t]]$x)
  ylim = range(ylim, d[[t]]$y)
}
plot(d[[1]], ylim=ylim, xlim=xlim, main="Specificity density")
if (T > 1) {
  for (t in 2:T) {
    lines(d[[t]], col=t)
  }
}

# test for difference in prevalence between populations
for (i in 1:(P-1)) {
  for (j in (i+1):P) {
    p <- sum(post[,i] > post[,j])/nrow(post);
    if (p > 0.5)
      p <- 1 - p
    cat("P(population", i, "and",j,"differ)=", p, "\n")
  }
}

# summary
tab <- apply(post, 2, function(x) { c(mean(x), sd(x), quantile(x, c(0.025, 0.975))) })
rownames(tab) <- c("mean", "sd", "2.5%", "97.5%")
print(tab)
```

Appendix 9.3-1 R script for latent class analysis in diagnostic testing

	Model 1[a]	Model 1[b]	Model 2[a]	Model 2[b]	Model 3[a]	Model 3[b]
Se_1	91.4 (84.6-96.5)	52.4 (30.8-83.0)	92.5 (86.5-96.9)	56.4 (31.8-87.1)	97.8 (93.8-99.7)	96.1 (86.8-99.8)
Sp_1	88.3 (83.1-92.8)	85.6 (68.9-99.3)	90.7 (85.9-94.5)	96.4 (89.4-99.8)	88.6 (82.8-93.4)	40.1 (14.6-86.5)
Se_2	62.9 (52.2-73.5)	34.2 (15.2-65.7)	65.3 (54.4-75.7)	30.6 (14.8-52.2)	69.1 (61.9-75.9)	78.5 (60.8-98.0)
Sp_2	95.7 (91.2-99.1)	94.0 (82.8-99.7)	97.7 (94.8-99.6)	97.7 (92.7-100.0)	97.8 (93.8-99.7)	77.3 (46.0-99.0)
Pr_1	16.4 (4.3-33.5)	22.6 (0.01-63.0)	30.6 (12.4-49.7)	60.6 (29.0-94.3)	81.7 (67.2-92.2)	68.1 (28.6-93.3)
Pr_2	40.7 (21.7-59.5)	70.8 (27.6-98.4)	6.3 (1.2-14.8)	0.05 (0.00-15.7)	81.2 (67.8-91.0)	69.2 (31.4-91.7)
Pr_3	20.4 (8.8-33.8)	28.6 (6.2-63.1)	24.7 (10.6-38.9)	42.8 (19.9-75.6)	81.2 (68.1-92.4)	63.9 (24.0-91.2)
P (Pr_1>Pr_2)	0.02	0.01	0.001	0	0.471	0.489
P (Pr_1>Pr_3)	0.334	0.367	0.292	0.15	0.472	0.371
P (Pr_2> Pr_3)	0.035	0.031	0.009	0	0.482	0.361

Appendix 9.3-2 True sensitivity and specificity of faecal DNA / PCR (Se_1/ Sp_1) and culture / PCR (Se_2/ Sp_2) for the detection of *Campylobacter jejuni* (model 1), *Campylobacter coli* (model 2) and *Campylobacter* sp. *nova 1* (model 3), with true prevalence in the three populations; breeding (Pr_1), wild (Pr_2) and insurance (Pr_3) was estimated by conditionally independent Bayesian latent class analysis with ([a]) informative and ([b]) non-informative priors, β (1,1). P is a Bayesian statistical probability estimating differences in prevalence (Pr) between populations where values close to 0 and 1 indicate potential significant differences.



Appendix 9.3-3 Multiple correspondence analysis (MCA) plots demonstrating a) covariate relationships between 118 takahe (*Porphyrio hochstetteri*) where coordinates of the first two dimensions (Dim.1 and Dim.2) explain most of the variation in the data represented. Observations are presented as grey dots on the plot, with density curves illustrating zones where individual observations overlap. Variable categories are assigned by name with subcategories of a variable being colour coordinated. Hierarchical clustering of MCA by b) population management and c) location betweenness.

