The Biology of *Avipoxvirus* in New Zealand Avifauna

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To My Beloved Family

사랑하는 가족에게 이 논문을 바칩니다

ABSTRACT

Avipoxvirus (APV) infection is a highly contagious disease of birds which is comparable to poxvirus infections in various mammalian species, including smallpox in humans. The infection has been reported in more than 200 bird species, affecting both domesticated and free-ranging birds around the world. The disease is associated with economic loss in the poultry industry and is implicated with the decline in biodiversity in free-ranging birds, particularly in island ecosystems. This study was the first investigation into APV infection in New Zealand free-ranging birds. The initial focus of this study was the phylogenetic analysis of APV in New Zealand. Avipoxvirus antibody was then detected using enzyme-linked immunosorbent assay (ELISA) in several introduced species and an endemic passerine species in New Zealand. The pathogenicity of two major APV strains isolated from New Zealand birds was evaluated and the safety and efficacy of a commercial fowlpox (FWPV) vaccine was investigated in a model passerine species.

This study confirms that various New Zealand birds including endangered species are susceptible to APV infection and that at least three different strains of APV are present in New Zealand, with overlaps in the geographic distributions between different strains. The results suggest that APV had been introduced to New Zealand through avian hosts, insect vectors or human intervention such as poultry vaccination. A high seroprevalence to APV has been observed in introduced and an endemic bird species in New Zealand, confirming that the virus is well established. A significant relationship between birds seropositive to APV and the ones positive to *Plasmodium* spp. has also been observed, both of which are known to be pathogens responsible for dramatic declines in island bird populations. Two major New Zealand APV strains isolated from clinical cases were pathogenic in Zebra finches (*Taeniopygia guttata*), which we used as

a model passerine species. A commercial FWPV vaccine was safe and effective in our model species against New Zealand APV isolates and I conclude that vaccination of native passerine birds using the FWPV vaccine could be an effective tool to reduce APV mortality, particularly in endangered species.

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THESIS STRUCTURE AND FORMAT

This thesis is written as a series of seven interrelated chapters, three of which (Chapters two, three and four) have been published in peer reviewed journals (Ha *et al*. 2011; Ha *et al*. 2012b; Ha *et al*. 2012a) and one of which (Chapter six) has been submitted to a peer reviewed journal.

Chapter one: Avipoxvirus – General Introduction introduces the main subject of my thesis by discussing the current knowledge on avipoxvirus (APV) in birds and reviewing the literature on various aspects of APV infecting domestic and wild birds. At the end of this first chapter, a brief summary of the specific aims of this thesis is presented.

Chapter two: The Phylogenetic Analysis of Avipoxvirus in New Zealand has been published in Veterinary Microbiology (Ha HJ, Howe L, Alley M, Gartrell B. 150, 80-7, 2011) and describes the results of the first phylogenetic analysis of APV in New Zealand.

Chapter three: Avipoxvirus Infections in Brown Kiwi (*Apteryx mantelli*) has been published in New Zealand Veterinary Journal (Ha HJ, Alley MR, Howe L, Castro I, Gartrell B. DOI:10.1080/00480169.2012.700629, 2012) and describes APV infections identified in two endemic brown kiwi. Not only it is the first APV reported in kiwi populations, it is the first viral disease documented.

Chapter four: The Seroprevalence of Avipoxvirus and Its Association with Avian Malaria (*Plasmodium* spp.) Infection in Introduced Passerine Birds in the Southern Regions of the North Island of New Zealand has been published in Avian Diseases (Ha HJ, Banda M, Alley M, Howe L, Gartrell BD. DOI: 10.1637/10285-061912-ResNote. 1, 2012).

Chapter five: The Detection of Avipoxvirus Antibody in North Island Robins (*Petroica australis longipes*) Demonstrates the Endemic Status of Avipoxvirus in Birds on an Island Refuge Used for Conservation describes the results of the first attempt to screen for APV antibody. This chapter further suggests the possibility of APV antibody screening in the event of translocation of endangered species.

Chapter six: Evaluation of the Pathogenicity of Avipoxvirus Strains Isolated from Wild Birds in New Zealand and the Efficacy of a Fowlpox Vaccine in Passerines has been submitted to Veterinary Microbiology (Ha HJ, Howe L, Alley M, Gartrell B.) and describes the results of challenge and vaccination studies carried out in a model passerine species.

Chapter seven: General Discussion summarises all information and puts it into context. The relevance of my findings is discussed and future research directions are suggested.

References: All references are listed at the end of the thesis to minimise repetition. All literature cited is consistent with the format used for the scientific journal: *New Zealand Veterinary Journal*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

Avipoxvirus – General Introduction

1.1. A HISTORY OF POXVIRUS IN HUMANS

Poxviruses are double-stranded DNA viruses that are specialised in animal hosts, including vertebrates and invertebrates (Wagner et al. 2008) The viruses, which can cause a variety of clinical syndromes ranging from debilitating cutaneous masses to severe septicaemic diseases, are unusual viruses in that infection often leads to high rates of mortality in the host population (Wagner et al. 2008). For instance, smallpox infection in humans, caused by variola virus, demonstrated this pathogenicity with 80% or higher mortality rate during the Middle Ages in Europe (Geddes 2006). This devastating smallpox infection stimulated remarkable achievements in our general understanding of virus epidemiology, virus-related immunity and vaccination. Smallpox infection has a long history with one of the earliest documented historical deaths being recorded in the Egyptian Pharaoh Ramses V who died from smallpox in 1157 BC (Fenner 1988). Evidence shows that smallpox had originated in Mesopotamia and then spread to neighbouring regions including Egypt and India (Geddes 2006). It was transferred to China from the South West in the first century BC then to Europe around the early 700s and to the Americas in the early 1500s (Geddes 2006). Although human populations introduced 'inoculation' using materials from smallpox patients as a preventative method against smallpox infection from the 13th century, the spread of smallpox throughout the world brought remarkable shifts in demographics (Fenner 1988). For example, smallpox outbreaks in Mexico resulted in the decrease of the population from 30 million to 2 million in 50 years (Geddes 2006).

In the late 18th century, Edward Jenner found that material from cowpox lesions could provide protection against smallpox which eventually led to the development of smallpox vaccines using a related vaccinia virus (Cartwright 2005). In 1967 the World Health Organization (WHO) initiated a smallpox eradication program with the ultimate

goal to eliminate the plague-like epidemic (Geddes 2006). Due to the intensive vaccination program in the late 60s and 70s which cost about 300 million US dollars, global smallpox eradication was achieved and the WHO officially claimed that "smallpox is dead" in 1980 (Geddes 2006).

This study will be focusing on avipoxviruses (APVs), a related and in many ways similar, viral pox disease. Avipoxvirus infection is a highly contagious disease of birds which is comparable to those poxvirus infections in various mammalian species (for example, swine pox, monkey pox and sheep sore-mouth) (Van Riper and Forrester 2007). With fewer historical records and far less money spent, the extent of APV infections and its toll on susceptible populations has only recently begun to be investigated. For New Zealand, with a relatively high number of potentially susceptible endemic avian species, a better understanding of APV and its potential threat is essential.

1.2. GENERAL CHARACTERISTICS OF POXVIRUSES

1.2.1. Structure

Poxviruses belong to the family Poxviridae and contain a haploid copy of linear, double-stranded DNA (Esposito and Fenner 2007). The poxvirus virions are brick shaped or ovoid and usually enveloped (Wagner et al. 2008). They are the largest animal viruses known and therefore, poxvirus was the first virus observed microscopically in 1886 (Smith and Kotwal 2002). There are two types of poxviruses; the internal mature virus (IMV) and the external enveloped virus (EEV), and both types can be extremely infectious (Esposito and Fenner 2007). The size measures approximately 220-450 nm long/140-260 nm wide/140-260 nm thick in brick shaped virions and 250-300 nm long/160-190 nm diameter in ovoid virions (Wagner et al. 2008) (Figure 1.1.).

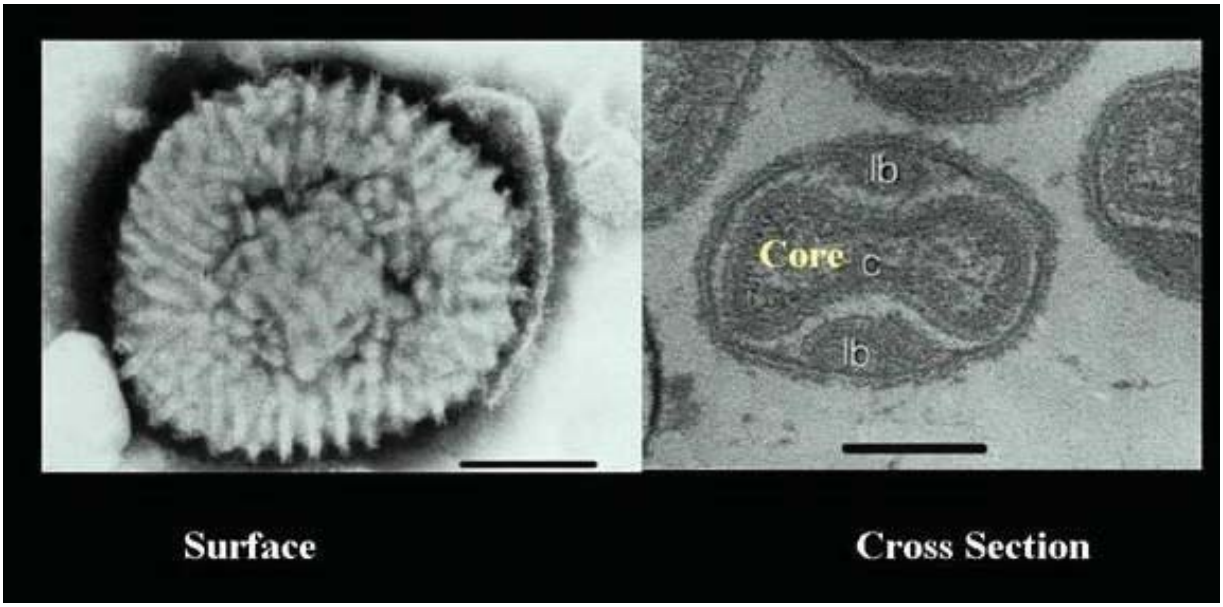

Figure 1.1. The electron microscopy of the surface and cross section of a poxvirus, c=core, lb=lateral bodies. (vaccinia virus, copyright of E. Niles).

Poxviruses are very stable and resistant to desiccation in the environment and can survive on fomites or in dried scabs for months or years (Tripathy and Reed 2008). With the exception of pigeon poxvirus (PGPV) which is resistant to both ether and chloroform, they are usually insensitive to ether but sensitive to common detergents, formaldehyde and oxidizing agents (Bolte et al. 1999). While 1% potassium hydroxide or heating at 50 °C for 30 minutes or 60 °C for eight minutes can inactivate the virus, it can withstand 1% phenol and 1:1,000 formalin for nine days (Andrews et al. 1978).

1.2.2. Replication and genome

Replication of poxvirus occurs in the cytoplasm of infected cells (Moss 2007). The virus enters the cytoplasm of host cells through receptor-mediated endocytosis (Wagner et al. 2008). Once a cell has been infected it takes a further 20-22 hours for the virus to complete its replication cycle, which has three distinguishable phases, 'early gene

expression', 'genome replication', and 'intermediate and late stages of replication' (Wagner et al. 2008). The replication of poxvirus always leads to cell lysis, releasing the mature viruses (Pastoret and Vanderplasschen 2003).

Similar to other poxviruses, APV replicates in the cytoplasm of infected cells (Wagner et al. 2008). Two distinct phases have been observed in the biosynthesis of fowlpox virus (FWPV): 1) a host response characterised by remarkable cellular hyperplasia and 2) synthesis of infectious virus, which takes place within the first 72 hours post infection (PI) and 72-96 hours PI, respectively (Cheevers et al. 1968). For the first 60 hours PI the virus is predominantly synthesising cellular DNA and remarkable epithelial hyperplasia can be observed between 36-48 hours PI (Cheevers et al. 1968). The replication of viral DNA in dermal epithelium initiates between 12 and 24 hours PI followed by the emergence of infectious virus (Cheevers et al. 1968). Viral DNA synthesis remains very low during the first 60 hours and then increases nearly five-fold between 60 and 72 hours which results in concomitant decrease in cellular DNA synthesis (Cheevers et al. 1968). During 72 and 96 hours PI, the synthesis of viral DNA becomes progressively more dominant and no more hyperplasia can be observed (Cheevers et al. 1968).

Poxvirus genomes vary from 130 kilobase pairs (kbp) to 380 kbp (Moss 2007). The central region of the genome of chordopoxviruses is highly conserved in gene content and the arrangement, except for some gene inversions in FWPV and species-specific gene insertion in FWPV and Molluscum contagiosum virus (Gubser et al. 2004). Highly conserved genes are only present in the central region in all chordopoxviruses and genes in terminal regions are divergent (Gubser et al. 2004). All chordopoxviruses share 90 conserved genes, which reduces to 49 when entomopoxviruses are included (Upton et al. 2003).

Avipoxviruses are the largest amongst the poxviruses, for example, FWPV is 288 kbp (Afonso et al. 2000) and canarypox (CNPV) is 365 kbp (Tulman et al. 2004). A comparison study of FWPV and other poxvirus members confirms that FWPV genome is the most divergent with the following major differences; large genome size; presence of 113 unique genes; and inversion of genes in the central region which are present in the terminal regions of other chordopoxviruses (Afonso et al. 2000; Gubser et al. 2004). The 1971 nucleotide long, highly conserved gene encoding FWPV 4b core protein with a molecular weight of 75.2 kDa has been identified (Binns et al. 1989), providing a useful signpost for polymerase chain reaction (PCR) and vaccine research.

1.2.3. Transmission

Transmission of vertebrate poxviruses occurs through aerosol, direct contact, arthropods or indirect contact via fomites (Wagner et al. 2008). The route of transmission is closely related to the mortality or pathogenicity in the host (Wagner et al. 2008). For example, smallpox infection spread by inhalation usually resulted in viraemia in the infected hosts and caused high mortality rates (Wagner et al. 2008). On the other hand, infection via skin break caused single or multiple localised infections (Esposito and Fenner 2007). Some poxviruses such as vaccinia, cowpox, and monkeypox virus have broad host ranges including humans however, most poxviruses have limited or single species host ranges (Esposito and Fenner 2007).

1.3. IMMUNITY TO POXVIRUS

The immune response to poxvirus infections is multifaceted. Usually, hosts surviving poxvirus infection develop life-long immunity to re-infection (Wagner et al. 2008). Once poxvirus enters the host cells, the innate immune response initiates. Non-specific

mechanisms including complement, interferon and natural killer (NK) cells work as a whole to prevent the infection from spreading to other cells and tissues (Smith and Kotwal 2002). A few days after infection, an adaptive immune response including humoral (B-cell immunity) and cell-mediated immune response (T-cell immunity) develops (Smith and Kotwal 2002). At this stage poxvirus antibodies are produced by plasma cells and poxvirus-specific cytotoxic T-lymphocyte (CTL)s are produced (Smith and Kotwal 2002). The antibodies play a critical role in resolving poxvirus infection although the importance of antibodies has been less appreciated than CTLs in poxvirus immunology research (Panchanathan et al. 2008). The antibodies bind to the poxviruses, causing aggregation and neutralisation of virus particles and disturb viral absorption and internalisation into the cell (Smith and Kotwal 2002). In addition, the antibodies generate complement activation and attach to infected host cells, leading to antibody-dependent cell-mediated cytotoxicity (ADCC) (Smith and Kotwal 2002). Poxvirus-specific CTLs play an active role in the later stage of immunity where they attack and kill virus-infected cells and as a result the viruses are eliminated (Smith and Kotwal 2002). Each part of the immune response is crucial in the control of poxvirus infections and should be taken into account in the design of poxvirus vaccines (Panchanathan et al. 2008).

When exposed to poxvirus, healthy individuals present ordinary-type poxvirus infection with the development of a vigorous cellular immune response (Esposito and Fenner 2007). Individuals with a defective immune response however, display a severe form of disease (Esposito and Fenner 2007). For instance, hemorrhagic smallpox caused extensive virus multiplication in the bone marrow in individuals with a defective immune response, resulting in blood coagulation defects (Esposito and Fenner 2007). Interestingly, while long-lasting immunity to reinfection to variola virus was observed in individuals recovered from smallpox infection, heterologous immunity such as

vaccination was found to be less durable (Esposito and Fenner 2007). This long-lasting immunity induced by poxvirus infections provides a convenient and reliable indicator for seroprevalence surveys.

1.4. TAXONOMY OF POXVIRUS

There are two subfamilies in Poxviridae family as Chordopoxvirinae and Entomopoxvirinae which infect vertebrates and insects, respectively (Esposito and Fenner 2007). Chordopoxvirinae includes eight genera: Orthopoxvirus, Parapoxvirus, Avipoxvirus (APV), Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus and Yatapoxvirus (Esposito and Fenner 2007). Each genus is genetically and antigenically related and has similar morphology and host range (Esposito and Fenner 2007). Entomopoxvirinae includes three genera: A, B & C (Gubser et al. 2004). Due to high sequence homology and antigenic similarity, humans are known to be susceptible to infection by various poxvirus species in Orthopoxvirus, Parapoxvirus, Molluscipoxvirus and Yatapoxvirus (Pastoret and Vanderplasschen 2003). While molluscum contagiosum virus, the sole member of Molluscipoxvirus is a human virus, APVs are known to cause productive infections only in avian species (Tripathy and Reed 2008). In this study, only APV will be discussed.

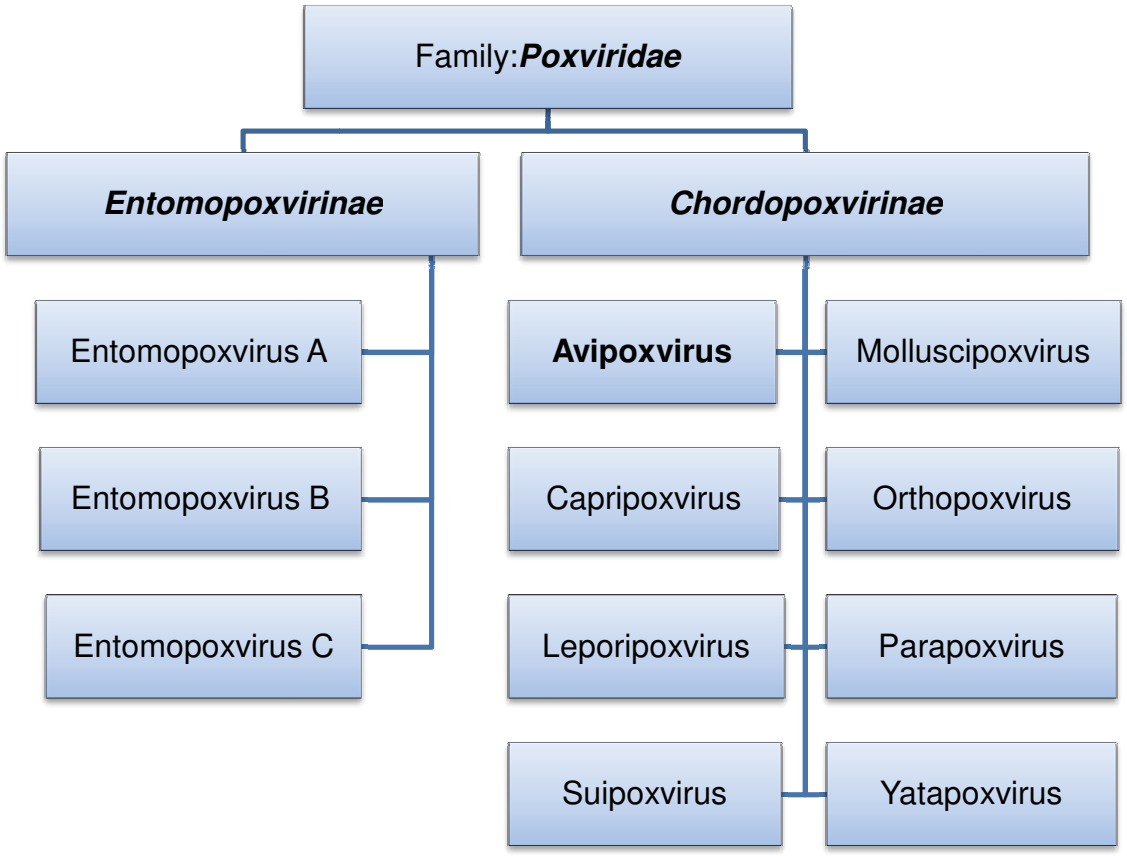

Figure 1.2. The taxonomic tree of the Poxviridae family.