Variable	Level	Coefficient (SE)	*p*-value	ChiSq *p*-value	
Sex	*Intercept* Female	-	-	0.11	*
	Male	0.62 (0.39)	0.12		
Age	*Intercept* Adult	-	-	0.76	
	Juvenile	-0.14 (0.45)	0.76		
Rearing	*Intercept* Wild	-	-	0.97	
	Foster	-0.24 (1.25)	0.85		
	Puppet	-0.07 (0.42)	0.87		
Nest site	*Intercept* North Island	-	-	0.53	
	South Island	0.28 (0.44)	0.53		
Nest site	*Intercept* Insurance	-	-	0.05	*
	Breeding	1.16 (0.54)	0.03		
	Wild	0.92 (0.46)	0.05		
Location	*Intercept* North Island	-	-	0.09	*
	South Island	0.83 (0.51)	0.11		
Location	*Intercept* Insurance	-	-	<0.01	*
	Breeding	0.36 (0.54)	0.50		
	Wild	1.65 (0.49)	<0.01		
Location	*Intercept* Offshore	-	-	0.01	*
	Mainland	1.22 (0.50)	0.02		
Translocation	*Intercept* No	-	-	0.84	
	Yes	-0.08 (0.41)	0.84		
In degree	*Intercept* High	-	-	0.01	*
	Low	-1.08 (0.44)	0.01		
Out degree	*Intercept* High	-	-	0.36	
	Low	0.37 (0.41)	0.37		
Betweenness	*Intercept* High	-	-	0.18	*
	Low	0.69 (0.45)	0.12		
	Medium	-0.08 (0.65)	0.90		
Location	*Intercept* Close to farms	-	-	0.18	*
	Remote from farms	0.54 (0.41)	0.18		
Nest site	*Intercept* Close to farms	-	-	0.50	
	Remote from farms	-0.29 (0.43)	0.50		
Sampling period	*Intercept* Period 1 (Mar-Apr 2012)	-	-	0.02	*
	Period 2 (Aug-Nov 2012)	-0.56 (0.77)	0.58		
	Period 3 (Feb-Apr 2013)	-2.75 (0.46)	0.01		

*variable was included in a multivariate model

Appendix 9.3-4 Table of *Campylobacter jejuni* univariate analyses

Variable	Level	Coefficient (SE)	*p*-value	ChiSq *p*-value	
Sex	*Intercept* Female	-	-	0.56	
	Male	0.39 (0.68)	0.57		
Age	*Intercept* Adult	-	-	0.59	
	Juvenile	0.38 (0.71)	0.60		
Rearing	*Intercept* Wild	-	-	0.66	
	Foster	14.50 (1385.38)	0.99		
	Puppet	0.30 (0.70)	0.67		
Nest site	*Intercept* North Island	-	-	0.19	*
	South Island	0.84 (0.63)	0.18		
Nest site	*Intercept* Insurance	-	-	0.34	
	Breeding	1.42 (1.11)	0.20		
	Wild	0.41 (0.65)	0.53		
Location	*Intercept* North Island	-	-	0.68	
	South Island	-0.33 (0.81)	0.69		
Location	*Intercept* Insurance	-	-	0.93	
	Breeding	0.14 (0.80)	0.86		
	Wild	-0.14 (0.71)	0.84		
Location	*Intercept* Offshore	-	-	0.92	
	Mainland	-0.07 (0.70)	0.92		
Translocation	*Intercept* No	-	-	0.47	
	Yes	-0.46 (0.62)	0.46		
In degree	*Intercept* High	-	-	0.96	
	Low	0.03 (0.65)	0.97		
Out degree	*Intercept* High	-	-	0.97	
	Low	-0.03 (0.65)	0.97		
Betweenness	*Intercept* High	-	-	0.65	
	Low	-0.03 (0.74)	0.97		
	Medium	-0.73 (0.88)	0.41		
Location	*Intercept* Close to farms	-	-	0.81	
	Remote from farms	-0.15 (0.65)	0.81		
Nest site	*Intercept* Close to farms	-	-	0.11	*
	Remote from farms	-1.42 (1.07)	0.18		
Sampling period	*Intercept* Period 1 (Mar-Apr 2012)	-	-	0.52	
	Period 2 (Aug-Nov 2012)	-0.01 (2306.1)	0.99		
	Period 3 (Feb-Apr 2013)	-0.39 (0.86)	0.70		

*variable was included in a multivariate model

Appendix 9.3-5 Table of *Campylobacter* sp. *nova 1* univariate analyses

Variable	Level	Coefficient (SE)	*p*-value	ChiSq *p*-value	
Sex	*Intercept* Female	-	-	0.25	
	Male	-0.51 (0.44)	0.25		
Age	*Intercept* Adult	-	-	<0.01	*
	Juvenile	1.39 (0.47)	<0.01		
Rearing	*Intercept* Wild	-	-	0.10	*
	Foster	2.16 (1.26)	0.09		
	Puppet	0.69 (0.46)	0.14		
Nest site	*Intercept* North Island	-	-	0.06	*
	South Island	-0.88 (0.46)	0.06		
Nest site	*Intercept* Insurance	-	-	0.03	*
	Breeding	-0.12 (0.54)	0.83		
	Wild	-1.30 (0.55)	0.02		
Location	*Intercept* North Island	-	-	0.29	
	South Island	-0.54 (0.28)	0.28		
Location	*Intercept* Insurance	-	-	<0.01	*
	Breeding	0.61 (0.49)	0.21		
	Wild	-2.91 (1.07)	0.01		
Location	*Intercept* Offshore	-	-	0.50	
	Mainland	0.34 (0.52)	0.51		
Translocation	*Intercept* No	-	-	0.65	
	Yes	0.21 (0.46)	0.65		
In degree	*Intercept* High	-	-	0.26	
	Low	0.51 (0.45)	0.25		
Out degree	*Intercept* High	-	-	<0.01	*
	Low	-1.30 (0.45)	<0.01		
Betweenness	*Intercept* High	-	-	<0.01	*
	Low	-1.61 (0.50)	<0.01		
	Medium	-1.02 (0.67)	0.13		
Location	*Intercept* Close to farms	-	-	<0.01	*
	Remote from farms	-2.23 (0.50)	<0.01		
Nest site	*Intercept* Close to farms	-	-	0.02	*
	Remote from farms	-1.1 (0.46)	0.02		
Sampling period	*Intercept* Period 1 (Mar-Apr 2012)	-	-	0.25	
	Period 2 (Aug-Nov 2012)	0.64 (0.78)	0.52		
	Period 3 (Feb-Apr 2013)	-1.35 (0.52)	0.18		

*variable was included in a multivariate model

Appendix 9.3-6 Table of *Campylobacter coli* univariate analyses

Variable	Level	Coefficient (SE)	*p*-value	ChiSq *p*-value	
Sex	*Intercept* Female	-	-	0.44	
	Male	-0.68 (0.89)	0.45		
Age	*Intercept* Adult	-	-	0.63	
	Juvenile	0.39 (0.80)	0.63		
Rearing	*Intercept* Wild	-	-	0.82	
	Foster	-14.86 (2284.1)	0.99		
	Puppet	-0.10 (0.86)	0.91		
Nest site	*Intercept* North Island	-	-	0.46	
	South Island	0.75 (1.10)	0.50		
Nest site	*Intercept* Insurance	-	-	0.44	
	Breeding	0.96 (0.95)	0.31		
	Wild	-0.18 (1.03)	0.86		
Location	*Intercept* North Island	-	-	0.63	
	South Island	0.54 (1.10)	0.65		
Location	*Intercept* Insurance	-	-	0.40	
	Breeding	-1.30 (1.14)	0.26		
	Wild	-0.85 (0.90)	0.34		
Location	*Intercept* Offshore	-	-	0.33	
	Mainland	-0.80 (0.79)	0.32		
Translocation	*Intercept* No	-	-	0.16	*