1.5. AVIPOXVIRUS

Avipoxvirus belongs to the virus subfamily Chordopoxvirinae, which is a member of Poxviridaefamily (Van Riper and Forrester 2007). Avipoxvirus shares similar morphology to other member viruses in the Poxviridae family (Tripathy and Reed 2008). It is known to be the one of the largest viruses among the Poxviridae family, measuring approximately 150 to 250 nm by 265 to 350 nm (Van Riper and Forrester 2007).

The genus Avipoxvirus contains a number of species. The viruses have been classified according to their hosts of origin such as fowlpox virus (FWPV), canarypox virus (CNPV) and pigeonpox virus (PGPV) (Van Riper and Forrester 2007). This terminology however

does not reflect the host range of the APV species since in general, APV strains are pathogenic for several species (Tripathy and Reed 2008). As a result of the recent development in molecular biology techniques and intensive research concerning APV infections in free-ranging birds, more APV species have been identified. Currently 16 species are included in the genus (Table 1.1.) (Van Riper and Forrester 2007; Tripathy and Reed 2008). The list of species includes APV strains isolated mainly in North America and Europe, implying that not much research has been conducted regarding APV infection outside of those regions.

Jarmin et al. (2006) and Manarolla et al. (2010) described three different APV clades identified as A, B and C based on the nucleotide sequences of the 4b core protein genes. Clade A, collectively known as the 'fowlpox virus clade' is further divided into four subclades; A1, A2, A3 and A4 and Clade B (canarypox virus clade) includes B1 and B2 subclades (Jarmin et al. 2006; Manarolla et al. 2010). Clade C represents psittacinepox virus (Jarmin et al. 2006; Manarolla et al. 2010).

Table 1.1. Avipoxvirus classification (Van Riper and Forrester 2007).

Genus	Species in the genus	
Avipoxvirus	Fowlpox virus Turkeypox virus Pigeonpox virus Canarypox virus Juncopox virus Psittacinepox virus Quailpox virus Sparrowpox virus Starlingpox virus	Listed in 1991 by Francki et al.
	Peacockpox virus Penguinpox virus Mynahpox virus Albatrosspox virus	Listed in 1993 by Tripathy
	Condorpox virus Alalapox virus Apapanepox virus	Listed in 2008 by Tripathy and Reed

1.5.1. Global distribution of APV

Since the first publication describing APV infection in 1844, it has been reported in more than 278 bird species in 20 orders throughout the world, both in free-ranging and captive birds (Bolte et al. 1999; Van Riper and Forrester 2007). Fowlpox infection of chickens and turkeys has a worldwide distribution (Tripathy and Reed 2008). Fowlpox

virus is the most well-known and studied APV species due to its economic importance, causing a decrease in egg production and growth rate and an increase in mortality rates in the poultry (Tripathy and Reed 2008).

Other APVs also display a global distribution including North and South America, Europe, Asia, Africa, Middle East, Oceania, Hawaii, Galapagos and Canary Islands (Bolte et al. 1999; Van Riper and Forrester 2007). The only exceptions are the Arctic or remote parts of the world where no records from wild birds exist. The recent isolation of the virus in the Southern giant petrel (*Macronectes giganteus*) from Antarctica shows this virus can withstand in the cold environment (Shearn-Bochsler et al. 2008). Although published information on APV infection in wild birds is limited to those areas where research on the virus has been actively carried out, it is known that APV infection in wild birds is more prevalent in temperate and warmer regions (Van Riper and Forrester 2007). Even within continental regions, APV infection is more frequent in moister and warmer areas (Forrester 1991). Avipoxvirus has special implications when introduced to remote islands where the hosts, vectors, and APVs have not necessarily co-evolved, the disease may have significant implications for population decline and extinction (Atkinson et al. 2005; Van Riper and Forrester 2007; Atkinson and LaPointe 2009). The disease spreads more rapidly in the native island avifauna than in the introduced avifauna, resulting in much higher prevalences (Van Riper et al. 2002; Atkinson et al. 2005; Smits et al. 2005; Zylberberg et al. 2012). Though its impact on wild birds is not as well recognised as in poultry, it is known to limit the survival of wild populations (Van Riper and Forrester 2007). This has been well-documented in Hawaiian islands where APV is known to be a major factor in population decline and extinction in Hawaiian bird species (Van Riper et al. 2002; Atkinson et al. 2005).

1.5.2. Transmission

The transmission of APV occurs mechanically through injured or lacerated skin (Tripathy and Reed 2008). The virus itself cannot penetrate intact skin, entering the hosts through wounds from insect bites or trauma (Adams et al. 2005). Insects such as mosquitoes, mites, midges or flies therefore play an important role in the transmission of APV (Van Riper and Forrester 2007). Most biting insects act as mechanical vectors, transferring the virus from infected individuals to susceptible birds (Van Riper and Forrester 2007). When the vector numbers are greatest, APV transmission is also the greatest (Van Riper and Forrester 2007). Transmission through direct contact with infected birds or contaminated food, water or other materials also occurs (Adams et al. 2005). Inhalation of virus particles though aerosols, foods or drinking water may lead to diphtheritic or systemic APV infection, particularly in confined environments such as aviaries (Lierz et al. 2007).

Avipoxviruses were believed to be host-specific or only infect closely related species but recent research has shown that most APVs infect several bird species (Tripathy and Hanson 1975; Weli et al. 2004; Van Riper and Forrester 2007). Birds living in areas of high population density, including captive birds, display a higher prevalence of APV infection than birds living in lower densities (Van Riper and Forrester 2007). In free-ranging low density populations, the infection rate is higher in warmer environment and lower altitude, where more vectors are likely to reside (Van Riper and Forrester 2007). The incubation period is usually less than one week but it may extend up to 30 days (Lierz et al. 2007). Longer incubation period of 90 to 150 days and 13 months have been reported in the house finch (*Carpodacus mexicanus*) and in the Northern flicker (*Colaptes auratus*), respectively (Van Riper and Forrester 2007).

Avipoxviruses are unable to complete the replication cycle in non avian species so they don't cause clinical infection in mammalian species (Tripathy and Reed 2008). While a report of poxvirus infection caused by FWPV from a rhinoceros exists, the isolated virus differed in several aspects from the generally known FWPV; the virus failed to propagate in cell cultures from chicken embryo fibroblasts with cytopathic effect; the pocks on the chorioallantoic membrane of the chicken embryo and the inflammatory reactions developed less intensively; and the incubation period in infected newborn chickens lasted 3 to 4 weeks showing a relatively high virulence (Mayr and Mahnel 1970). Attenuated strains of recombinant APVs have been widely used as successful vaccine delivery vectors for a number of mammalian pathogens (Baxby and Paoletti 1992; Beukema et al. 2006) but little information exists as to their efficacy or safety in avian species outside of the poultry industry (Skinner et al. 2005; Beukema et al. 2006).

1.5.3. Pathogenesis of APV infection

Avipoxvirus infection has two primary forms: the cutaneous and the diphtheritic forms and also a rare systemic form (Adams et al. 2005). The cutaneous form (dry form) is characterised as the development of nodular or tumour lesions on unfeathered parts of the skin, e.g., feet, legs, head and eyelids (Tripathy 1993; Adams et al. 2005). Lesions on eyelids are common features of the cutaneous form which may cause closure of the eyes (Tripathy 1993). Birds infected by the cutaneous form are more likely to recover and develop long-lasting immunity (Van Riper and Forrester 2007). The diphtheritic form (wet form) causes diphtheritic mucosal plaques in the upper respiratory and gastrointestinal tracts (Adams et al. 2005). Quite often birds affected with the diphtheritic form also present with clinical signs consistent with the cutaneous form (Bolte et al. 1999). The mortality rate is higher in diphtheritic or mixed forms than cutaneous forms (Bolte et al. 1999). Infected birds may have difficulties in feeding and

in respiration resulting in death (Tripathy 1993). Rarely, the systemic form of APV infection might be observed, especially in canaries (Van Riper and Forrester 2007; Shivaprasad et al. 2009). Where the acute systemic form occurs, the birds may display distinguishable lesions such as fibrinous inflammation on serous membranes, liver degeneration or necrosis, oedema and hyperplasia of lungs, or fibrinous pneumonia (Van Riper et al. 2002; Shivaprasad et al. 2009). Canaries infected by cutaneous form may present with skin lesions even on feathered parts of the body (Van Riper and Forrester 2007).

The virulence of the virus is linked to concurrent environmental or physical stress, strain of virus, route of infection, presence of other infections, bird species and the age of birds infected (Adams et al. 2005). Some species of birds including Phasianidae and Emberizidae were found to be more susceptible to poxvirus infection than other species (Van Riper and Forrester 2007). Avipoxvirus infection has never been reported in the tinamous (Tinamiformes), loons (Gaviiformes), nightjars (Caprimulgiformes), and kingfishers (Coraciiformes) (Van Riper and Forrester 2007). Pigeons, quails, and canaries infected with APV show high mortality rates (Tripathy 1993). Canarypox infection is the most fatal among all poxvirus infections (Tripathy 1993); canaries and some finches display extremely high mortality rates, up to 80 to 100% (Bolte et al. 1999).

Figure 1.3. A silvereye (*Zosterops lateralis*) affected by APV

(Dunedin, New Zealand, courtesy of P. Sorrell, 2005).

1.6. DIAGNOSIS OF APV INFECTION

Traditionally APV was diagnosed by the combination of clinical signs of disease and a range of adjunctive tests including: histopathological examination to detect typical eosinophilic intracytoplasmic inclusions (Bollinger's inclusion bodies); electron microscopy; virus isolation on chorioallantoic membrane (CAM) of embryonated chicken eggs or cell cultures; serological methods; and DNA probes (Tripathy 1993). These methods may be useful to detect APV but provide no information as to the difference between species. At present, PCR of 4b core protein gene is believed to be the most sensitive method to detect various APV species (Luschow et al. 2004). In particular, PCR in conjunction with restriction enzyme analysis (REA) has been found to efficiently differentiate closely related virus species (Luschow et al. 2004).

1.6.1. Microscopic examination

Avipoxvirus infection always causes hyperplasia of the epithelium and enlargement of cells accompanied by inflammatory changes regardless of the form of the infection (Tripathy and Reed 2008). When replicating in the cytoplasm of epithelial cells, APV produces large acidophilic A-type cytoplasmic inclusion bodies (Bollinger bodies), containing elementary (Borrel) bodies (Tripathy 1993). Due to the large virion size, APV elementary bodies can be seen in smears by light microscopy with Wright's stain or Gimenez stain (Tripathy and Hanson 1976). Virus particles can be detected in lesions or exudates by negative staining or ultrathin sections of the infected tissues using electron microscopy (Tripathy and Reed 2008). Electron microscopy has a few advantages. It is rapid and simple and also differentiates poxvirus infection from other avian diseases which display similar clinical signs and histological changes (Tripathy 1993).

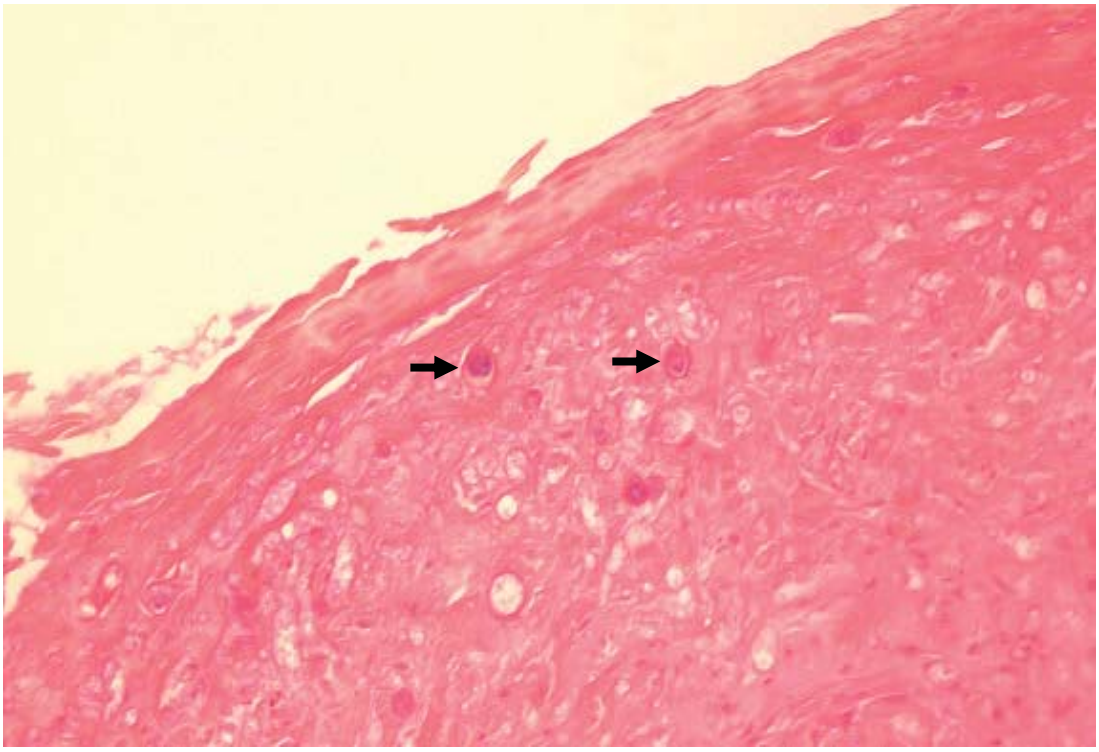

Figure 1.4. Poxvirus specific inclusion bodies

(H&E stain, New Zealand shore plover (*Thinornis novaeseelandiae*),

2006, courtesy of B. Gartrell).

1.6.2. Virus isolation

Avipoxvirus can be isolated in the chorioallantoic membranes of chicken embryos, cell cultures, and other avian species (Tripathy 1993). The inoculation of APV suspect materials on the CAM of 9-12-day-old chicken embryos from a specific pathogen-free (SPF) flock is the method of choice for virus isolation (Tripathy 1993). When incubating at 37°C, pocks on the CAM can be observed 5 to 7 days post inoculation (Tripathy 1993). Some APVs can be differentiated according to the characteristics and size of pocks (Tripathy 1993). Some poxviruses, especially from free-ranging or pet birds, have been observed not to grow on the CAM while some of them also fail to grow in cell culture (Tripathy 1993). Chicken embryo, chicken embryo kidney, chicken embryo dermis, duck embryo, or quail cells are used to isolate APVs by cell culture (Tripathy 1993). Cytoplasmic inclusion bodies may be observed 30 to 48 hours post inoculation and a cytopathic effect (CPE) may be observed in 4-6 days (Tripathy 1993). Although cell cultures are very useful to differentiate antigenic and genetic characteristics of APVs, many strains of APVs fail to produce CPE in the initial inoculation (Tripathy and Reed 2008). The bird inoculation method provides crucial information on the host range and pathogenicity for APVs, especially some strains which fail to grow on the CAM (Tripathy 1993). A suspension of tissue samples suggestive of APV infection can be inoculated through scarification of the comb or unfeathered parts of skin, denuded feather follicles or by wing-web stick method (Tripathy 1993). Inoculated birds may present typical cutaneous lesions after 5-7 days but in atypical cases other diagnostic methods such as histopathological examination should be carried out as well (Tripathy and Reed 2008).

1.6.3. Serological and protection tests

Serological tests to detect antigen or antibodies to APVs include neutralization test, complement fixation, immunodiffusion, passive hemagglutination, agar gel precipitation tests (AGPT), fluorescent antibody, immunoperoxidase (IPOX) tests, immunoblotting, and enzyme-linked immunosorbent assays (ELISA) (Tripathy 1993; Tripathy and Reed 2008). ELISA is the most favoured method to evaluate humoral antibody responses due to the convenience and sensitivity (Tripathy and Reed 2008). Currently, as no commercial ELISA test kits are available for APV infection, the test requires the precise preparation of APV antigen and use of both positive and negative sera to achieve the best sensitivity and reliability. The relationships between APV strains also can be identified though serological tests, examining the antigenic properties, host susceptibility and cross-protection among different APV isolates (Adams et al. 2005).

Protection tests are used to estimate the immunogenicity of APV vaccines such as FWPV and pigeonpox vaccines (Van Riper and Forrester 2007). An established protocol involves the use of vaccinated SPF birds and nonvaccinated birds (Van Riper and Forrester 2007). Three weeks after vaccination, the birds are challenged with a different strain of APV capable of developing clinical signs in control birds (Van Riper and Forrester 2007). When a minimum of 90% of the controls develop lesions of APV and a minimum of 90% of the vaccinated birds do not develop any lesions, the vaccine is qualified suitable to use for immunisation (Van Riper and Forrester 2007).

1.6.4. Molecular methods

Molecular methods have been found to be more sensitive and useful than traditional diagnostic methods such as histological examination, virus isolation or serological tests for the detection of APV (Van Riper and Forrester 2007). Polymerase chain reaction by amplifying various sizes of APV DNA can detect the virus even when the amount of virus is extremely small (Tripathy and Reed 2008). It is also useful in case of mixed infections since different sizes of fragments can be amplified in a single PCR by using pathogen-specific primers (Tripathy and Reed 2008). The technique, as well as the development of primers, has been progressed mainly to detect FWPV DNA (Lee and Lee 1997; Singh et al. 2000; Kim and Tripathy 2001). Amongst APVs, only the genomes of FWPV and CNPV have been completely sequenced and data regarding other APVs are limited (Afonso et al. 2000; Tulman et al. 2004). A PCR assay designed to amplify APV 4b core protein gene, which is a well conserved region within FWPV and CNPV virus, has been found to be effective to detect APVs in various free-ranging bird species (Luschow et al. 2004; Weli et al. 2004; Adams et al. 2005; Jarvi et al. 2008). Recent developments in molecular techniques enable us not only to identify the presence of APV but also to differentiate closely related strains (Van Riper and Forrester 2007). Polymerase chain reaction in conjunction with restriction enzyme analysis (REA) has been found to efficiently differentiate even closely related virus species (Luschow et al. 2004). By comparing their nucleotide or protein variation, the relationships among APV isolates can be determined (Luschow et al. 2004). Molecular methods can also be applied for the differentiation of APV infection from diseases like infectious laryngotracheitis virus infection, which can display similar symptoms to the diphtheritic form of APV infection (Tripathy and Reed 2008).