	Yes	1.11 (0.79)	0.16		
In degree	*Intercept* High	-	-	0.19	*
	Low	1.02 (0.79)	0.20		
Out degree	*Intercept* High	-	-	0.23	*
	Low	1.18 (1.10)	0.28		
Betweenness	*Intercept* High	-	-	0.51	
	Low	0.76 (1.14)	0.51		
	Medium	1.42 (1.26)	0.26		
Location	*Intercept* Close to farms	-	-	0.65	
	Remote from farms	0.38 (0.86)	0.66		
Nest site	*Intercept* Close to farms	-	-	0.30	
	Remote from farms	0.85 (0.80)	0.29		
Sampling period	*Intercept* Period 1 (Mar-Apr 2012)	-	-	0.56	
	Period 2 (Aug-Nov 2012)	-0.01 (2306.1)	0.99		
	Period 3 (Feb-Apr 2013)	-0.39 (0.86)	0.70		

*variable was included in a multivariate model

Appendix 9.3-7 Table of unidentified *Campylobacter spp.* univariate analyses

Variable	Level	Coefficient (SE)	*p*-value	ChiSq *p*-value	
Sex	*Intercept* Female	-	-	0.92	
	Male	0.04 (0.38)	0.92		
Age	*Intercept* Adult	-	-	0.04	*
	Juvenile	0.92 (0.45)	0.04		
Rearing	*Intercept* Wild	-	-	0.07	*
	Foster	16.72 (1385.38)	0.99		
	Puppet	0.44 (0.41)	0.28		
Nest site	*Intercept* North Island	-	-	0.75	
	South Island	-0.13 (0.42)	0.75		
Nest site	*Intercept* Insurance	-	-	0.23	*
	Breeding	0.83 (0.52)	0.11		
	Wild	0.11 (0.42)	0.26		
Location	*Intercept* North Island	-	-	0.75	
	South Island	0.15 (0.45)	0.75		
Location	*Intercept* Insurance	-	-	0.63	
	Breeding	0.44 (0.47)	0.35		
	Wild	0.29 (0.44)	0.51		
Location	*Intercept* Offshore	-	-	0.05	*
	Mainland	0.85 (0.43)	0.05		
Translocation	*Intercept* No	-	-	0.90	
	Yes	0.05 (0.39)	0.90		
In degree	*Intercept* High	-	-	0.36	
	Low	-0.36 (0.39)	0.36		
Out degree	*Intercept* High	-	-	0.52	
	Low	-0.25 (0.39)	0.52		
Betweenness	*Intercept* High	-	-	0.50	
	Low	-0.18 (0.42)	0.68		
	Medium	-0.69 (0.59)	0.25		
Location	*Intercept* Close to farms	-	-	0.11	*
	Remote from farms	0.61 (0.39)	0.12		
Nest site	*Intercept* Close to farms	-	-	0.01	*
	Remote from farms	1.08 (0.45)	0.02		
Sampling period	*Intercept* Period 1 (Mar-Apr 2012)	-	-	-	*
	Period 2 (Aug-Nov 2012)	0.18 (0.77)	0.86		
	Period 3 (Feb-Apr 2013)	-2.55 (0.42)	0.01		

*variable was included in a multivariate model

Appendix 9.3-8 Univariate analyses of variables in respect to multiple carriage of *Campylobacter* spp.

9.4. Chapter 4 supplementary information

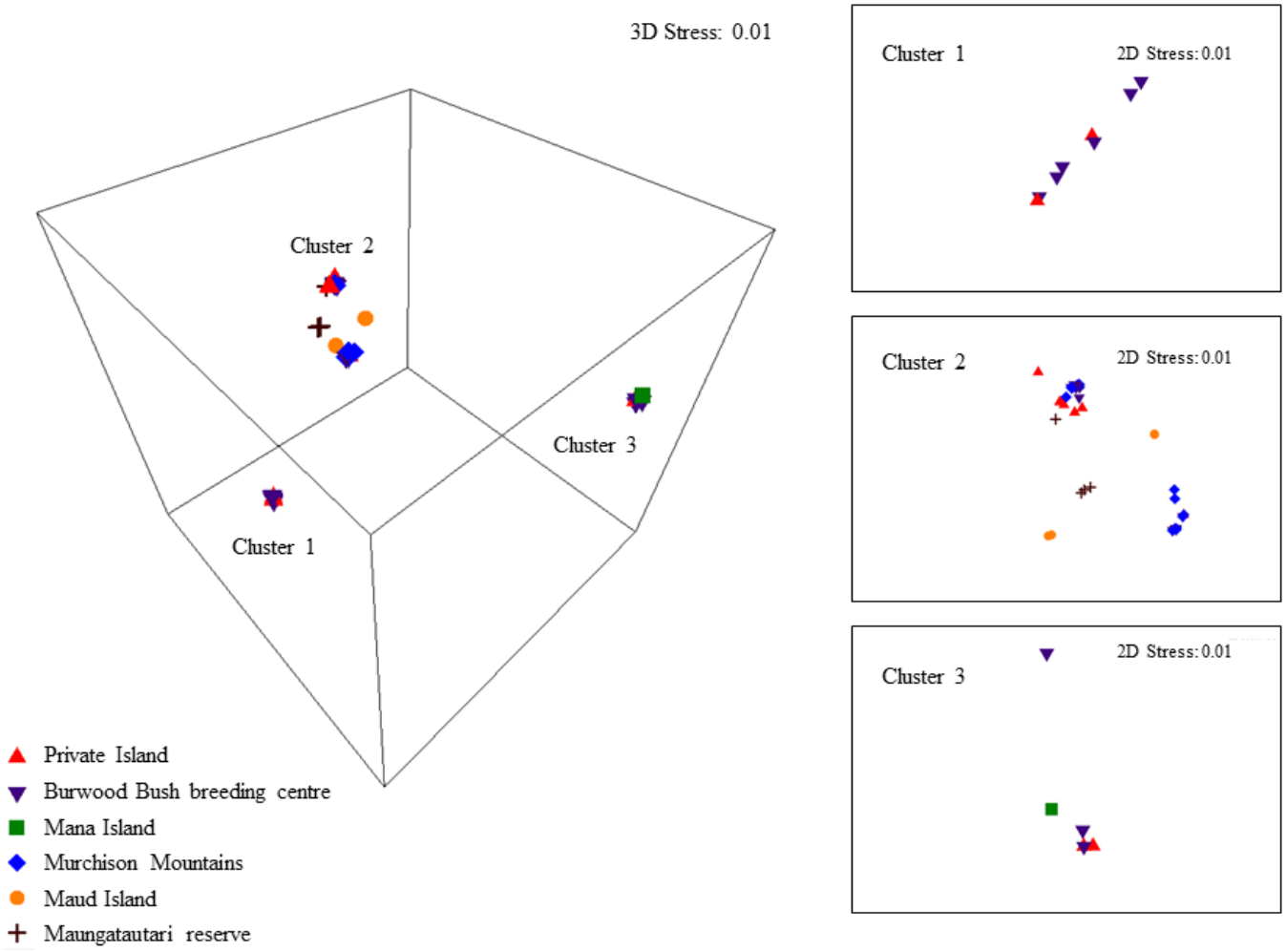
See excel spreadsheet for tables of factors, cofactors and allelic profiles per *C.* sp. *nova 1* genome. Additional sheets contain distance matrices for: *Campylobacter* sp. *nova 1* allele, uncorrected P distance and GTR distance matrices, and the uncorrected P distance matrix for the combined *C.* sp. *nova 1* and publically available *Campylobacter* spp. rMLST analysis.

Appendix 9.4-1 Electronic supplementary material

***Campylobacter* species**	**Strain**	**GenBank assembly ID**
Campylobacter coli	15-537360	GCA_000494775.1
Campylobacter coli	76339	GCA_000470055.1
Campylobacter coli	RM1875	GCA_000583755.1
Campylobacter coli	RM4661	GCA_000583775.1
Campylobacter coli	CVM N29710	GCA_000465235.1
Campylobacter coli	1091	GCA_000253675.2
Campylobacter coli	1098	GCA_000253695.2
Campylobacter coli	1417	GCA_000253735.2
Campylobacter coli	1957	GCA_000253875.2
Campylobacter coli	2680	GCA_000253515.2
Campylobacter coli	111-3	GCA_000253415.2
Campylobacter coli	151-9	GCA_000254095.2
Campylobacter coli	84-2	GCA_000253615.2
Campylobacter coli	202-04	GCA_000253915.2
Campylobacter coli	H9	GCA_000254195.2
Campylobacter coli	JV20	GCA_000146835.1
Campylobacter coli	K3	GCA_000505605.1
Campylobacter coli	RM2228	GCA_000167415.1
Campylobacter coli	RM5611	GCA_000583795.1
Campylobacter coli	Z163	GCA_000253455.2
Campylobacter concisus	13826	GCA_000017725.1
Campylobacter concisus	UNSWCD	GCA_000259315.1
Campylobacter concisus	UNSW3	GCA_000466645.1
Campylobacter concisus	UNSW1	GCA_000466665.1
Campylobacter concisus	UNSWCS	GCA_000466685.1
Campylobacter concisus	ATCC51561	GCA_000466705.1
Campylobacter concisus	UNSW2	GCA_000466725.1
Campylobacter concisus	ATCC51562	GCA_000466745.1
Campylobacter cuniculorum	DSM23162	GCA_000621005.1
Campylobacter curvus	525.92	GCA_000017465.1
Campylobacter curvus	DSM6644	GCA_000376325.1
Campylobacter fetus subsp. *fetus*	82-40	GCA_000015085.1
Campylobacter fetus subsp. *testudinum*	03-427	GCA_000495505.1
Campylobacter fetus subsp. *venerealis*	Azul-94	GCA_000174675.1
Campylobacter fetus subsp. *venerealis*	NCTC 10354	GCA_000222425.1
Campylobacter fetus subsp. *venerealis* bv. *Intermedius*	99541	GCA_000414135.1
Campylobacter fetus subsp. *venerealis* bv. *Intermedius*	cfvi03/293	GCA_000512745.1
Campylobacter gracilis	RM3268	GCA_000175875.1
Campylobacter hominis	BAA-381	GCA_000017585.1
Campylobacter hyointestinalis subsp. *hyointestinalis*	DSM_19053	GCA_000705275.1
Campylobacter jejuni subsp. *doylei*	49349	GCA_000701745.1
Campylobacter jejuni subsp. *doylei*	269.97	GCA_000017485.1
Campylobacter jejuni subsp. *jejuni*	RM1221	GCA_000011865.1
Campylobacter jejuni subsp. *jejuni*	NCTC11168	GCA_000009085.1
Campylobacter jejuni subsp. *jejuni*	00-2425	GCA_000468915.1