1.7. IMMUNITY TO APV AND VACCINATION

Birds which have recovered from poxvirus infections or have been vaccinated usually develop long-lasting immunity to the same virus strain (Lierz et al. 2007). Transovarial transmission of immunity has not been observed (Van Riper and Forrester 2007). Cross-immunity to several strains has been demonstrated and reciprocal immunisation using APV from one host to another host species is possible (Van Riper and Forrester 2007). For example, a CNPV strain has been found to infect chickens, quails and turkeys but not house sparrows (*Passer domesticus*) and rock doves (*Columba livia*) while another CNPV did infect chickens, rock dove and house sparrow (Karstad 1971). Many APV isolates from wild birds are known to be non-pathogenic for chickens (Van Riper and Forrester 2007; Tripathy and Reed 2008).

In general, live attenuated or nonattenuated vaccines are used in poxvirus vaccination (Tripathy and Reed 2008). Currently FWPV, PGPV, CNPV, quailpox and turkeypox vaccines are commercially available (Tripathy and Reed 2008). Cell culture attenuated CNPV vaccine and chicken embryo adapted CNPV vaccine have been used in some countries (Hitchner 1981; Tripathy 1993). Commonly used methods of administrating the vaccines include the wing-web, thigh stick or oral vaccination methods (Tripathy and Reed 2008). Mass vaccination was conducted for FWPV via drinking water and aerosol but the immunisation effects varied (Nagy et al. 1990; Ariyoshi et al. 2003). It appears that to achieve a reasonable protection in mass vaccination the vaccine must contain a high concentration of virus (Tripathy 1993). With the exception of FWPV and pigeonpox vaccine, APV vaccines are only applied to their original hosts (Tripathy and Reed 2008). Fowlpox vaccine can be used in chickens and turkeys and pigeonpox vaccine can be used in chickens, turkeys and pigeons (Tripathy and Reed 2008). When chickens and turkeys are vaccinated with pigeonpox vaccine which is known to be less

pathogenic in chickens and turkeys, it stimulates the birds' immunity to APV infection instead of developing disease (Tripathy and Reed 2008). Recently, the possibility of *in-ovo* vaccination of FWPV has been suggested (Avakian et al. 2000).

Unlike other viruses, poxviruses have defence methods to protect themselves from the host immune mechanisms by carrying a range of proteins involved in immune evasion and immune modulation (Smith and Kotwal 2002). The viruses encode proteins involved in obstructing many of the strategies deployed by the host to combat viral infections, including proteins that block the activity of many chemokines, cytokines, serine proteases and even complement (Smith and Kotwal 2002). Fowlpoxvirus infections have been reported in previously vaccinated flocks which demonstrate the limitations of the use of vaccination as a preventative method (Singh et al. 2000). However, vaccination remains the most effective preventative method when there is no treatment available for APV infection. Increased concerns about the impacts of APV infection on rare or endangered native bird species around the world have resulted in the urgent requirement for more APV vaccines that are applicable in various bird species (Adams et al. 2005). The development of APV vaccines that can provide safe and effective protection in a variety of bird species will be of great benefit in the intensive management of many endangered bird species.

1.8. AVIPOXVIRUS AND CONSERVATION OF AVIAN SPECIES

Increasing numbers of free-ranging bird species are being found to be susceptible to APV infection. This includes the Galapagos mockingbird (*Nesominus parvulus*) (Thiel et al. 2005), the Darwin's finch (*Geospiza* spp.) (Kleindorfer and Dudaniec 2006), the Hawaiian goose (*Branta sandvicensis*) (Kim and Tripathy 2006a), white-tailed laurel-pigeon (*Columba junoniae*) (Medina et al. 2004), the Southern giant petrel (Shearn-

Bochsler et al. 2008), short-toed lark (*Calandrella rufescens*) (Smits et al. 2005) and Berthelot's pipit (*Anthus bertheloth*) (Smits et al. 2005; Illera et al. 2008). Poxvirus infection might be associated with pairing failure, impaired flight, reduced foraging and hatching rates, diminished immunological defence and reduced fitness in free-ranging birds (Kleindorfer and Dudaniec 2006). Although it is difficult to evaluate the cost poxvirus infection might inflict on free-ranging bird populations, it is suggested that exotic pathogens are implicated in the extinction of various bird species (Illera et al. 2008).

The introduction of pathogens into a new environment or novel host species is of great concern for the conservation of biodiversity. Avipoxvirus, which is generally slow-spreading and self-limiting in mainland avifauna, can spread rapidly when introduced to remote island ecosystems (Van Riper and Forrester 2007). For instance 50% of short-toed larks in the Canary Islands were infected by APV within a few years of its first report in 2000 (Smits et al. 2005). This rapid transmission on introduction is also well-documented in avian species in the Galapagos Islands where APVs are now well established (Wikelski et al. 2004; Gottdenker et al. 2008). In general, avian populations on isolated islands show a high prevalence of APV infection (Van Riper and Forrester 2007). Bird species endemic to isolated islands are known to have diminished natural immunity to introduced diseases (Kleindorfer and Dudaniec 2006). Likely contributing to this, island species are more likely to show reduced genetic diversity, particularly at the MHC locus which can result in increased susceptibility to a range of pathogens (Hale and Briskie 2009). Also, the island species are less likely to be exposed to a higher diversity of pathogens, which in turn makes those populations vulnerable to the introduction of new pathogens (Kleindorfer and Dudaniec 2006). For example, Atkinson et al (1995) found that Hawaiian bird species were more susceptible to APV infection than the same species of birds in mainland USA (Atkinson et al. 1995). A

recent study in Galapagos finches confirmed an increasing overall prevalence of APV over the last decade, with a variation between species in immune responses (Zylberberg et al. 2012).

The introduction of a pathogen from naturally occurring host species to naïve and susceptible host populations can cause disastrous results (Sandro 2008). A well-documented non-avian example is the decline of European red squirrels (*Sciurus vulgaris*) in the British Isles that were exposed to parapoxvirus infection introduced by North American eastern grey squirrels (*Sciurus carolinensis*) (Sandro 2008). While eastern grey squirrels presented a high prevalence of homologous antibodies to parapoxvirus infection, the development of antibodies to this pathogen was observed only in few red squirrels (Sandro 2008). The poor immune response to the virus in conjunction with the susceptibility to serious parapoxvirus disease has been predicted to lead to extinction of the red squirrels in the next 20 years (Sandro 2008). This highlights a particular concern to New Zealand avian conservation due to the uniqueness and vulnerability of New Zealand avifauna. The isolation of psittacine poxvirus in captive eastern rosellas (*Platycercus eximius*) provoked serious concerns about the impacts that this exotic poxvirus strain might cause to native bird species (Gartrell et al. 2003). If native New Zealand birds are susceptible to this exotic poxvirus infection and develop poor immunity against it then entire species might be at risk.

1.9. AVIPOXVIRUS INFECTION IN NEW ZEALAND BIRDS

Information regarding APV infection in New Zealand birds is extremely limited, not only in free-ranging birds but also in the poultry industries. With ongoing vaccinations, APV infection is not a major concern in the poultry industry in New Zealand (Pacificvet Limited 2007) however, the virus has more serious implications on the future of free-

ranging birds. It has been identified in various New Zealand birds including endangered species, making New Zealand avifauna more vulnerable (Alley 2002). The first official record of APV infection in New Zealand describes the infection in Richard's pipit (*Anthus novaeseelandiae*) in 1953 (Westerskov 1953). Two more APV suspected or confirmed cases were reported in the 1970s in another pipit and a silvereye (*Zosterops lateralis*) (Quinn 1971; Austin et al. 1973). More recently, it has been reported in black robin (*Petroica traversi*) (Tisdall and Merton 1988), oyster catcher (*Haematopus unicolor*) (Johnstone and Cork 1993), kereru (New Zealand pigeon, *Hemiphaga novaeseelandiae*) (Alley 2002), North Island robin (*Petroica australis*), weka (*Gallirallus australis*), and song thrush (*Turdus philomelos*) (Gartrell et al. 2003). Avipoxvirus is an ongoing cause of mortality in black robin and shore plover (*Thinornis novaeseelandiae*) populations (Gartrell et al. 2003) whose conservation status has been claimed as 'Endangered' by the International Union for Conservation of Nature (IUCN. 2012). Sandflies (*Simuliidae* spp.) were considered to be the major possible vector in New Zealand (Gartrell et al. 2003) however recent global warming has resulted in an increase in number and range of more insect vectors for APV including the introduced mosquito *Culex quinquefasciatus* and the native mosquito *Culex pervigilans*, posing greater threats to New Zealand birds (Derraik 2004; Tompkins and Gleeson 2006).

Following the first identification of psittacine poxvirus in New Zealand (Gartrell et al. 2003), poxvirus infection has been gaining more attention in conservation management of New Zealand biodiversity (Stone and Forbes 2002-2003). It is currently uncertain if these APV infections represent native strains affecting depleted populations, or were introduced with their European hosts and played a role in the decline of avian biodiversity. To our knowledge, no research has been conducted concerning the phylogenetic relationships between APV strains in New Zealand. Van Riper and Forrester (2007) pinpointed that the prevalence of APV in New Zealand was

greater than 10%. However, this is based on fairly old data (Westerskov 1953) and the original source is unclear. To our knowledge reliable information on the current prevalence or seroprevalence of APV in New Zealand is lacking.

1.10. OBJECTIVES OF THE STUDY

There are four main objectives for this study. The first objective is to examine the genetic diversity of APVs affecting native and introduced bird species in New Zealand (Chapter 2 and 3). Secondly, to determine how widespread APV is in New Zealand bird populations (Chapter 4 and 5). The third objective is to identify the pathogenicity of New Zealand APV strains (Chapter 6). Lastly, the safety and efficacy of a commercially available APV vaccine in New Zealand is investigated (Chapter 6).

CHAPTER TWO

The Phylogenetic Analysis of Avipoxvirus in New Zealand

2.1. ABSTRACT

Avipoxvirus is known to be endemic in New Zealand and it is a cause of ongoing mortalities in the endangered black robin and shore plover populations. There is no information on the strains of avipoxvirus occurring in New Zealand and their likely origin or pathogenicity. This study was designed to identify the phylogenetic relationships of pathogenic avipoxvirus strains infecting introduced, native, and endemic bird species in New Zealand. Avipoxvirus 4b core protein gene was detected in tissue samples from 25/48 birds (52.1%) from 15 different species in New Zealand. Bootstrap analysis of avipoxvirus 4b core protein gene revealed that the New Zealand avipoxvirus isolates was comprised of three different subclades. The majority of New Zealand avipoxvirus isolates (74%) belonged to A1 subclade which shared 100% genetic similarity with the fowlpox HPB strain. An isolate from a wood-pigeon (kereru) belonged to subclade A3, displaying 100% sequence homology to albatrosspox virus. An additional group, isolated from two shore plovers and one South Island saddleback, grouped within subclade B1 and presented 99% sequence homology to European PM33/2007 and Hawaiian HAAM 22.10H8 isolates. The results suggest that a variety of New Zealand bird species are susceptible to avipoxvirus infection, that there are more than two distinctive avipoxvirus subclades in New Zealand, and that the most prevalent A1 strain may have been introduced to New Zealand through introduced avian hosts such as passerines or poultry.

Key words: avipoxvirus, 4b core protein, New Zealand birds, phylogenetic analysis

Abbreviations: PCR = polymerase chain reaction; bp = base pairs; DNA = deoxyribonucleic acid; APV = avipoxvirus; FWPV = fowlpox virus; ABPV = albatrosspox virus; CNPA = canarypox virus; PGPV = pigeonpox virus; FLPV = falconpox virus; PRPV = parrotpox virus

2.2. INTRODUCTION

Avipoxvirus (APV) infection is a common viral disease of birds which has been identified in more than 278 bird species in 20 orders (Van Riper and Forrester 2007). Since the first publication reporting APV infection in 1844, it has been isolated throughout the world both in free-ranging and captive birds (Bolte et al. 1999). The virus belongs to the subfamily of *Chordopoxvirinae* of the family *Poxviridae* (Tripathy and Reed 2008). Currently, 16 species comprise the genus APV which are named after their host species: fowlpox, turkeypox, pigeonpox, canarypox, and psittacinepox (Tripathy and Reed 2008). Avipoxvirus infection has two primary clinical forms: a dry cutaneous form and a wet diphtheritic form (Van Riper and Forrester 2007). The cutaneous form is characterised as the development of nodular or tumour lesions on unfeathered parts of the skin, e.g., feet, legs, head, and eyelids (Adams et al. 2005). Birds infected by the cutaneous form are more likely to recover and develop long-lasting immunity, however, many individuals die from secondary bacterial or fungal infections of the primary pox lesions (Van Riper and Forrester 2007). The diphtheritic form of APV infection causes diphtheritic lesions in the upper respiratory and gastrointestinal tracts (Adams et al. 2005). Often, birds affected with the diphtheritic form also present clinical signs consistent with the cutaneous form and the mortality rate is higher in diphtheritic or mixed forms than the cutaneous form (Bolte et al. 1999). Rarely, a systemic form of APV infection might be observed, especially in canaries (Van Riper and Forrester 2007). The virulence of the virus is linked to several factors including environmental or physical stress, the strain of virus, the route of infection, the presence of other infections, and the species and age of birds infected (Adams et al. 2005; Tripathy and Reed 2008).

While APV infection causes a significant worldwide economic loss in the poultry industry, it is also implicated in the decline of biodiversity in endemic and native bird populations, as demonstrated in Hawaii and Galapagos Islands (Thiel et al. 2005; Jarvi et al. 2008). On these islands, increasing numbers of free-ranging birds are found to be susceptible to APV infection, including the Galapagos mockingbird (*Nesominus parvulus*) (Thiel et al. 2005), Darwin's finch (*Geospiza* spp.) (Kleindorfer and Dudaniec 2006), Hawaiian goose (*Branta sandvicensis*) (Kim and Tripathy 2006a), white-tailed laurel-pigeon (*Columba junoniae*) (Medina et al. 2004), southern giant petrel *(Macronectes giganteus)* (Shearn-Bochsler et al. 2008), short-toed lark (*Calandrella rufescens*) (Smits et al. 2005) and Berthelot's pipit (*Anthus bertheloth*) (Illera et al. 2008). Sub-lethal APV infections in birds may be associated with pairing failure, impaired flight, reduced foraging and hatching rates, diminished immunological defence, and reduced fitness (Kleindorfer and Dudaniec 2006). Although it is difficult to evaluate the full costs of APV infection in free-ranging bird populations, it is likely that APV strains have been a factor in the extinction of a range of bird species (Thiel et al. 2005; Illera et al. 2008; Jarvi et al. 2008).

Avipoxvirus is known to be endemic in New Zealand birds and there are several official records of APV infections in New Zealand silvereye (*Zosterops lateralis*) (Austin et al. 1973) and Richard's pipit (*Anthus novaeseelandiae*) (Westerskov 1953; Quinn 1971). It is believed to be a cause of ongoing mortalities in the endangered black robin (*Petroica traversi*) and shore plover (*Thinornis novaeseelandiae*) populations which are being intensively managed by the Department of Conservation (Gartrell et al. 2003). Recently, APV has been gaining more recognition in New Zealand after an incursion of psittacinepox virus which was isolated and contained in 2003 (Gartrell et al. 2003). Although psittacinepox remains an exotic disease to New Zealand, detailed information on endemic APV strains in New Zealand is extremely limited. Of relevance to this study,

there is no information on the strains of APV occurring in New Zealand and their likely origin or pathogenicity. Thus, this study was designed to identify the phylogenetic relationships of pathogenic APV strains infecting introduced, native, and endemic bird species in New Zealand.

2.3. MATERIALS AND METHODS

2.3.1. Sample collection

Overall, 48 birds from 20 species were sampled (Table 2.1.). Forty tissue samples were collected from cutaneous or mucosal lesions suggestive of APV on gross and histological examination from birds submitted for post mortem examination at the Institute of Veterinary Animal and Biomedical Sciences (IVABS), Massey University between 1992 and 2009. Fresh tissue samples were available only from five birds and sample collection from the remaining 35 birds was from archived paraffin-fixed tissues. The collected tissue samples were mainly skin lesions around the eye, head, or legs except for five birds that displayed either the diphtheritic or mixed form of APV infections. In addition, eight suspect APV cases were identified in live birds and fresh tissue samples from biopsies of cutaneous lesions were preserved in 10% formalin or 70% ethanol solution. Most of the species are classified as native or endemic to New Zealand except canary (*Serinus canaria*), house sparrow (*Passer domesticus*), song thrush (*Turdus philomelos*), and turkey (*Meleagris gallopavo*) (Table 2.1.). Six species, such as black robin, shore plover, wrybill (*Anarhynchus frontalis)*, black stilt (*Himantopus novaezelandiae*), Chatham Island taiko (*Pterodroma magentae*), and North Island brown kiwi (*Apteryx mantelli*), are classified as vulnerable to critically endangered by the International Union of the Conservation of Nature (IUCN. 2012). Published sequences from GenBank including vaccinia virus (M11079), fowlpox FWPV

HP-B (AY530302), pigeonpox PGPV TP-2 (AY530303), albatrosspox ABPV 353/87 (AM050392), falconpox FLPV GB362-02 (AY530306), canarypox CNPV 1445 (AM050375), avipoxvirus isolate PM33/2007 (EF634350), avipoxvirus Hawaiian strain HAAM 22.10H8 (EF568395), pigeonpox PGPV B7 (AY453177), and parrotpox PRPV 364/89 (AM050383) were selected for sequence comparison.

2.3.2. DNA extraction and APV specific PCR

Avipoxvirus DNA was extracted from a fowlpox vaccine, Poxine (Duphar; Fort Dodge), and used in the PCR assay as a positive control. Thirty five paraffin embedded tissues were cut at 10 µm for DNA extraction using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Victoria, Australia). For fresh samples, 25 mg of pox-suspicious lesions were collected for DNA extraction. The DNA extraction followed the manufacturer's protocol for paraffin-embedded tissue or formalin-fixed tissue with a slight modification. After overnight incubation at 56°C in 180 µl of Buffer ATL and 20 µl of proteinase K, some samples needed additional digestion. The samples were mixed with another 20 µl of proteinase K and incubated for 3 more hours at 56°C. The DNA extraction proceeded as described in the manufacturer's protocol with addition of 200 µl of absolute ethanol.

PCR was performed for the detection of the APV 4b core protein gene DNA as described by Jarvi et al (2008), with minor modifications. Two and a half µl of DNA was added to a mixture of 0.8 mM deoxynucleoside triphosphate, 2 mM $MgCl_2$, 10 X PCR Buffer, 0.8 mM of each primer and 1 unit of Platinum Taq DNA polymerase (Invitrogen, California, USA) to make a total volume of 25µl. The PCR program began with an initial denaturation at 94°C for 4 min, then 40 cycles of denaturation for 30 sec at 94°C, annealing for 1 min at 53°C, extension for 1 min at 72°C; with a final extension at 72°C for 7 min. All PCR products were visualised by electrophoresis on a 1% (w/v) ultra-pure

agarose gel (Invitrogen, California, USA) containing Ethidium bromide.

2.3.3. Gene sequencing and phylogenetic analysis

All APV positive PCR amplicon samples were purified using PureLink PCR purification kit (Invitrogen, California, USA) and subjected to automatic dye-terminator cycle sequencing with BigDye™ Terminator version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyser (Applied Biosystems Inc, Foster City, California, USA) to confirm genomic sequences. The sequences were compared to published sequences available from GenBank using NCBI Blast. The GenBank assession numbers included in this study were vaccinia (M11079), fowlpox FWPV HP-B (AY530302), pigeonpox PGPV TP-2 (AY530303), albatrosspox ABPV 353/87 (AM050392), falconpox FLPV GB362-02 (AY530306), canarypox CNPV 1445 (AM050375), avipoxvirus isolate PM33/2007 (EF634350), avipoxvirus Hawaiian strain HAAM 22.10H8 (EF568395), pigeonpox PGPV B7 (AY453177), and parrotpox PRPV 364/89 (AM050383). Alignments were performed on the trimmed sequences (420 bp) using Clustal W (Thompson et al. 1994) with gaps ignored and a phylogenetic tree was generated using a Jukes-Cantor distance model and neighbour-joining method in Geneious Pro 4.5.4™ (Biomatters Ltd, Auckland, New Zealand). Bootstrap testing of phylogeny was performed with 1000 replications and values equal to or greater than 50 are indicated on the branches. The sequence divergence between and within the different lineages was calculated using a Jukes-Cantor model of substitution implemented in the program PAUP* 4.0 Beta version 10 (Swofford 2002). Avipoxvirus clade and subclade labelling was performed as previously described by Jarmin et al. (2006) and Manarolla et al. (2010).

2.4. RESULTS

2.4.1. PCR and phylogenetic analysis

Avipoxvirus 4b core protein gene was detected in tissue samples from 25/48 birds (52.1%) from 15 different species (Table 2.2.). These included three formalin or ethanol fixed tissue samples and 22 paraffin-fixed samples (Table 2.2.). The PCR assay used in this study was sensitive enough to amplify APV 4b core protein gene from tissue samples embedded in paraffin for prolonged periods. The oldest APV isolate dated to 1992 from a variable oyster catcher (*Haematopus unicolor*) (variable oyster catcher 1:HQ701720) (Table 2.2.). In most of the species that were found APV positive by PCR in this study, APV infection has never been or only infrequently reported (North Island robin (*Petroica australis longipes*), black robin, North Island saddleback (*Philesturnus carunculatus rufusater*), South Island saddleback (*Philesturnus carunculatus carunculatus*), shore plover, wrybill, variable oyster catcher, paradise shelduck (*Tadorna variegate*), North Island brown kiwi, kereru (*Hemiphaga novaeseelandiae*)) (Van Riper and Forrester 2007). Along with repeating PCR for a number of samples which presented negative results in PCR, histological examinations were carried out, too. The presence of inclusion bodies was not obvious in most cases, including PCR positive samples, and it was decided to focus on PCR results only.

Among the 25 birds positive by PCR, nucleotide sequences from 17 cases were trimmed to 420 bp for phylogenetic analysis. Three distinctive clades; A, B, and C, with several subclades have been described in Jarmin et al (2006) and Manarolla et al (2010) (Figure 2.1.). The 17 New Zealand APV isolates identified in this study belonged to subclades A1, A3, and B1 (Figure 2.1.). The results of the NCBI Blast and phylogenetic analysis of the 17 420 bp fragments revealed that the isolates from one song thrush

(HQ701723), one North Island saddleback (HQ711987), one house sparrow (HQ701716), one black robin (HQ701721), one silvereye (HQ701724), three shore plovers (HQ701717, HQ711988, HQ711989), one North Island robin (HQ701722), two variable oyster catchers (HQ701720, HQ711990), one paradise shelduck (HQ701719), and one South Island saddleback (HQ701718) belonged to subclade A1, sharing 100% genetic similarities with fowlpox virus HPB (AY530302) (Figure 2.1., Table 2.2.). One kereru isolate (HQ701713) had 100% sequence homology with albatrosspox virus ABPV 353/87 (AM050392) and comprised subclade A3 (Figure 2.1., Table 2.2.). Two shore plover isolates (HQ701714, HQ711991) originating from an offshore Island and one South Island saddleback isolate (HQ701715) belonged to a further subclade B1 (Figure 2.1., Table 2.2.). These isolates presented 99% sequence homology with published sequences of the APV 4b core protein gene from APV subclade B1 isolates such as APV isolate PM33/2007 (EF634350) and APV Hawaiian strain HAAM 22.10H8 (EF568395).

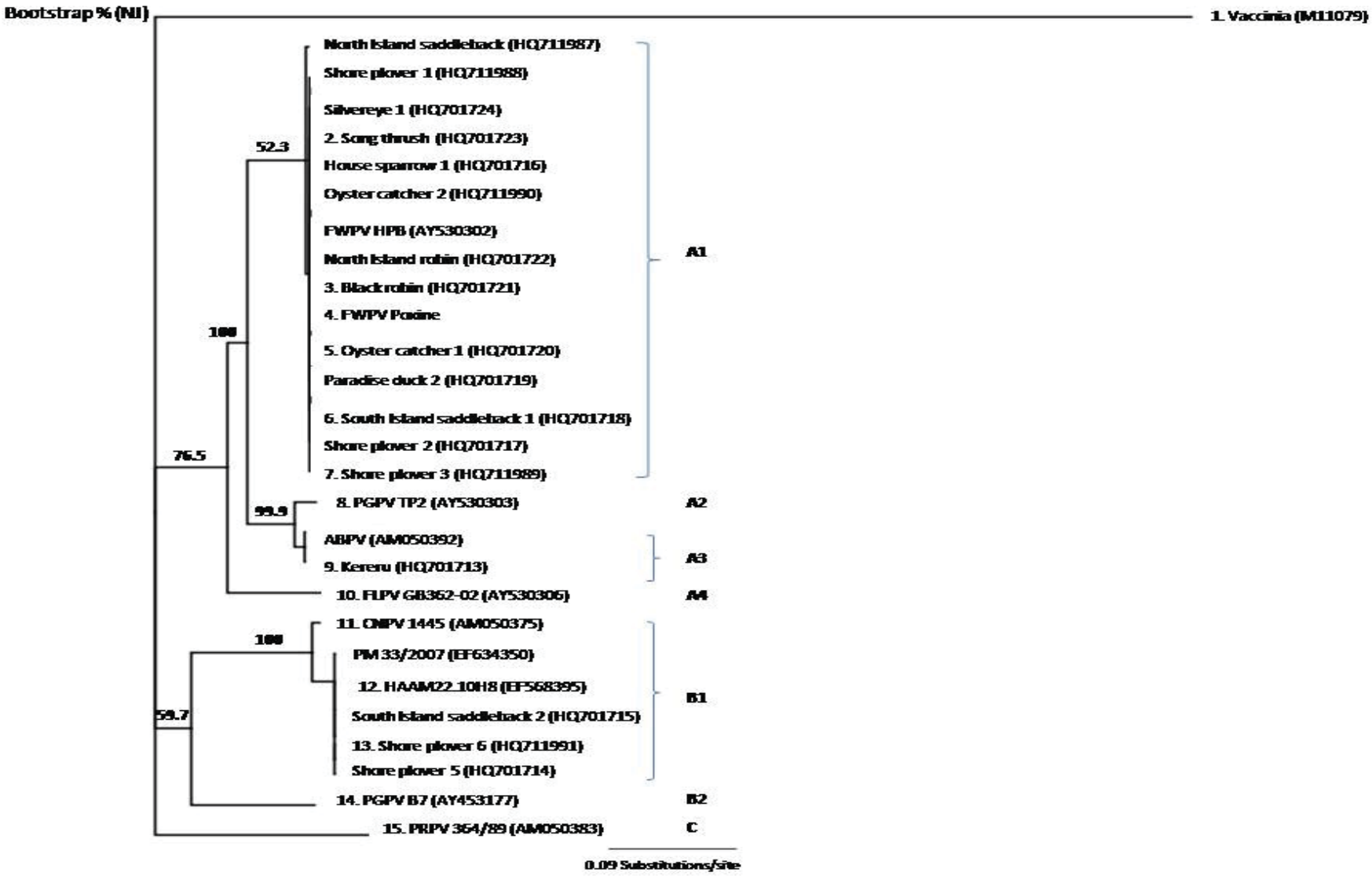
Bootstrap % (NJ)
1. Vaccinia (M11079)
North Island saddleback (HQ711987)
Shore plover 1 (HQ711988)
Silvereye 1 (HQ701724)
2. Song thrush (HQ701723)
House sparrow 1 (HQ701716)
Oyster catcher 2 (HQ711990)
FWPV HP8 (AY530302)
North Island robin (HQ701722)
3. Black robin (HQ701721)
4. FWPV Poxine
5. Oyster catcher 1 (HQ701720)
Paradise duck 2 (HQ701719)
6. South Island saddleback 1 (HQ701718)
Shore plover 2 (HQ701717)
7. Shore plover 3 (HQ711989)
A1
8. PGPV TP2 (AY530303)
A2
ABPV (AM050392)
9. Kereru (HQ701713)
A3
10. FLPV GB362-02 (AY530306)
A4
11. ONPV 1445 (AM050375)
PM 33/2007 (EF634350)
12. HAAM22.10H8 (EF568395)
South Island saddleback 2 (HQ701715)
13. Shore plover 6 (HQ711991)
Shore plover 5 (HQ701714)
B1
14. PGPV B7 (AY453177)
B2
15. PRPV 364/89 (AM050383)
C
52.3
100
76.5
99.9
100
59.7
0.09 Substitutions/site

Figure 2.1. Phylogenetic tree of nucleotide sequences of the 4b core protein gene of APV isolated in this study, reference APV sequences and the Vaccinia orthologue sequence, rooted on Vaccinia. Only full sequences (420 bp) were used for the generation of phylogenetic tree. Bootstrap testing of phylogeny was performed with 1000 replications and values equal to or greater than 50 are indicated on the branches. Numbers in front of genome species names correspond to the numbers of 4b protein gene lineages in Table 2.3. GenBack accession numbers are given in parenthesis for reference APV sequences. Subclade grouping is indicated as suggested by Jarmin et al. (2006) and Manarolla et al. (2010).

Nucleotide sequencing of the isolates from a canary and a turkey was not successful. The sequencing results presented evidence of multiple infections. As turkey and canary are not the species of concern in this study, it was decided not to conduct further tests. The smaller fragments (238 to 373 bp) from the remaining six isolates (one wrybill, one shore plover, one silvereye, one house sparrow, one paradise shelduck, and one kiwi) were used for the identification of clades. Three isolates; one shore plover (shore plover 4), one silvereye (silvereye 2), and one house sparrow (house sparrow 2) belonged to subclade A1 (Table 2.2.), with 100% sequence homology to FWPV HPB (AY530302). Two APV isolates from one kiwi and one paradise shelduck (paradise duck 1) belonged to subclade A3, with sequence homology of 93% and 100% to ABPV 353/87 (AM050392), respectively (Table 2.2.). The wrybill isolate belonged to subclade B1 with 95% sequence homology to European strain PM33/2007 (EF634350) and APV Hawaiian strain HAAM 22.10H8 (EF568395) (Table 2.2.).

Among the 13 New Zealand APV isolates comprising subclade A1, only the song thrush isolate (HQ701723) displayed a minor level of divergence (0.2%) when compared to other members of the same group (Table 2.3.). The kereru isolate (HQ701713) in the A3 subclade presented a greater genetic similarity to albatrosspox than pigeonpox virus as the mean divergence percentage between a kereru isolate and pigeonpox virus PGPV TP 2 (AY050303) was 2.4% (Table 2.3.). The three New Zealand B1 APV isolates comprising subclade B1 presented 2.1% sequence divergence from canarypox virus CNPV 1445 (AM050375) and 0.0% sequence divergence from Hawaiian strain HAAM 22.10H8 (EF568395) (Table 2.3.). New Zealand APV isolates comprising subclade B1 displayed 23.3% - 23.6% sequence divergence from APV isolates grouped in subclade A1 (Table 2.3.). Mean divergence percentage between two shore plover isolates, one belonging to subclade A1 (HQ711989) and the other belonging to subclade B1 (HQ711991), was 23.6% (Table 2.3.). Mean divergence percentage between Clade A

and Clade B and between Clade A and Clade C were greater than 20.5% and 23.8%, respectively (Table 2.3.).

Spatial distribution of the clades on APV isolated from pox-affected birds in New Zealand is displayed in figure 2.2. Subclade A1 had been identified throughout New Zealand including Chatham Islands, which is more than 850 km east of the New Zealand mainland. Subclade A3 and subclade B1 displayed similar geographic distributions within the northern part of the North Island and on the offshore islands between the North and South Island.

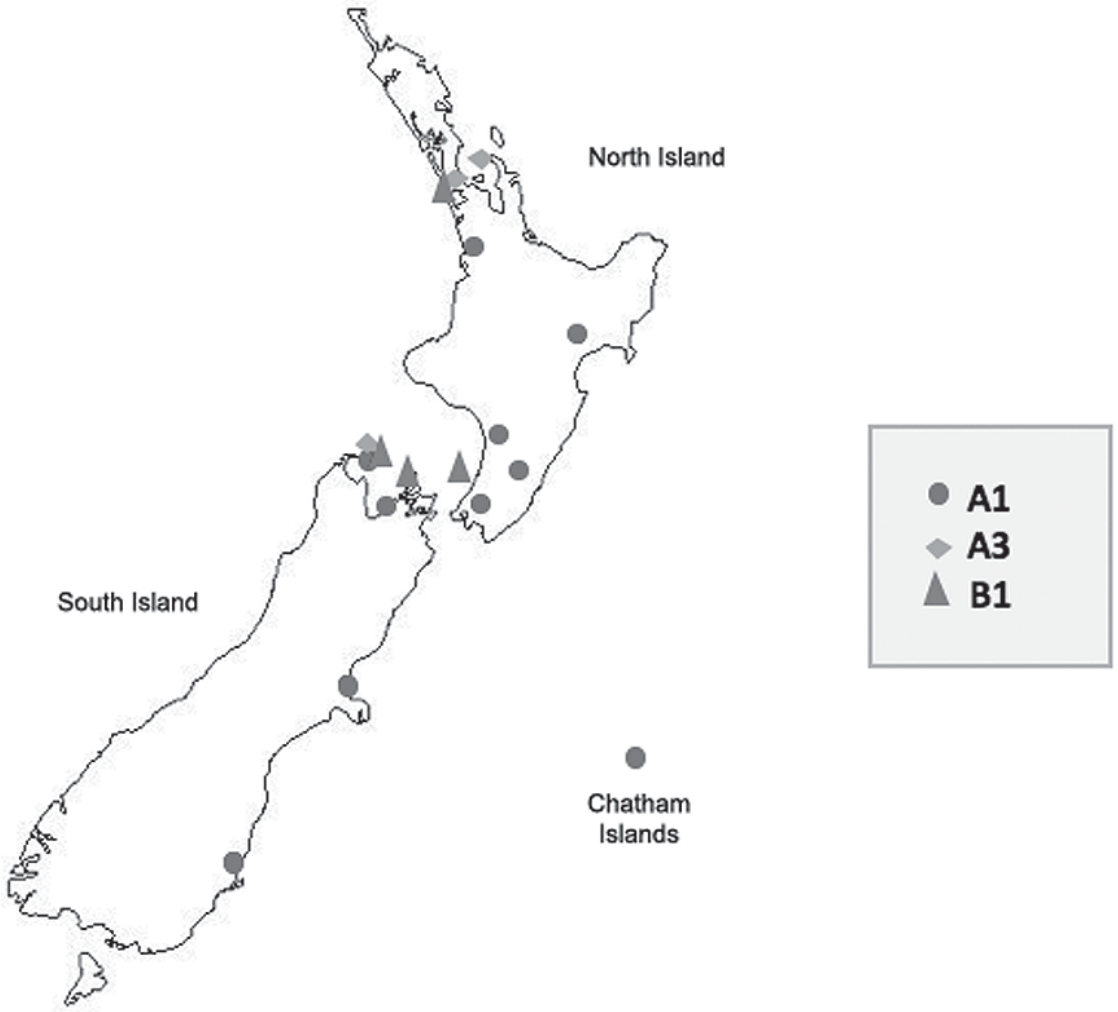

Figure 2.2. Spatial distribution of the clades on APV isolated from pox-affected birds in New Zealand.

Table 2.1. The Order, Family, scientific names, the origin, and sample preservation method of bird species sampled in this study.

[A] Classification: I = introduced species, N = native species, E = endemic species.

[B] The origin of birds sampled: NI = North Island, SI = South Island, OSI = Offshore Island.

Order	Family	Scientific name	Common name (I, N, E) [A]	Region[B]			The No. of samples[C]	
				NI	SI	OSI	Paraffin-embedded	Fixed
Galliformes	Phasianidae	*Meleagris gallopavo*	Turkey (I)	1	-	-	1	-
Passeriformes	Fringillidae	*Serinus canaria*	Canary (I)	-	1	-	-	1
	Passeridae	*Passer domesticus*	House sparrow (I)	2	-	-	2	-
	Turdidae	*Turdus philomelos*	Song thrush (I)	1	-	-	1	-
	Zosteropidae	*Zosterops lateralis*	Silvereye (N)	2	3	-	5	-
	Petroicidae	*Petroica australis*	North Island robin (E)	1	-	-	1	-
	Petroicidae	*Petroica traversi*	Black robin (E)	-	-	2	1	1
	Callaeidae	*Philesturnus carunculatus rufusater*	North Island saddleback (E)	1	-	-	1	-
		Philesturnus carunculatus carunculatus	South Island saddleback (E)	-	-	2	2	-

Order	Family	Scientific name	Common name (I, N, E) [A]	Region[B]			The No. of samples[C]	
				NI	SI	OSI	Paraffin-embedded	Fixed
Charadriiformes	Charadriidae	*Thinornis novaeseelandiae*	Shore plover (E)	15	-	3	13	5
		Anarhynchus frontalis	Wrybill (E)	1	-	-	1	-
	Recurvirostridae	*Himantopus novaezelandiae*	Black stilt (E)	-	-	1	-	1
	Haematopodidae	*Haematopus unicolor*	Variable oyster catcher (E)	1	1	-	2	-
Procellariiformes	Procellariidae	*Macronectes giganteus*	Southern Giant petrel (N)	1	-	-	-	1
		Pterodroma magentae	Chatham Island taiko (E)	-	-	1	-	1
Gruiformes	Rallidae	*Gallirallus philippensis assimilis*	Banded rail (N)	1	-	-	-	1
Anseriformes	Anatidae	*Tadorna variegata*	Paradise duck (E)	2	-	-	2	-

Order	Family	Scientific name	Common name (I, N, E) [A]	Region[B]			The No. of samples[C]	
				NI	SI	OSI	Paraffin-embedded	Fixed
Apterygiformes	Apterygidae	*Apteryx mantelli*	North Island brown kiwi (E)	-	-	1	1	-
Columbiformes	Columbidae	*Hemiphaga novaeseelandiae*	Kereru (New Zealand woodpigeon, E)	1	1	1	2	1
Psittaciformes	Psittacidae	*Cyanoramphus* spp.	Kakariki (N)	-	1	-	-	1
Total				**30**	**9**	**9**	**35**	**13**

[C] Sample preservation method: paraffin embedded, fixed = tissues preserved in 10% formalin or 70% ethanol.