***Campylobacter* species**	**Strain**	**GenBank assembly ID**
Campylobacter jejuni subsp. *jejuni*	4031	GCA_000493495.1
Campylobacter jejuni subsp. *jejuni*	84-25	GCA_000168195.1
Campylobacter jejuni subsp. *jejuni*	CF93-6	GCA_000168115.1
Campylobacter jejuni subsp. *jejuni*	HB93-13	GCA_000168175.1
Campylobacter jejuni subsp. *jejuni*	D2600	GCA_000234545.1
Campylobacter jejuni subsp. *jejuni*	DFVF1099	GCA_000184805.2
Campylobacter jejuni subsp. *jejuni*	1336	GCA_000163975.1
Campylobacter jejuni subsp. *jejuni*	IA3902	GCA_000025425.1
Campylobacter jejuni subsp. *jejuni*	ICDCCJ07001	GCA_000184085.1
Campylobacter jejuni subsp. *jejuni*	M1	GCA_000148705.1
Campylobacter jejuni subsp. *jejuni*	NW	GCA_000234525.1
Campylobacter jejuni subsp. *jejuni*	R414	GCA_000163995.1
Campylobacter jejuni subsp. *jejuni*	S3	GCA_000184205.1
Campylobacter jejuni subsp. *jejuni*	PT14	GCA_000302555.2
Campylobacter jejuni subsp. *jejuni*	81-176	GCA_000015525.1
Campylobacter jejuni subsp. *jejuni*	81116	GCA_000017905.1
Campylobacter jejuni subsp. *jejuni*	10186	GCA_000686225.1
Campylobacter jejuni subsp. *jejuni*	260.94	GCA_000168135.1
Campylobacter jejuni subsp. *jejuni*	ATCC33560	GCA_000251165.2
Campylobacter jejuni subsp. *jejuni*	CG8486	13826
Campylobacter lari	RM2100	GCA_000019205.1
Campylobacter mucosalis	DSM21682	GCA_000705255.1
Campylobacter rectus	RM3267	GCA_000174175.1
Campylobacter showae	RM3277	GCA_000175655.1
Campylobacter showae	CSUNSWCD	GCA_000313615.1
Campylobacter showae	CC57C	GCA_000344295.1
Campylobacter upsaliensis	RM3195	GCA_000167395.1
Campylobacter upsaliensis	JV21	GCA_000185345.1
Campylobacter upsaliensis	DSM5365	GCA_000620965.1
Campylobacter ureolyticus	DSM20703	GCA_000374605.1
Campylobacter ureolyticus	ACS-301-V-Sch3b	GCA_000413435.1
Campylobacter ureolyticus	CIT007	GCA_000597825.1

Appendix 9.4-2 List of publicly available *Campylobacter* spp. genomes used for rMLST comparison to *Campylobacter* sp. *nova* *1*



Appendix 9.4-3 Multidimensional scaling plot of 70 *Campylobacter* sp. *nova 1* 52 gene rMLST haplotypic profiles, coloured by location of the takahe host

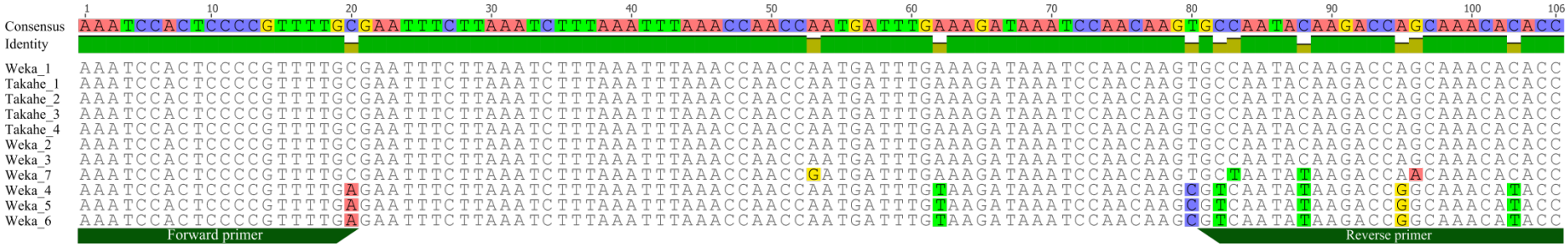
Factor	d.f	SS	Estimate of variation	Pseudo-F	P(perm)	Permutations
Sampling location	5	4.99E-03	8.28E-05	6.4102	0.0001	9921
Cofactors						
North vs South Island	1	3.00E-04	9.12E-06	1.3932	0.2101	6984
Wild vs Captive breeding and Insurance	1	1.83E-03	5.08E-05	9.4558	0.0004	9958
Captive breeding vs Insurance	1	4.52E-04	1.01E-05	2.0075	0.1265	9947