Table 2.2. GeneBank accession numbers, year of sample collection, sample preservation method, type of APV infection, and clade description of 25 APV isolates identified in this study.

Common name	Positive/tested (%)	PCR positive Samples	GeneBank Accession No. [A]	Sampling year	Preservation method	Infection type	Clade[B]
Turkey	1/1 (100%)	Turkey	N/A	2009	paraffin	cutaneous	N/I
Canary	1/1 (100%)	Canary	N/A	2009	10% formalin	cutaneous	N/I
House sparrow	2/2 (100%)	House sparrow 1	HQ701716	1997	paraffin	cutaneous	A1
		House sparrow 2	N/A	2002	paraffin	cutaneous	A1
Song thrush	1/1 (100%)	Song thrush	HQ701723	2002	paraffin	cutaneous	A1
Silvereye	2/5 (40%)	Silvereye 1	HQ701724	2007	paraffin	cutaneous	A1
		Silvereye 2	N/A	2008	paraffin	mixed	A1
North Island robin	1/1 (100%)	North Island robin	HQ701722	2002	paraffin	cutaneous	A1
Black robin	1/2 (50%)	Black robin	HQ701721	2005	paraffin	diphtheritic	A1
North Island saddleback	1/1 (100%)	North Island saddleback	HQ711987	2004	paraffin	cutaneous	A1
South Island saddleback	2/2 (100%)	South Island saddleback 1	HQ701718	2006	paraffin	cutaneous	A1
		South Island saddleback 2	HQ701715	2007	paraffin	cutaneous	B1
Variable oyster catcher	2/2 (100%)	Variable oyster catcher 1	HQ701720	1992	paraffin	mixed	A1
		Variable oyster catcher 2	HQ711990	2002	paraffin	cutaneous	A1

Common name	Positive/tested (%)	PCR positive Samples	GeneBank Accession No. [A]	Sampling year	Preservation method	Infection type	Clade[B]
Shore plover	6/18 (33%)	Shore plover 1	HQ711988	2002	Paraffin	cutaneous	A1
		Shore plover 2	HQ701717	2005	Paraffin	cutaneous	A1
		Shore plover 3	HQ711989	2007	Paraffin	cutaneous	A1
		Shore plover 4	N/A	2008	Paraffin	cutaneous	A1
		Shore plover 5	HQ701714	2009	70% ethanol	cutaneous	B1
		Shore plover 6	HQ711991	2009	70% ethanol	cutaneous	B1
Wrybill	1/1 (100%)	Wrybill	N/A	2004	paraffin	cutaneous	B1
Paradise duck	2/2 (100%)	Paradise duck 1	N/A	2006	paraffin	mixed	A3
		Paradise duck 2	HQ701719	2008	paraffin	cutaneous	A1
North Island brown kiwi	1/1 (100%)	North Island brown kiwi	N/A	2006	paraffin	cutaneous	A3
Kereru	1/3 (33%)	Kereru	HQ701713	2002	paraffin	mixed	A3

Table 2.3. The sequence divergence (in percentage) between 15 4b protein gene lineages of avian poxvirus spp.

	Lineage														
	1[A]	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1 Vaccinia (M11079)	0.0[B]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 Song thrush (HQ701723)	55.0	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-
3 Black robin (HQ701721)	54.8	0.2	0.0	-	-	-	-	-	-	-	-	-	-	-	-
4 Fowlpox FWVP	54.8	0.2	0.0	0.0	-	-	-	-	-	-	-	-	-	-	-
5 Variable oyster catcher 1 (HQ701720)	54.8	0.2	0.0	0.0	0.0	-	-	-	-	-	-	-	-	-	-
6 South Island saddleback 1 (HQ701718)	54.8	0.2	0.0	0.0	0.0	0.0	-	-	-	-	-	-	-	-	-
7 Shore plover 3 (HQ711989)	54.8	0.2	0.0	0.0	0.0	0.0	0.0	-	-	-	-	-	-	-	-
8 Pigeonpox TP2 (AY530303)	54.4	9.1	9.3	9.3	9.3	9.3	9.3	0.0	-	-	-	-	-	-	-
9 Kereru (HQ701713)	55.1	8.1	8.3	8.3	8.3	8.3	8.3	2.4	0.0	-	-	-	-	-	-
10 Falconpox (AY530306)	54.9	11.7	11.9	11.9	11.9	11.9	11.9	11.2	11.7	0.0	-	-	-	-	-
11 Canarypox 1445 (AM05375)	53.4	22.1	22.4	22.4	22.4	22.4	22.4	23.8	24.0	22.3	0.0	-	-	-	-
12 Avipoxvirus (EF568395)	53.9	23.3	23.6	23.6	23.6	23.6	23.6	24.0	24.8	23.6	2.1	0.0	-	-	-
13 Shore plover 6 (HQ711991)	54.0	23.3	23.6	23.6	23.6	23.6	23.6	24.1	24.8	23.6	2.1	0.0	0.0	-	-
14 Pigeonpox B7 (AY453177)	55.2	21.7	21.9	21.9	21.9	21.9	21.9	20.8	21.0	20.5	17.6	15.9	16.0	0.0	-
15 Parrotpox (AM050383)	58.4	24.0	23.8	23.8	23.8	23.8	23.8	24.5	25.0	26.2	24.0	23.3	23.3	22.9	0.0

[A] Numbers correspond to the numbers of 4b protein gene lineages in Figure 2.1., in which the genospecies of the virus and the GenBank accession numbers of the lineages are given.

[B] The sequence divergence was calculated with the use of a Jukes-Cantor model of substitution.

2.5. DISCUSSION

Avipoxvirus 4b core gene was detected in 25 birds (52.1%) among 48 free-ranging birds in New Zealand. The birds comprised of 15 avian species including 10 endemic, one native, and four introduced species. Among the 25 birds positive by PCR, 17 cases were fully sequenced and six were partially sequenced. The unsuccessful sequencing results might be because of (i) the deterioration of DNA during purification process, (ii) the technical errors during sequencing process, (iii) inappropriate PCR primers, (iv) the divergence of those isolates, or (v) infection by multiple species of APV in the same sample.

All of the 23 APV isolates sequenced fully or partially in this study belonged to the three subclades of avipoxviruses described in Manarolla et al. (A and B) (2010). Subclades A1 and B1 appeared to be the major APV groups existing in New Zealand. One isolate from kereru (HQ701713) comprised subclade A3 along with albatrosspox virus ABPV (AM050392). The majority (74%) of New Zealand APV isolates from 10 bird species belonged to the same subclade (A1), displaying 100% genetic homology between the isolates and with the attenuated fowlpox vaccine (FWPV Poxine) used by the poultry industry in New Zealand (Pacificvet Limited 2007). The results indicate that a wide variety of New Zealand free-ranging birds are susceptible to the specific A1 strain of fowlpox, which is used throughout the world as an attenuated fowlpox vaccine. A previous study showed that an APV isolate from a free-ranging bird presented a genetic similarity with FWPV and turkeypox virus (Luschow et al. 2004). Our study is unprecedented in detecting sixteen isolates of APV from ten species of birds in the same clades as FWPV (Luschow et al. 2004; Jarmin et al. 2006). It is likely that this strain of APV has been well established throughout New Zealand (Figure 2.2.). This strain of AVP has also been identified in an endangered black robin from Chatham

Islands, which is more than 850 km away from the New Zealand mainland. This results in the speculation that FWPV, which may have been introduced by poultry to New Zealand, has become endemic among New Zealand birds. Yet, the 4b core genes compared in this study are only parts of the entire APV genomes and the virulence of FWPV in various New Zealand birds has not been identified. Further work is required to investigate the role of poultry or poultry vaccination in the spread of the FWPV throughout New Zealand avifauna.

Other New Zealand APV isolates appear to be closely related to overseas isolates, in particular European isolates (A3 & B1 subclades). For instance, a kereru (HQ701713), a kiwi isolate, and a paradise shelduck isolate presented genetic similarities with albatrosspox ABPV (AM050392) which has been previously identified in passerine in Europe (A3 subclade). This suggests that some APV strains might have been introduced to New Zealand from Europe with the introduction of European bird species. It is interesting to note that a kereru isolate (HQ701713) presented a greater genetic similarity to albatrosspox ABPV (AM050392) than pigeonpox virus PGPV TP2 (AY530303). The mean distance percentage between a kereru isolate (HQ701713) and pigeonpox PGPV TP 2 (AY530303) was 2.4%. A South Island saddleback isolate (HQ701715) and two shore plover isolates (HQ701714, HQ711991) comprised subclade B1, along with canarypox CNPV 1445 (AM050375), APV isolate PM33/2007 (EF634350) and APV Hawaiian strain HAAM 22.10H8 (EF568395). This also suggests the possible introduction of APV strains from overseas with the introduction of exotic bird species such as passerines. It is interesting that New Zealand APV isolates presented great genetic similarities with APV Hawaiian isolate (EF568395). This might be due to not only the similar introduction of exotic passerines or game birds but also the recent introduction of mosquito (*Culex quinquefasicatus*). Two different subclades (A1 & B1) were isolated from South Island saddlebacks and shore plovers. The shore plover

isolates (HQ701714, HQ711991), belonging to subclade B1, were from an offshore island and had 23.6% sequence divergence from the remaining shore plover isolates grouped in subclade A1 (HQ701717, HQ711988, HQ711989). Two South Island saddleback isolates comprising two different subclades (HQ701718: A1; HQ701715: B1) were from the same region. The variation in APV isolates within a single species is similar to previous studies (Luschow et al. 2004; Jarmin et al. 2006; Manarolla et al. 2010). Jarmin et al (2006) suggested that this makes it difficult to introduce a host-species-based approach to the taxonomy of APVs.

The results of this study also confirm that many New Zealand native bird species are susceptible to pathogenic APV infection. Among fifteen species of birds tested positive for APV DNA by PCR assay, eleven species were considered as endemic or native in New Zealand. Avipoxvirus infection is not considered as a disease of concern in the poultry industry in New Zealand and fowlpox vaccination is only carried out in the northern part of the North Island (Pacificvet Limited 2007). However, it has previously been suggested that APV infection is endemic in New Zealand free-ranging birds and may be playing a role in the decline of biodiversity in New Zealand (Gartrell et al. 2003). This study confirms the hypothesis that there have been previous introductions of exotic strains of APV into the New Zealand avifauna that were probably concurrent with the deliberate introduction of northern hemisphere birds. What remains unresolved is what impact these exotic APV strains have had on the dramatic decline of native biodiversity seen over this period. While the introduction of mammalian predators is without doubt the key factor in the decline of New Zealand's avifauna, this study suggests the possibility that APV may have played a previously unrecognized role in the loss of species diversity.

Sandflies (family Simuliidae), introduced mosquito such as *Culex quinquefasicatus*, and

the native mosquito *Culex pervigilans* are the likely vectors for APV infection (Gartrell et al. 2003). Due to warmer summers, the number of vector species is increasing even in the southern part of New Zealand (Tompkins and Gleeson 2006) and as a result, the incidence of insect-vectored diseases such as APV is likely to increase. Therefore, understanding the taxonomic variation and pathogenicity of APV in New Zealand and the susceptibility of native species is key information required for the intensive conservation management of New Zealand's threatened avifauna.

It was noteworthy that some APV isolates in this study were related to translocation of the animals, such as the isolates from South Island saddlebacks (HQ701715, HQ701718) and shore plovers (HQ701714, HQ711991). Each of the host birds had a history of translocation from New Zealand mainland to an offshore island prior to the development of clinical signs consistent with APV infection. These birds displayed no clinical abnormalities in the quarantine procedure carried out before translocations. It is probable that two shore plovers became infected by APV after being transferred from captivity to an offshore island. The supporting evidence for this conclusion is that: 1) they presented no abnormalities before translocation, 2) they developed similar clinical signs 4-15 days after translocation, and 3) the strain of APV isolated in them differed from APV isolated in the source population. In contrast, APVs isolated from South Island saddleback 1 (HQ701718) and 2 (HQ701715) were different strains despite the fact that their origins and translocation sites were the same. This suggests that there may be overlap in the geographic distribution of APV strains within New Zealand. A number of New Zealand endemic bird species have been successfully managed through offshore island or mainland island translocation programs (Department of Conservation 1994, 2001) and disease risk control is an important component of translocation planning. Our results revealed that there was more than one APV subclade present in New Zealand and the geographic overlap was greater in the areas

where movement of animals frequently occurred. The key risks associated with APV for wildlife translocations is either the unintentional transfer of APV into new geographic areas with the movement of the host species, or the movement of susceptible host species into an area with pathogenic strains of APV. If these risks are to be managed, then clarification of the geographic distribution of APV and host susceptibility to APV strains should be taken into account in the conservation management of endangered bird species.

2.6. CONCLUSION

In summary, based on our phylogenetic analysis of APV isolates, we conclude that many of New Zealand endemic and native bird species are susceptible to pathogenic APV infection, and that there are more than two distinctive APV subclades in New Zealand. In addition, the most prevalent strains were introduced to New Zealand through either introduced avian hosts or insect vectors. The results raise the speculation that human intervention such as poultry vaccination might have caused the spread of a specific APV strain in New Zealand avifauna.

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

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: Hye Jeong HA

Name/Title of Principal Supervisor: Associate Professor Brett Gartrell

Name of Published Research Output and full reference: The phylogenetic analysis of avipoxvirus in New Zealand.

Ha HJ, Howe L, Alley M, Gartrell B. Veterinary Microbiology 150, 80-7, 2011

In which Chapter is the Published Work: Chapter two

Please indicate either:

- **The percentage of the Published Work that was contributed by the candidate:** 80%

  and / or

- **Describe the contribution that the candidate has made to the Published Work:**

Hye Jeong Ha
Candidate's Signature

3/10/2012
Date

Brett Gartrell
Principal Supervisor's signature

3/10/2012
Date

GRS Version 3– 16 September 2011

CHAPTER THREE

Avipoxvirus Infections in Brown Kiwi (*Apteryx mantelli*)

3.1. ABSTRACT

3.1.1. Case history

Nodular lesions were found on the skin of two immature brown kiwi (*Apteryx mantelli*) less than six months of age living freely on Ponui island off the North Island of New Zealand. The lesions were observed during routine external examination undertaken as a part of the management of other research projects, one in 2006 and the other in 2011. Apart from the skin lesions both birds showed no ill effects and the lesions resolved spontaneously over a two month period.

3.1.2. Pathological findings

The first case showed several 3mm diameter firm, brown nodules located on the skin below the hock of both legs. The second case had a single 7 x 20 mm multinodular mass on the base of the bill. A proportion of the mass and scab samples were collected for diagnosis. Histological examination of the nodules revealed severe ballooning degeneration of keratinocytes and epithelial hyperplasia. Round eosinophilic structures resembling avipoxvirus (APV) intracytoplasmic inclusion bodies (Bollinger bodies) were observed in the layers of keratinocytes. In deeper layers of the epidermis, there was evidence of secondary bacterial growth and inflammation.

3.1.3. Diagnosis

DNA was extracted from tissue samples and subjected to PCR analysis. Avipoxvirus 4b core protein gene was detected in both samples by PCR. Bootstrap analysis of APV 4b core protein gene revealed that APV isolates from two kiwi comprised two different

subclades. One isolate displayed 100% sequence homology to subclade B1, and the other presented 100% sequence homology to subclade A3.

3.1.4. Clinical relevance

It is confirmed that kiwi are susceptible to APV infection and that at least two different strains of APV are present in the population examined. Since there is no information on the origin, virulence, or prevalence of APV in kiwi a study of the seroprevalence would be useful to elucidate the degree of exposure and immune response to APV. This would allow a more informed approach to risk management of the disease in wild and captive populations.

Keywords: Avipoxvirus, Brown kiwi, proliferative dermatitis

Abbreviations: APV = avipoxvirus; PCR = polymerase chain reaction; Bp = base pairs; FWPV = fowlpox virus; ABPV = albatrosspox virus; PGPV = pigeonpox virus; PRPV = parrotpox virus; NCBI = National Center for Biotechnology Information; GTR+I = general time-reversible model including invariable sites

3.2. INTRODUCTION

Kiwi (*Apteryx spp.*) are a group of five endemic bird species thought to have resided in New Zealand for more than 60 million years (Haddrath and Baker 2001). Although they once thrived throughout the forests, the number of kiwi has decreased with the arrival of humans (Holzapfel et al. 2008). The five species have been intensively managed by the New Zealand Department of Conservation, however, the overall population is still in decline (Holzapfel et al. 2008). The main reasons for the decline of kiwi populations is predation by introduced mammalian predators, but recently, there has been an increased awareness of the threats posed by exotic avian diseases (Holzapfel et al. 2008). Yet, the information on diseases in kiwi populations is extremely limited, probably due to the species' nocturnal nature. As a result, the impact of disease on kiwi populations has been considered a low priority as there is little evidence of disease affecting the species on a population scale.

Avipoxvirus (APV) infection is a common disease of birds reported in more than 200 bird species representing 20 genera (Van Riper and Forrester 2007). The virus is a double-stranded DNA virus that belongs to the virus family *Poxviridae* and to date, at least 10 species have been identified (Van Riper and Forrester 2007). The virus is very resistant in the environment and can be transmitted by insect bites, by direct contact with infected birds, by contaminated food, water, or by exposure to virus particles in the environment (Tripathy and Reed 2008). The infection is known to be host specific with three different forms of avian disease documented: the cutaneous, diphtheritic, and systemic form (Van Riper and Forrester 2007). Since the cutaneous form is the most common, the disease is typically characterised by the presence of proliferative dermal nodules or tumour lesions on unfeathered parts of the body (Adams et al. 2005). The virulence of the virus can vary depending on the species of bird, its age, the

strain of virus, route of infection, the presence of other pathogens, or other stress factors (Tripathy and Reed 2008).

The effects of APV in wild birds are not as well-known as in poultry, however, it is known to play a role in biodiversity decline (Van Riper and Forrester 2007). Recent research revealed that APV can affect survival, reproduction, and fledging rates in birds (Vanderwerf 2009). Island-endemic birds are known to be more susceptible to APV than widespread continental or seabirds, with greater than 80% prevalence in some species (Parker et al. 2011). The virulence, prevalence and seroprevalence of APV in New Zealand birds are yet to be investigated.

This paper describes two cases of APV infection in brown kiwi (*Apteryx mantelli*), one of which was reported previously by the authors in the phylogenetic study of APV in New Zealand birds (Ha et al. 2011; Chapter two). To the authors' knowledge, no APV disease has been documented in kiwi populations prior to these studies.

3.3. CASE HISTORY

Two free-living kiwi, residing on Ponui Island (1770 ha; 36 55' S, 175 11' E) an offshore island located near Auckland in the North Island of New Zealand were found with lesions suggestive of APV infection. Samples of nodule tissue from both cases were submitted to the New Zealand Wildlife Health Centre at Massey University for diagnosis. Apart from the skin lesions both birds showed no ill effects and the lesions resolved spontaneously over a two month period.

Case 1. A juvenile kiwi weighing 800 grams, aged five months was captured in March 2006 and physically examined before attaching a radio transmitter and releasing.

Several 5 mm diameter firm brown nodules were found on the skin of the medial aspects of both legs. One of the skin nodules fell off during handling and it was placed in Seutin's buffer solution.

Case 2. In January 2011 a kiwi chick aged two months was found with a single irregular multinodular mass located at the base of the bill to the right of the midline closely attached to the lateral cere (Figure 3.1.). It measured 7 mm (width) by 5 mm (thick) by 20 mm (length), was cream/brown in colour and had a firm texture with a slightly roughened surface. Scab samples were collected using forceps and kept in a polystyrene sterile container for transportation to the laboratory.

Figure 3.1. The head and beak of a juvenile kiwi (Case No 2) showing the multinodular pox lesion firmly attached to the beak and cere. Note the presence of several ticks on the face.

3.4. PATHOLOGICAL FINDINGS

Tissues of birds' nodules were fixed in 10% formalin and embedded in paraffin wax. These were then cut at 4 µm and stained with haematoxylin and eosin (H&E) for histological examination.

Histologically, the nodules consisted of disorganised layers of proliferating keratinocytes, many of which showed severe ballooning degeneration (Figure 3.2., A). In some of the layers of keratinocytes, round eosinophilic structures resembling APV intracytoplasmic inclusion bodies (Bollinger bodies) were observed (Figure 3.2., B). These inclusion bodies were often poorly stained which was likely to be the result of poor sample preservation. In some areas, there were layers of exudate in which necrotic inflammatory cells mixed with colonies of bacteria were also present (Figure 3.2., A).

3.5. MOLECULAR BIOLOGY

DNA was extracted from paraffin embedded tissue (kiwi 1) or fresh tissue samples (kiwi 2) using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Victoria, Australia). Paraffin embedded tissue was cut at 10 µm for DNA extraction. For fresh samples, approximately 25 mg of tissue was used. The DNA extraction followed the manufacturer's protocol for animal tissue for paraffin-embedded tissue or fresh animal tissue with a slight modification as described in Ha et al. (2011; Chapter two). After overnight incubation at 56 °C in 180 µl of Buffer ATL and 20 µl of proteinase, the samples were mixed with another 20 µl of preoteinase K and incubated for 3 more hours at 56 °C for further lysis. The DNA extraction proceeded as described in the manufacturer's protocol with the addition of 200 µl of ethanol.

PCR to detect the APV 4b core protein gene DNA was carried out as described by Ha et al. (2011; Chapter two). A total volume of 25 µl, containing 0.8 mM deoxynucleoside triphosphate, 2 mM $MgCl_2$, 10 X PCR Buffer, 0.8 mM of each primer, 1 unit of Platinum Taq DNA polymerase (Invitrogen, California, USA), and 2.5 µl of DNA was used. The primers were P1 and P2 (Lee and Lee 1997). The samples were subjected to an initial denaturation at 94 °C for 4 min, then 40 cycles (denaturation for 30 sec at 94 °C; annealing for 1 min at 53 °C; extension for 1 min at 72 °C), and a final extension at 72 °C for 7 min. A volume of 10 µl of each PCR product was separated on a 1% (w/v) ultra-pure agarose gel (Invitrogen, California, USA) containing ethidium bromide.

The purification of the PCR amplicons and gene sequencing were carried out as documented in Ha et al. (2011; Chapter two). Two APV PCR amplicons were purified using PureLink PCR purification kit (Invitrogen, California, USA) and subjected to automatic dye-terminator cycle sequencing with BigDye™ Terminator version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyser (Applied Biosystems Inc, Foster City, California, USA) to confirm genomic sequences.

Resulting sequences were submitted to the National Center for Biotechnology Information (NCBI) blast nucleotide database and compared with previously published sequences. The NCBI GenBank accession numbers included in this study were vaccinia (M11079), fowlpox (FWPV) HP-B (AY530302), pigeonpox (PGPV) TP-2 (AY530303), albatrosspox (ABPV) 353/87 (AM050392), avipoxvirus isolate PM33/2007 (EF634350), pigeonpox B7 (AY453177), and parrotpox (PRPV) 364/89 (AM050383). The APV 4b core protein genes from the two kiwi and the seven GenBank sequences were trimmed to the same length (320 bp) using Geneious Pro 4.5.4™ (Biomatters Ltd, Auckland, New Zealand) and aligned using Clustal W (Thompson et al. 1994). A Bayesian phylogenetic tree was generated in MrBayes version 3.1 (Ronquist and Huesenbeck 2003) using a

general time-reversible model including invariable sites (GTR+I). The Bayesian phylogeny was obtained using one cold and three hot Monte Carlo Markov chains, which were sampled every 1,000 generations over 1 million generations. Of these trees, the first 25% were discarded as burn-in material. The remaining 750 trees were used to construct a majority consensus tree. Bootstrap percentages from the Bayesian analysis were added to the tree at the appropriate nodes. Subclade grouping is indicated as described in Ha et al. (2011; Chapter two).

Avipoxvirus 4b core protein gene was detected in both samples using PCR. The APV sequences from the two kiwi were genetically distinct (Figure 3.3.). The kiwi 1 isolate displayed 100% genetic homology to albatrosspox (ABPV) 353/87 (AM050392) and 97% genetic homology to pigeonpox (PGPV) TP-2 (AY530303). However, the isolate from kiwi 2 clustered in the B1, which is generally referred as 'canarypox' group (Figure 3.3.). The isolate presented 100% sequence homology to APV isolate PM33/2007 (EF634350).

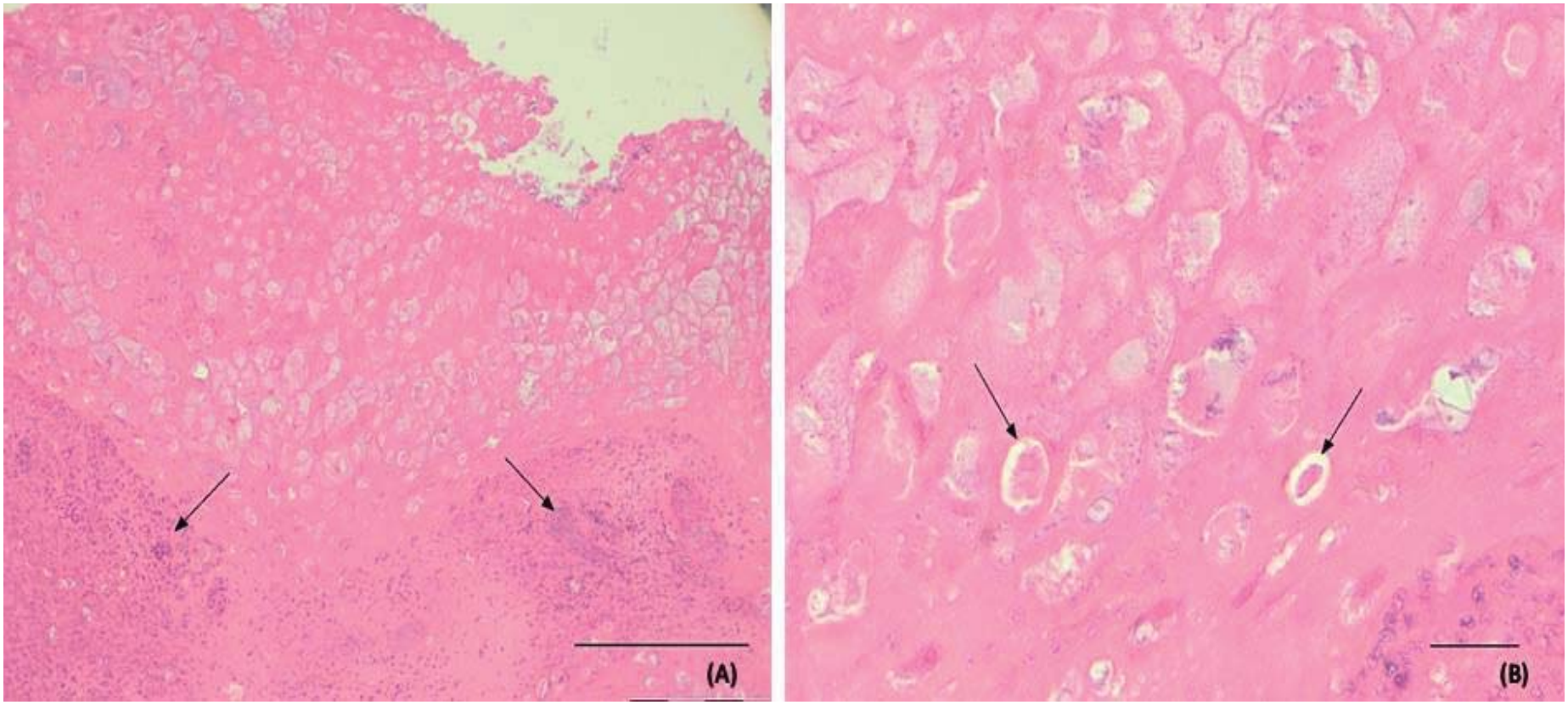

Figure 3.2. Light photomicrographs of the skin lesion from Case 1 showing (A) Horizontal layers of hyperplastic epithelial cells undergoing keratinisation which show ballooning degeneration. Beneath this are layers of exudate in which necrotic inflammatory cells mixed with colonies of bacteria are present (arrows)High H&E Scale bar = 20 µm. (B): power of the same lesion showing the remnants of intracytoplasmic inclusion bodies (arrows) within the ballooned keratinocytes. H&E Scale bar = 100 µm.

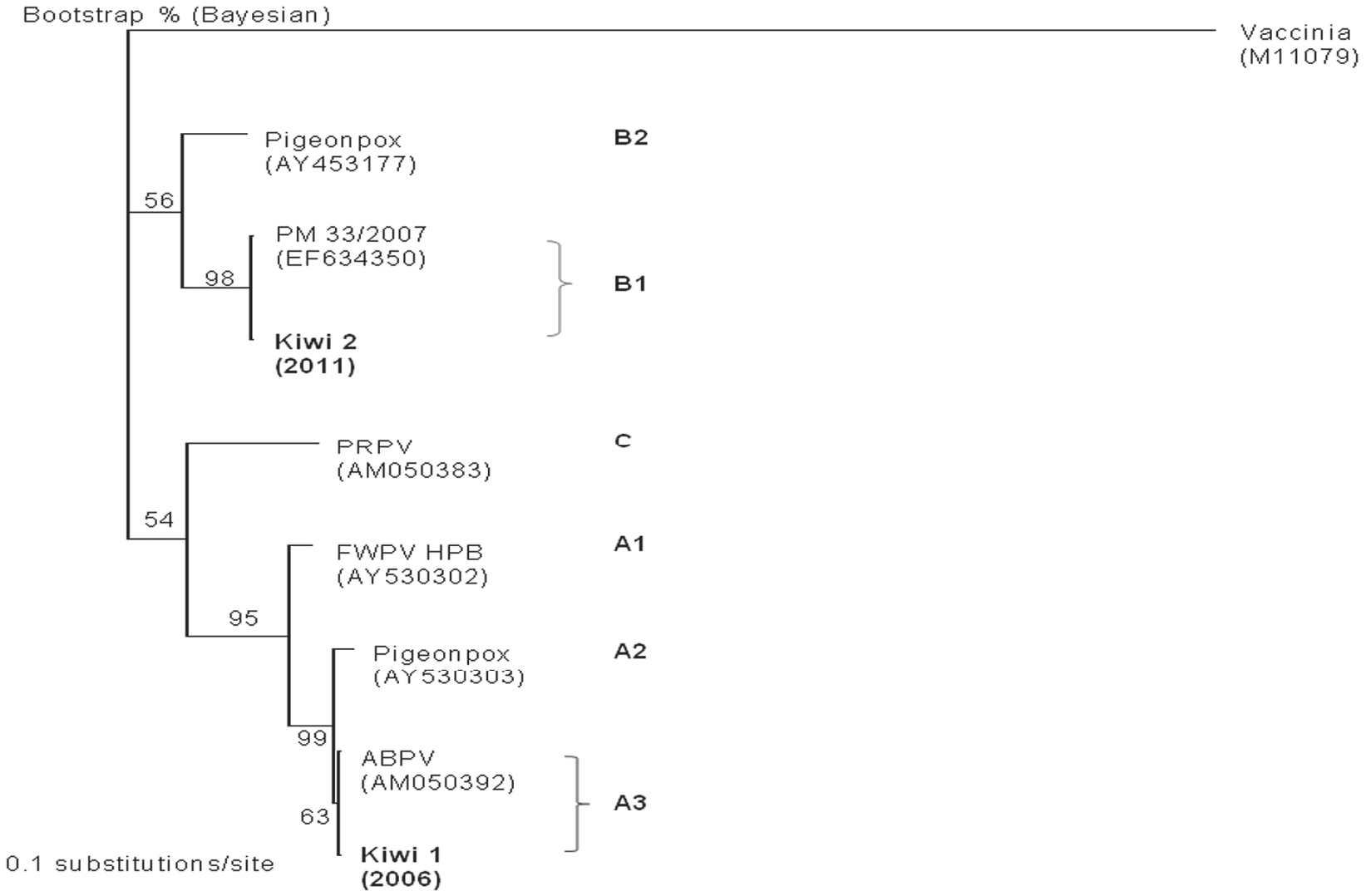
Bootstrap % (Bayesian)
Vaccinia
(M11079)
Pigeonpox
(AY453177)
B2
56
PM 33/2007
(EF634350)
98
B1
Kiwi 2
(2011)
PRPV
(AM050383)
C
54
FWPV HPB
(AY530302)
A1
95
Pigeonpox
(AY530303)
A2
99
ABPV
(AM050392)
A3
63
Kiwi 1
(2006)
0.1 substitutions/site

Figure 3.3. Phylogenetic tree of nucleotide sequences of the 4b core protein gene of APV isolated in two kiwi, six reference APV sequences and the Vaccinia orthologue sequence. The tree is rooted on Vaccinia. A Bayesian phylogenetic tree was generated in MrBayes version 3.1 (Ronquist and Huesenbeck 2003) using a general time-reversible model including invariable sites (GTR+I). The Bayesian phylogeny was obtained using one cold and three hot Monte Carlo Markov chains, which were sampled every 1,000 generations over 1 million generations. Of these trees, the first 25% were discarded as burn-in material. The remaining 750 trees were used to construct a majority consensus tree. GenBank accession numbers of the sequences are provided after the names of APV isolates when available.

3.6. DISCUSSION

Viruses of subclades A3 and B1, to which the two kiwi isolates belong, have previously been identified in New Zealand birds (Ha et al. 2011; Chapter two), suggesting that the viruses are widespread. The subclade A3 APV has been identified in New Zealand infecting a kereru (*Hemiphaga novaeseelandiae*) and subclade B1 APV has been found in shore plover (*Thinornis novaeseelandiae*) and South Island saddleback (*Philesturnus carunculatus carunculatus*) (Ha et al. 2011; Chapter two).

Veterinary interest in New Zealand avifauna dates back more than 60 years and was initiated by concerns about the health of kiwi populations (Alley 2002). There are reports of several bacterial or parasitic diseases in the species (Alley 2002), but no viral disease had been documented prior to the identification of APV in New Zealand birds in 2011 (Ha et al. 2011; Chapter two). Although both kiwi infected by APV have recovered from the infection, the virulence of the virus in kiwi remains unclear. In the best case scenario, APV infection might not pose a significant threat to kiwi, however, immune suppression caused by poxvirus infection cannot be overlooked as it is well established that APV infection often results in a secondary bacterial or fungal infection (Wang et al. 2006).

Mixed infection by avirulent pathogens can result in the alteration of virulence, creating highly virulent pathogens (Thomas et al. 2003). For example, mortalities by mixed infection by APV and avian malaria have been documented in endemic New Zealand birds (Alley et al. 2010). Given that APV can be transmitted by insect vectors, and that the number of insect vectors is increasing, including the introduced mosquito *Culex quinquefasicatus*, the potential threat posed by APV to New Zealand birds, including kiwi, is likely to increase

(Tompkins and Gleeson 2006; Van Riper and Forrester 2007).

The mode of transmission of the disease in this population has yet to be confirmed but large numbers of ticks (*Haemaphysalis longicornis and Ixodes anatis*) are often present all over the body of the birds (Castro 2006) (Figure 3.1.). In addition, other biting ectoparasites such as fleas (*Pygiopsylla phiola*) and trombiculid mites (*Guntheria (Derrickiella) apteryxi;* I. Castro[1.] pers. obs.) are also common and abundant in this population, raising the possibility that these may be involved in APV transmission. Avipoxvirus transmission can also occur through direct contact between the birds. Brown kiwi pairs are known to share burrows (Potter 1989) and burrows may be sequentially and concurrently occupied by several birds (Ziesemann 2011). Although difficult to observe, physical interactions amongst kiwi including fighting and copulation (Cunningham and Castro 2011), may offer opportunities for pathogen transmission.

The fact that a ratite bird can be infected by a passerine APV strain supports the claim by Jarmin et al. (2006) that the evolution of the host may not be the main key in the evolution of APV strains. Despite both kiwi in this study having originated from the same offshore island, the two APV isolates identified were of different subclades. Kiwi were introduced to the island from various source populations 47 years ago, raising the possibility of accidental introduction of different strains of APV onto the island. Alternatively, different strains of APV might have been introduced by poultry, passerine or migratory birds. The existence of two strains raises the question of whether additional strains exist on this and other islands. Further work, including more intensive monitoring of APV in kiwi populations is required in order to provide valuable information on the virulence, prevalence, and the potential impacts of APV on New Zealand's endangered kiwi.

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A study of the seroprevalence of APV in kiwi populations is necessary to clarify the degree of exposure and immune response to APV. This would allow a more informed approach to risk management of the disease in wild and captive populations.