Island vs Mainland	1	4.14E-04	7.25E-06	1.9389	0.1301	9958
Residuals	64	9.96E-03	1.56E-04			
Total	69	1.50E-02				

Appendix 9.4-4 PERMANOVA results from 9,999 permutations (perm) of location factors using an uncorrected P measure matrix from 52 rMLST nucleotide comparison of 70 takahe (*Porphyrio hochstetteri*) *Campylobacter* sp. *nova 1*

Factor	d.f	SS	Estimate of variation	Pseudo-F	P(perm)	Permutations
Sampling location	5	1.63E-02	2.41E-04	4.0546	0.0001	9866
Cofactors						
North vs South Island	1	1.66E-03	7.47E-05	1.7148	0.1333	7053
Wild vs Captive breeding and Insurance	1	5.78E-03	1.52E-04	6.3545	0.0002	9922
Captive breeding vs Insurance	1	1.83E-03	3.68E-05	1.8267	0.0864	9931
Island vs Mainland	1	1.97E-03	3.62E-05	2.0374	0.0636	9922
Residuals	64	5.14E-02	8.03E-04			
Total	69	6.77E-02				

Appendix 9.4-5 PERMANOVA results from 9,999 permutations (perm) of location factors using a GTR matrix from 52 rMLST nucleotide comparison of 70 takahe (*Porphyrio hochstetteri*) *Campylobacter* sp. *nova 1*

9.5. Chapter 5 supplementary information



Appendix 9.5-1 Sequence alignment of the *in silico* PCR target region for *Campylobacter* sp. *nova 1* isolates identified by *in vitro* PCR. Nucleotide polymorphisms which differ from the consensus sequence have been highlighted. Nucleotide bases are colour coded as follows: A = Yellow, T = Green, C = Blue, G = Yellow.

O what a joyous joyous day
Is that on which we come
At the recess from school away,
Each lad to his own home!

What though the coach is crammed full,
The weather very warm;
Think you a boy of us is dull,
Or feels the slightest harm?

The dust and sun is life and fun;
The hot and sultry weather
A higher zest gives every breast,
Thus jumbled all together.

Sometimes we laugh aloud aloud,
Sometimes huzzah, huzzah.
Who is so buoyant, free, and proud,
As we home-travellers are?

But sad, but sad is every lad
That day on which we come,
That last last day on which away
We all come from our home.

The coach too full is found to be:
Why is it crammed thus?
Now every one can plainly see
There's not half room for us.

Soon we exclaim, O shame, O shame,
This hot and sultry weather,
Who but our master is to blame,
Who pack'd us thus together!

Now dust and sun does every one
Most terribly annoy;
Complaints begun, soon every one
Elbows his neighbour boy.

Not now the joyous laugh goes round,
We shout not now huzzah;
A sadder group may not be found
Than we returning are.

---The Journey to and from school
Mary Lamb