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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: Hye Jeong HA

Name/Title of Principal Supervisor: Associate Professor Brett Gartrell

Name of Published Research Output and full reference:

Avipoxvirus infections in brown kiwi (Apteryx mantelli)

Ha HJ, Alley M, Howe L., Castro I., Gartrell B. New Zealand Veterinary Journal (2012)
DOI:10.1080/00480169.2012.700629

In which Chapter is the Published Work: Chapter three

Please indicate either:

- **The percentage of the Published Work that was contributed by the candidate:** 80%

  and / or

- **Describe the contribution that the candidate has made to the Published Work:**

Hye Jeong Ha
Candidate's Signature

3/10/2012
Date

Brett Gartrell
Principal Supervisor's signature

3/10/2012
Date

GRS Version 3– 16 September 2011

CHAPTER FOUR

The Seroprevalence of Avipoxvirus and Its Association with Avian Malaria (*Plasmodium* spp.) Infection in Introduced Passerine Birds in the Southern Regions of the North Island of New Zealand

4.1. SUMMARY

Blood samples were collected from 65 free-ranging birds from six species in the southern North Island of New Zealand. Sera from the birds were tested for the presence of avipoxvirus (APV) antibodies by ELISA and blood cells from 55 birds were also tested for *Plasmodium* spp. by PCR. Forty five birds (69.2%) tested seropositive to APV. Song Thrushes (*Turdus philomelos*) presented the highest seroprevalence at 100% (4/4), followed by Eurasian Blackbirds (*Turdus merula*) (96.86%, 31/32), Chaffinches (*Fringilla coelebs*) (54.55%, 6/11), Starlings (*Sturnus vulgaris)* (25%, 3/12), Greenfinches (*Carduelis chloris)* (25%, 1/4), and European Goldfinches (*Carduelis carduelis)* (0%, 0/2). *Plasmodium* spp. DNA was detected in 15/55 birds (27.3%), including 11 Eurasian Blackbirds, one Song Thrush, and three Starlings. Eight Eurasian Blackbird isolates (73%) grouped within the subgenus *Novyella*. Two Eurasian Blackbird isolates and the Song Thrush isolate clustered within a different group with previously reported lineages LINN1 and AFTRU5. In addition, all three Starling isolates clustered within the well-characterized lineage *Plasmodium (Huffia) elongatum* GRW06. All *Plasmodium* positive Eurasian Blackbirds and the Song Thrush were seropositive to APV while only 67% of *Plasmodium* positive Starlings showed evidence of previous exposure to APV. A significant relationship between birds seropositive to APV and birds infected by *Plasmodium* spp. was observed in the Chi-Square test ($\chi^2 = 5.69$, df1, $p = 0.0086$). To the authors' knowledge this is the first report describing the seropevalence of APV and its association with *Plasmodium* spp. infection in introduced bird species in New Zealand.

Keywords: Avipoxvirus, Seroprevalence, Avian malaria *Plasmodium* spp.

Abbreviations: APV = avipoxvirus; ELISA = enzyme-linked immuosorbent assay; FWPV = fowlpox; BSA = bovine serum albumin; PBS = phosphate-buffered saline; PCR = polymerase chain reaction

4.2. INTRODUCTION

Avipoxvirus (APV) and avian malaria due to *Plasmodium* spp. are known to have played a significant role in the extinction of endemic Hawaiian birds (Atkinson and LaPointe 2009). Both diseases have also been documented as causing mortalities in endangered bird species in New Zealand (2, 4, 18). For example, concurrent infection by APV and *Plasmodium* spp. has caused mortalities in translocated South Island Saddlebacks (*Philesturnus carunculatus carunculatus*) (Alley et al. 2010). Although these pathogens are distinct from each other, one being virus and the other being parasite, concurrent infections appear to be common because they share the same insect vectors (Atkinson et al. 2005; Illera et al. 2008; Alley et al. 2010). In New Zealand, recent warmer summer temperatures have caused an increase in population number and expansion of range of such insect vectors, including sandflies (family Simuliidae), introduced mosquitoes such as *Culex quinquefasicatus*, and the native mosquito *Culex pervigilans* (Tompkins and Gleeson 2006; Ha et al. 2011; Chapter two). This has resulted in an increase in the threats posed by insect-borne pathogens to many native New Zealand birds.

Avian pox is caused by viruses of the genus *Avipoxvirus* which comprises the virus family Poxviridae (Tripathy and Reed 2008). To date, 16 species have been identified in the genus APV including fowlpox, turkeypox, pigeonpox, sparrowpox and psittacinepox (Van Riper and Forrester 2007). Avipoxviruses were previously thought to be host-specific or only infect closely related species however; recent research reveals that most APVs infect multiple bird species (Tripathy and Reed 2008). Its distribution is worldwide and various bird species are susceptible to the disease (Van Riper and Forrester 2007). Regardless of the virus strains the disease is usually manifested as either cutaneous or diphtheritic

clinical forms but mixed lesions can also be found in the same bird (Tripathy and Reed 2008). In the cutaneous form proliferative lesions develop on featherless parts of the skin and in the diphtheritic form, lesions develop on the mucosal surfaces of the upper digestive and respiratory tracts (Tripathy and Reed 2008). The mortality rate is generally low in the cutaneous form but it can be high when the infection is diphtheritic or mixed or when the animals are exposed to a secondary infection (Tripathy and Reed 2008). A rare form of infection, the systemic form, has also been reported in some species, especially in canaries (Van Riper and Forrester 2007). In New Zealand, both the cutaneous and diphtheritic forms of APV infection, along with mixed forms, have been reported in various native and introduced birds and at least three different strains of APV are present (Ha et al. 2011; Chapter two).

Avian malaria is caused by various lineages of protozoans of the genus *Plasmodium* in the order Haemosporida (Tompkins and Gleeson 2006). These cosmopolitan parasites may display low host specificity and a great genetic diversity and phylogenetic complexity (Howe et al. 2011). The most widely distributed are *Plasmodium (Haemamoeba) relictum*, along with *Plasmodium (Huffia) elongatum,* and *Plasmodium (Noveylla)* spp. (Valkiunas 2004). Studies in New Zealand have shown *Plasmodium* spp*.* are able to infect a wide range of endemic avifauna (Baillie and Brunton 2011; Castro et al. 2011; Howe et al. 2011) and in some cases have caused mortality in threatened species such as New Zealand Dotterel (*Charadrius obscures)* (Alley 2002), Hihi (*Notiomystis cincta)* (Alley et al. 2008), Brown Kiwi (*Apteryx mantelli*), Great Spotted Kiwi (*Apteryx haastii*), and Mohua (*Mohoua ochrocephala*) (Howe et al. 2011). It has been suggested that the combined establishment of the introduced mosquito *Culex quinquefasicatus* in New Zealand and widespread establishment of introduced Passeriformes such as Eurasian Blackbirds (*Turdus merula*)

and house sparrows (*Passer domestica*) has resulted in the establishment of avian malaria reservoirs (Tompkins and Gleeson 2006).

Both APV and *Plasmodium* spp. are considered as 'diseases of concern' in conservation management in New Zealand (Jakob-Hoff 2001; Biosecurity New Zealand 2006), however, detailed information on both diseases is limited. For example, there have been no studies on the virulence of New Zealand APV isolates, the seroprevalence of APV or the frequency of co-infection with *Plasmodium* spp.. Current sampling of wild birds which is usually undertaken after a disease outbreak, is not likely to provide adequate information for long-term conservation management. Consequently, this study was designed to provide baseline information on the seroprevalence of APV and degree of commonality with *Plasmodium* spp. in free-ranging introduced bird species in the southern region of the North Island of New Zealand. The study is an integral part in the development of successful conservation and disease management programs for New Zealand's threatened and endangered avifauna.

4.3. MATERIAL AND METHODS

4.3.1. Ethics approval and permits

The use of live animals in this study was approved by Massey University Animal Ethics Committee (MUAEC 10/11 & MUEAC 11/17). Banding permit No. was 2008/052.

4.3.2. Field sampling

Bird captures were carried out during April and June 2011, around Palmerston North (40° 35'S, 175° 61'E), New Zealand, using polyester mistnets (38 mm mesh/ 4 shelves/ 75 denier/ 2-ply, Avivet Inc., Dryden, NY, USA). A total of 65 birds from six species were captured, including 32 Eurasian Blackbirds (*Turdus merula*), 11 Chaffinches (*Fringilla coelebs*), two European Goldfinches (*Carduelis carduelis)*, four Greenfinches (*Carduelis chloris)*, four Song Thrushes (*Turdus philomelos*), and 12 Starlings (*Sturnus vulgaris)*. Every captured bird was examined for feather condition, evidence of skin lesions, external injury and general body condition. After banding with numbered metal bands to prevent re-sampling, blood samples were collected into heparinised capillary tubes from the brachial vein and the birds were released. A small drop of blood from each bird was fixed as a thin blood smear to examine for the presence of *Plasmodium* spp. and the remaining sample was transferred into heparinised BD Microtainer® blood collection tubes (BD, Franklin Lakes, NJ, USA). The samples were centrifuged at 2,500 X g for 30 min and separated plasma and blood cells were kept at -20°C for later use in APV ELISA and *Plasmodium* spp. PCR screening, respectively. Samples from ten birds including four Eurasian Blackbirds, one Chaffinch, one European Goldfinch, two Song Thrushes, and two Starlings were excluded from *Plasmodium* spp. PCR screening due to improper sample preservation.

4.3.3. Fowlpox antigen preparation

Fowlpox antigen was prepared as described by Singh et al. (2003). Lyophilized fowlpox (FWPV) vaccine, Poxine (Duphar; Fort Dodge, IA, USA), was resuspended in extraction buffer (20 mM Tris, pH 8.0, 20 mM KCl, 10% sodium dodexyl sulfate, 2% desoxycholate, 0.5%

ß-mercaptoethanol). Proteolytic inhibitor cocktail was added to a final concentration of 1% and the lysates were rotated for 2 hours at 25 °C. After a centrifugation at 1000 x *g* at 25 °C for 10 min, supernatants were collected and used as a source of antigens for ELISA and Western Blotting.

4.3.4. Positive/negative control sera preparation

Positive/negative control sera were prepared from four chickens *(Gallus gallus domesticus*) from a research colony which was known to be FWPV free and had no history of FWPV vaccination. Blood samples were collected at two weeks old and two of the birds were subsequently vaccinated with Poxine immediately after blood sampling. Following vaccination blood samples were collected from all four birds weekly until three weeks post vaccination when all birds were exsanguinated. Each blood sample was centrifuged at 2,500 X g for 30 min and resulting separated serum was kept at -80°C for later use. The sera from the vaccinated and unvaccinated chickens were used as positive and negative control sera, respectively, for ELISA and Western Blotting.

4.3.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA assay was performed as described by Singh and Tripathy (2000) on sera from 65 birds with the following minor modifications. Briefly, Immulon Maxi sorp 96-well microtiter plates (NUNC A/S, Roskilde, Denmark) were coated with 1 µg of antigen in 100 µl of carbonate-bicarbonate buffer, pH 9.6, per well and incubated overnight at 4 °C. The coated

wells were washed once with 300 µl/well wash solution (phosphate-buffered saline (PBS), pH 7.4, 0.1% Tween 20) using ELISA plate washer (BioTek, Winooski, VT, USA). Non specific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS (pH 7.4) containing 0.1% Tween 20 (PBST) for 1 hr at 37 °C. After an additional single wash, test sera from free-ranging birds, diluted 1:100 in PBST containing 1% BSA, were added to each well. The plates were incubated for 2 hr at 37 °C. Following incubation and three washes with wash solution, 100 µl of 1/1000 dilution (in PBST containing 1% BSA) of horseradish peroxidase-conjugated goat anti-avian immunoglobulin G (IgG) antibodies (Lifespan Biosciences, Seattle, WA, USA) was added to each well and incubated for further 1 hr at 37 °C. The secondary antibody has been verified to cross-react with a variety of bird species including dove, duck, sparrow, chicken, turkey, emu, ostrich, quail, macaw, and cockatiel (Lifespan Biosciences, Seattle, WA, USA). After three washes, bound antibodies were detected with a tetramethylbenzidene (TMB) substrate kit (Thermo Scientific, Rockford, IL, USA) as per the manufacturer's instructions. The reactions were stopped by addition of 1N sulphuric acid. The absorbance at 450 nm was measured using ELISA plate reader (BioTek, Winooski, VT, USA). An $OD_{450} \geq 0.6$ was considered positive for APV antibodies. ELISA assays were carried out twice, in duplicate, for a total of four assays per sample. In the case of the finch species the assays were conducted once in duplicate due to limited serum volume.

4.3.6. Western immunoblotting

Western blot analysis was performed as described by Kim and Tripathy (2006b) on sera from 12 birds with the following minor modifications. Briefly, prepared antigen (1 µg/1 µl) was denatured using 2X Laemmli sample buffer containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl (Sigma, St Louis, MO,

USA) by heating at 100ºC for 10 minutes. The denatured protein and broad range protein standards (Bio-Rad, Hercules, CA, USA) were then resolved by 15% sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) in a Miniprotein II cell (Bio-Rad, Hercules, CA, USA) at 200V for 45 min at room temperature. The separated proteins were transferred to nitrocellulose membrane with a Mini Trans-blot Cell (Bio-Rad, Hercules, CA, USA) at 100 V for 1 hr at 4 °C. Nonspecific binding sites were blocked overnight with 3% BSA in PBST at 4 °C. The membranes were washed three times for 5 min in PBST and then incubated with test sera from free-ranging birds, diluted 1:100 in PBST containing 1% BSA, for 2 hr at room temperature. After three washes for 10 min each, the membranes were incubated with horseradish peroxidase-conjugated goat anti-bird immunoglobulin G (IgG) (H+L) antibodies (Novus Biologicals, Littleton, CO, USA), diluted 1:1000 in PBS containing 1% BSA, for 1 hr at room temperature. After final three washes, bound secondary antibodies were detected using an ECL substrate kit (Thermo Scientific, Rockford, IL, USA) as per manufacturer's instructions and exposed to film.

4.3.7. DNA extraction, PCR amplification and sequencing

DNA was extracted from 55 red blood cell pellets using a Qiagen DNeasy Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions for nucleated whole blood. The presence of the cytochrome b gene of *Plasmodium* spp. was identified using a nested PCR and the nested primer sets HaemNF1/HaemNR3 and HaemF/HaemR2 as described by Hellgren et al. (2007). To confirm successful amplification 10µl of the final PCR product was run on a 1.5% agarose gel containing ethidium bromide prior to purification and sequencing. A known *Plasmodium* positive blood sample, confirmed by sequencing, was used as a positive control and water blanks were included as negative controls.

All *Plasmodium* positive PCR amplicon samples were purified using a PureLink PCR purification kit (Invitrogen, Carlsbad, CA, USA) and subjected to automatic dye-terminator cycle sequencing with BigDye^{TM} Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA) to confirm genomic sequence using both the forward and reverse primers. Chromatograms were examined for conspicuous overlapping peaks suggestive of *Plasmodium* spp. co-infection. The *Plasmodium* isolate sequences obtained were compared to the MalAvi database (Bensch et al. 2009) and by NCBI Blast to other published sequences available from GenBank.

4.3.8. Blood smear preparation and examination

Blood smear microscopy was undertaken in order to confirm the presence of *Plasmodium* spp.. The slides were fixed in absolute methanol for three minutes in the laboratory before subsequently staining with Geimsa (diluted 1:10 for 45-60 minutes). Each smear was examined under a light microscope for a minimum of 15 minutes; initially at low power (200 X) for 3 minutes then 1000 X under oil immersion and the observed blood parasites were counted and photographed.

4.3.9. Statistical analysis

Correlation between birds seropositve to APV and birds positive to *Plasmodium* spp. was determined using the Chi-Square test (Kuzma 1998).

4.4. RESULTS

4.4.1. Seroprevalence of APV

No gross abnormalities or clinical signs suggestive of APV infection were observed in the 65 birds captured. Overall, 69.2% of birds (45/65) were seropositive to APV (Table 4.1.). Song Thrushes presented 100% seroprevalence (4/4), followed by Eurasian Blackbirds (96.86%, 31/32), Chaffinches (54.55%, 6/11), Starlings (25%, 3/12), Greenfinches (25%, 1/4), and European Goldfinches (0%, 0/2). Samples from 12 birds which had sufficient remaining sera were subjected to western immunoblotting to confirm their sero-status. Sera from five birds (one Eurasian Blackbird and four Starlings) with an ELISA $OD_{450} < 0.6$ showed no antibody response to the AVP antigens presented. However, the remaining seven sera (6 Eurasian Blackbirds and one Song Thrush) with an ELISA $OD_{450} \geq 0.6$ contained antibodies which reacted to an 80 kDa APV antigen. In addition, other immunodominant antigens (39 kDa, 46 kDa and 60 kDa) were also observed in the seven ELISA positive samples. The results of the western immunoblotting confirmed the validity of using the ELISA $OD_{450} \geq 0.6$ cutoff to detect sero-positive samples.

4.4.2. Molecular studies

In these studies, we detected *Plasmodium* DNA in 15/55 (27.3%) of samples which included samples from 11 Eurasian Blackbirds, one Song Thrush, and three Starlings (Table 4.1.). None of the samples from the Chaffinches (n = 10), Greenfinches (n = 4) and European Goldfinches (n = 2) captured were positive for presence of *Plasmodium* spp. DNA (Table 4.1.). All 15 *Plasmodium* spp. positive PCR products had sufficient amplification to be sequenced.

The resulting chromatograms revealed that one Eurasian Blackbird sample had conspicuous overlapping peaks suggestive of a mixed infection and was thus removed from further analysis. The remaining 14 samples did not reveal any evidence of mixed infections and were submitted for BLAST analysis to compare the isolates against the NCBI GenBank and MalAvi databases.

The majority (8/11, 72.7%) of the Eurasian Blackbird isolates grouped with well characterised lineages of the subgenus Novyella (Figure 4.1.). All eight Eurasian Blackbird isolates had 100% sequence homology with the published *Plasmodium* spp. lineage SYATO5 (GenBank DQ847271) previously detected in New Zealand Eurasian Blackbirds, Kereru (New Zealand Pigeon *Hemiphaga* novaeseelandiae) and Tomtits (*Petroica macrocephala*) (Howe et al. 2011; MalAvi database). Sequence divergence analysis revealed 0.47% divergence between the Eurasian Blackbird isolates and *Plasmodium* lineages collected from members of the Turdidae family such as the African Thrush (*Turdus pelios)* lineage W38 (GenBank EU810633, MalAvi AFTRU08), and 1.1% divergence with lineage W37 (GenBank EU810632, MalAvi AFTRU4).

In addition, one Eurasian Blackbird and the Song Thrush had 100% sequence homology to lineage LINN1 (MalAvi database) and lineage AFTRU5 from an African Thrush (GenBank EU81610) (Figure 4.1.). An additional Eurasian Blackbird also clustered in this group with 99% sequence homology (0.2% sequence divergence) to the LINN1 and AFTRU5 lineages. These lineages have been previously detected in New Zealand in the North Island Saddleback (*Philiesturnus carunculatus rufaster*), Eurasian Blackbird, Great Spotted Kiwi, Bellbird (*Anthornis melanura*), and Song Thrush (Castro et al. 2011; Howe et al. 2011; MalAvi database).

All three Starling isolates had a 100% homology to previously published and well characterized lineages of *Plasmodium (Huffia) elongatum* GRW06 (GenBank DQ368381) (Figure 4.1.). This lineage has been previously identified in various New Zealand introduced, native, and endemic avifauna including Eurasian Blackbirds, Silvereyes (*Zosterops lateralis*), South Island Saddleback, North Island Robins (*Petroica australis*), Bellbirds and Brown Kiwi (Baillie and Brunton 2011; Castro et al. 2011; Howe et al. 2011).

All eleven (100%) Eurasian Blackbirds, 2/3 (67%) Starlings and the one Song Thrush blood sample which were PCR positive for *Plasmodium* spp. were also seropositive for APV.

Table 4.1. Birds sampled and the results of ELISA for APV antibody, PCR for *Plasmodium*, number of *Plasmodium* positive birds seropositive to APV, and *Plasmodium* lineages identified.

Species	Common Name	APV ELISA Positive (%)	*Plasmodium* PCR Positive (%)	No. of *Plasmodium* positive birds seropositive to APV (%)	*Plasmodium* lineages identified (GenBank no.)
Turdus merula	Eurasian Blackbird	31/32 (97%)	11/28 (39.2%)	11/11 (100%)	Mixed (unresolved) *P. (Novyella) spp. SYATO5* (DQ847271) *Plasmodium* spp AFTRU5 (DQ847263)
Fringilla coelebs	Chaffinch	6/11 (54.6%)	0/10 (0%)	0/0 (0%)	-
Careulis chloris	European Goldfinch	0/2 (0%)	0/2 (0%)	0/0 (0%)	-
Cardeulis cardeulis	Greenfinch	1/4 (25%)	0/4 (0%)	0/0 (0%)	-
Turdus philomelos	Song Thrush	4/4 (100%)	1/1 (100%)	1/1 (100%)	*Plasmodium* spp AFTRU5 (DQ847263)
Sturnus vulgaris	Starling	3/12 (25%)	3/10 (30%)	2/3 (67%)	*P. (Huffia) elongatum* GRW6 (DQ368381)
	Total	**45/65 (69.2%)**	**15/55 (27.3%)**	**14/15 (93.3%)**	

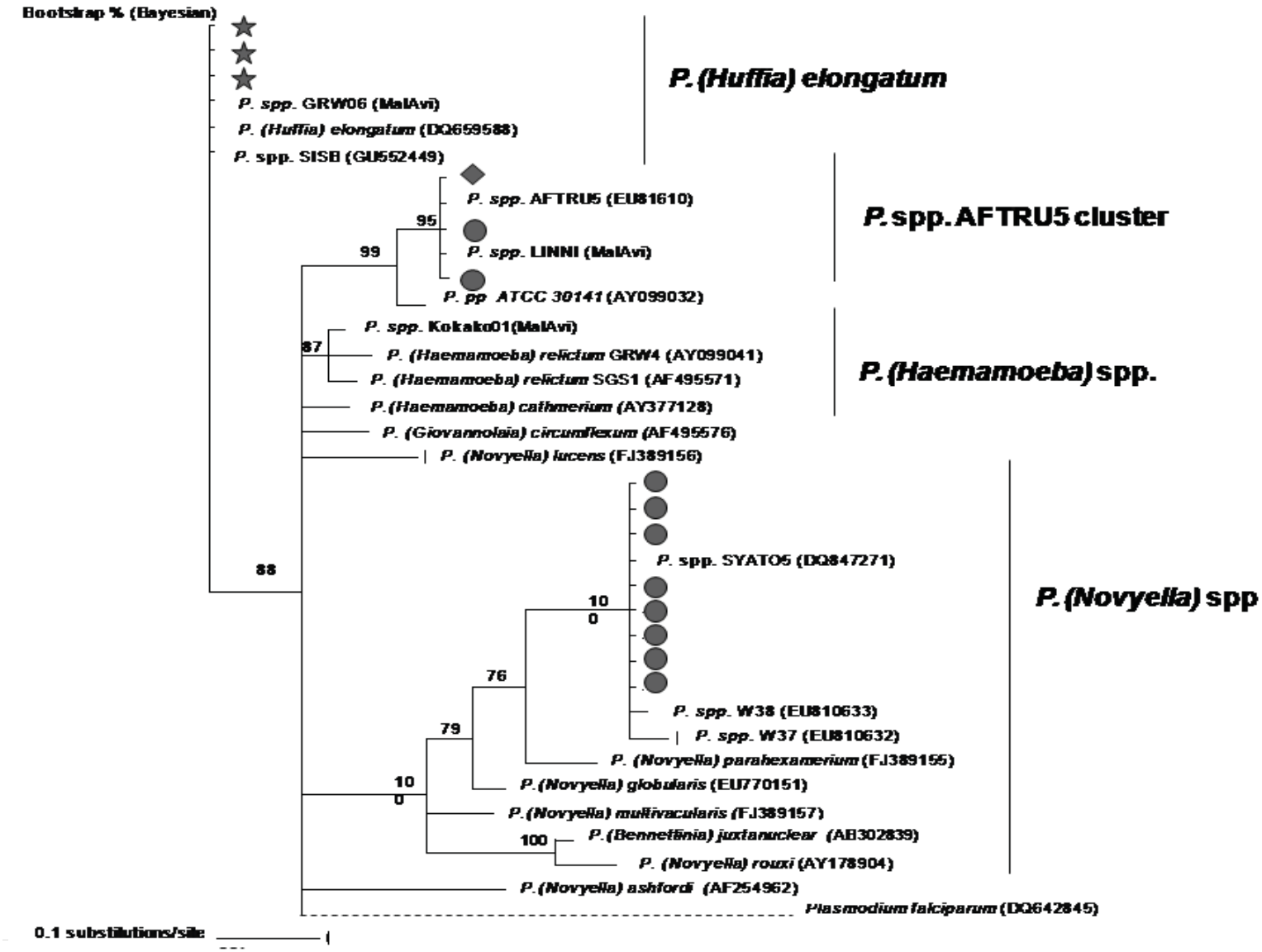
Bootstrap % (Bayesian)
P. spp. GRW06 (MalAvi)
P. (Huffia) elongatum (DQ659588)
P. spp. SISB (GU552449)
P. (Huffia) elongatum
P. spp. AFTRU5 (EU81610)
P. spp. LINNI (MalAvi)
P. pp ATCC 30141 (AY099032)
P. spp. AFTRU5 cluster
P. spp. Kokako01(MalAvi)
P. (Haemamoeba) relictum GRW4 (AY099041)
P. (Haemamoeba) relictum SGS1 (AF495571)
P. (Haemamoeba) cathemerium (AY377128)
P. (Haemamoeba) spp.
P. (Giovannolaia) circumflexum (AF495576)
P. (Novyella) lucens (FJ389156)
P. spp. SYATO5 (DQ847271)
P. spp. W38 (EU810633)
P. spp. W37 (EU810632)
P. (Novyella) parahexamerium (FJ389155)
P. (Novyella) globularis (EU770151)
P. (Novyella) multivacularis (FJ389157)
P. (Bennettinia) juxtanuclear (AB302839)
P. (Novyella) rouxi (AY178904)
P. (Novyella) ashfordi (AF254962)
P. (Novyella) spp
Plasmodium falciparum (DQ642845)
95
99
87
88
10
0
76
79
10
0
100
0.1 substitutions/site

Figure 4.1. Phylogenetic analysis and comparison of 14 *Plasmodium* spp. isolates from introduced avian species and previously published *Plasmodium* spp. sequences present in the GenBank and/or MalAvi database. Bayesian phylogeny of mitochondrial cytochrome *b* gene from *Plasmodium falciparum* used as an outgroup, 21 previously published *Plasmodium* spp. submitted to Genbank or the MalAvi database, and the 14 *Plasmodium* spp. isolates from this study. Lineages of malaria parasites to *P. (Huffia) elongatum, P.* spp AFTRU5 cluster, P. *(Haemamoeba) relictum*, and *P. (Novyella)* spp. are indicated. Positive avian species are represented by ● **(**Eurasian Blackbird), ★ **(**Starling) and ◆ **(**Song Thrush). Numbers above or below branch nodes indicate bootstrap support based on 1.5 million generations. Names of the lineages (when available) and GenBank accession numbers of the sequences are given after the species names of the parasites. The branch lengths are drawn proportionally to the amount of changes (scale bar shown).

4.4.3. Microscopy

Fifteen thin blood smears from the captured birds which were positive for *Plasmodium* spp. by PCR were examined. Parasites were not always detected in the thin blood smears of infected birds, nor were all developmental stages represented when parasites were seen. The most common developmental stages were trophozoites and thus speciation based on microscopy could not be determined. Seventy-three percent (11/15) of the blood slides were positive for very low level peripheral parasitemia (0-3 parasites observed during 10 minutes of observation). The remaining four slides could not be assessed due to poor blood smear quality.

4.4.4. Statistical analysis

There was a significant relationship between birds seropositive to APV and birds infected by *Plasmodium* spp. ($\chi^2 = 5.69$, df1, $p = 0.0086$).

4.5. DISCUSSION

Most sampling for disease surveillance in New Zealand is opportunistic and has taken the form of case reports (Westerskov 1953; Austin et al. 1973; Alley et al. 2010). The present study is the first to determine the seroprevalence of APV and the degree of association with *Plasmodium* spp. infection in free-ranging introduced bird species in the southern regions of the North Island of New Zealand. The seroprevalence of APV in 65 captured birds was 69.2% (n = 65) with the highest seroprevalence of APV observed in ground feeding species such as the Song Thrush (100%, n = 4) and Eurasian Blackbird (96.86%, n =

32). This unexpectedly high seroprevalence suggests that APV is well established among introduced bird populations in New Zealand. Based on this, it could be assumed that APV is also well established among native bird species in New Zealand as native and introduced species often display considerable overlapping of territory in the wild (Chambers 2009).

Despite the high seroprevalence of APV, no birds with clinical signs suggestive of APV infection were caught during sampling. This suggests that most of the birds examined had previously been infected and recovered from APV infection. Given that the prevalence of APV is generally high in late summer and autumn in temperate climates (Van Riper and Forrester 2007), seasonal variation might also be a reason for the low occurrence of clinical disease as our sampling period was in late autumn and early winter. Alternatively, biased sampling methodology could contribute to the absence of skin lesions. Birds acutely infected by APV are not likely to have the same flight ability as healthy and active individuals and may therefore be less likely to fly into mistnets. Further, our sampling was carried out only in introduced bird species, which are reported to have lower prevalence of APV infection than native species in several island populations (Van Riper et al. 2002; Thiel et al. 2005). Finally, it is possible that APV infection is only mildly pathogenic in many introduced bird species in New Zealand and this had led to the low frequency of clinical lesions observed but allowed the development of an appropriate immune response (Tompkins and Gleeson 2006).

Interestingly, all the eleven Eurasian Blackbirds and one Song Thrush which tested positive to *Plasmodium* spp. also showed a high antibody titer to APV. Sixty seven per cent of *Plasmodium* positive Starlings were also seropositive to APV. It is possible that these birds were infected by both APV and *Plasmodium* spp. via a common vector either at the same

time or at discrete time points. This high commonality of seroprevalence of APV and *Plasmodium* spp. infection in Eurasian Blackbirds and Starlings exceeds the degree of co-infection reported in other island bird populations and suggests that in New Zealand these birds have a high natural exposure and recover from these pathogens. This raises the possibility that the birds may be a reservoir of infection for both organisms. Such a possibility is in line with several other studies which have suggested that Eurasian Blackbirds might play a role as reservoirs of *Plasmodium* spp. because of the high prevalence of infection in the population (Tompkins and Gleeson 2006; Baillie and Brunton 2011; Howe et al. 2011). Likewise, they might act as APV reservoirs by providing insect vectors the opportunity to mechanically transmit the virus to a wider range of bird populations.

There are 16 species of mosquito present in New Zealand; 12 endemic and four introduced (Derraik 2004). A *Plasmodium* spp. isolated from a native *C. pervigilans* blood-engorged female indicates that this species is a vector for avian malaria (Massey et al. 2007). Two other mosquito species including *Aedes albopictus* and *C. quinquefasciatus* are known to be vectors for APV in the Hawaiian islands (Fonseca et al. 2000; Van Riper et al. 2002). The greatest prevalence of APV and avian malaria at 1,000 – 1,200 m and below 600 m in elevation may be attributed to *C. quinquefasciatus*, which is abundant at elevations of less than 1,500 m above sea level (Van Riper et al. 2002; Ahumada et al. 2004; Aruch et al. 2007). The mosquito *C. quinquefasciatus* was introduced into northern New Zealand about 30 years ago and has recently extended its distribution throughout the country (Tompkins and Gleeson 2006). On the other hand, the forest day mosquito *A. albopictus* is yet to become established in New Zealand but this species has been detected during biosecurity control (Derraik 2004). These introductions of invasive mosquitoes are of particular

concern as they could pose a further threat to already endangered endemic New Zealand birds (Lapointe 2008).

It is of note that the birds that had evidence of infection with both APV and *Plasmodium* spp. had only low levels of parasitemia at the time of capture. This is in contrast to the report of South Island Saddlebacks which were concurrently infected by both *P. elongatum* and APV, resulting in diseases consistent with co-infection and subsequent death of several birds post translocation (Alley et al. 2010). Concurrent infection was thought to be responsible for the decline of 60% of the local Saddleback population in the Marlborough Sounds during the same period (Alley et al. 2010). *Plasmodium elongatum* is considered to be endemic in New Zealand and has been identified in a wide range of introduced and native birds (Massey et al. 2007). Generally, this subgenus of *Plasmodium* appears to be endemic in the population and has a wide host diversity of infection and produces a disease, if present that is chronic in nature. Death due to *P. elongatum* has been reported in not only South Island Saddlebacks but also a Eurasian Blackbird (Howe et al. 2011). In contrast, the *P.* (*Novyella*) spp. SYATO5 commonly indentified in Eurasian Blackbirds, appear to be less pathogenic, have a high level of host specificity and rarely seems to infect endemic New Zealand passerines or cause mortality (Beadell et al. 2009; Howe et al. 2011).

Little is known about the *Plasmodium* lineages AFTRU5/LINN1 observed in a Eurasian Blackbird and a Song Thrush in this study and related lineages within the group. Lineages within this group have typically demonstrated a high level of specificity at the avian host family level (Beadell et al. 2009) and a high level of host sharing between the *Culex* vectors (Kimura et al. 2009). In New Zealand, death has been reported in both a Eurasian

Blackbird and an endemic Great Spotted Kiwi, suggesting that this lineage can be pathogenic and its prevalence should be closely monitored (Howe et al. 2011).

Climate change, pathogens and predators can each play an important role in regulating the distribution and abundance of wildlife populations (Hobbelen et al. 2011). For example, given potential for immune-compromise by APV (Tripathy and Reed 2008), concurrent infections with *Plasmodium* spp. are reported to result in amplified severity of acute avian malarial infections. This can lead to the possibility of increased virulence and spread of both pathogens with the potential of relapsing or chronic infections, population decreases or even extinctions (Van Riper et al. 2002; Atkinson et al. 2005). Of the two pathogens APVs are reported to be host specific especially in wild birds (Smits et al. 2005; Kleindorfer and Dudaniec 2006) where as *Plasmodium* spp. are reported to switch hosts in wild avifauna (Krizanaskiene et al. 2006; Martinsen et al. 2008). To understand the epidemiology of AVP and *Plasmodium* spp. and the role of introduced avian species in their transmission, continuous sampling of both native and introduced species, in multiple regions, and at different times of the year will be necessary. Comprehensive knowledge of the abundance of the vectors and pathogens will allow the reduction in potential population loss which can be caused by these infectious diseases.

In New Zealand, re-introduction of endangered species has been increasingly used as an important management tool to forestall extinctions of most of the endangered avifauna (Armstrong et al. 2007). Undetected asymptomatic carriers of both *Plasmodium* and APV may pose a threat during translocations due to source or destination species' naivety to both pathogens, risking immune suppression, stress and other negative impacts on the

captive management programs. Asghar *et al*. (2011) further points out that there are subtle health effects with chronic Haemosporidan infection, which may influence host fitness and breeding and could directly affect the viability of management programs such as translocations.

We suggest that further sampling of native bird species in New Zealand for the surveillance of APV infection or antibodies is required. This would provide information on the susceptibility and the immune response to APV and, when correlated with the growing data on *Plasmodium* in New Zealand bird species, would be beneficial in estimating the likelihood and managing the impact of *Plasmodium*, APV co-infections in future conservation management programs.

Table 4.2. Summary of results of APV ELISA and *Plasmodium* spp. PCR in sampled birds. The resulting χ^2 value by the Chi-Square test was 5.69, indicating that there is correlation between birds seropositive to APV and birds infected by *Plasmodium* spp.. *Not tested: please note that only 55 birds were tested for *Plasmodium* spp. PCR while 65 birds were tested for APV antibodies due to improper sample preservation.

		***Plasmodium* spp.**		
		Positive	Negative	Total
APV ELISA	Positive	14	24	38 (*7)
	Negative	1	16	17 (*3)
	Total	15	40	55 (*10)

DRC 16

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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: Hye Jeong HA

Name/Title of Principal Supervisor: Associate Professor Brett Gartrell

Name of Published Research Output and full reference:
The seroprevalence of avipoxvirus and its association with avian malaria (Plasmodium spp.) infection in introduced passerine birds in the southern regions of the North Island of New Zealand

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In which Chapter is the Published Work: Chapter four

Please indicate either:

- **The percentage of the Published Work that was contributed by the candidate:** 80%

  and / or

- **Describe the contribution that the candidate has made to the Published Work:**

Hye Jeong Ha
Candidate's Signature

3/10/2012
Date

Brett Gartrell
Principal Supervisor's signature

3/10/2012
Date

GRS Version 3– 16 September 2011

CHAPTER FIVE

The Detection of Avipoxvirus Antibody in North Island Robins (*Petroica australis longipes*) Demonstrates the Endemic Status of Avipoxvirus in Birds on an Island Refuge Used for Conservation

5.1. ABSTRACT

Avipoxvirus (APV) is known to be a major factor in population decline and extinction in Hawaiian bird species. It is believed to cause mortalities in endangered New Zealand birds such as black robin (*Petroica traversi*) and shore plover (*Thinornis novaeseelandiae*). Previous studies revealed that various New Zealand bird species were susceptible to APV infection and at least three different strains of APV were present. A high seroprevalence of APV (69.2%) was observed in introduced bird species however, no information regarding the seroprevalence of APV in endemic birds in New Zealand exists. To our knowledge, this is the first study to detect APV antibodies in endemic birds in New Zealand. Blood samples were collected from 15 North Island robins (*Petroica australis longipes*) from Kapiti Island prior to translocation. Plasma from each bird was tested for the presence of APV antibodies by ELISA. Eight birds (53.3%) tested seropositive to APV. Given the high seroprevalence of APV in the robin population sampled, it is likely that APV is well-established in bird populations on Kapiti Island.

Keywords: avipoxvirus, New Zealand, antibody, North Island robins (*Petroica australis longipes*), ELISA

5.2. INTRODUCTION

Avipoxvirus (APV) infection is a common disease caused by viruses of the genus *Avipoxvirus* of the virus family Poxviridae, characterised by proliferative lesions on the skin, unfeathered parts of the body, and/or mucous membranes of the mouth and upper respiratory tract (Tripathy and Reed 2008). Its distribution is worldwide and various bird species are susceptible to the disease (Van Riper and Forrester 2007). The virus has been isolated from bird populations from tropical islands to Antarctica indicating how wide-spread and stable the virus is in the environment (Tripathy et al. 2000; Thiel et al. 2005; Shearn-Bochsler et al. 2008). The most common form of disease transmission is by biting insects including mosquitoes, mites, or flies (Tripathy and Reed 2008). The successful spread of the virus is generally associated with host density, host susceptibility, vector numbers, warm and moist weather and numbers of APVs present in the area (Van Riper and Forrester 2007). Consequently, disease outbreaks often occur in birds under captive management and the prevalence of APV is much higher in the warmer and mesic regions of the world (Van Riper and Forrester 2007). In a temperate climate, higher prevalence of APV has been reported during summer and early autumn when insect vectors are abundant and active (Van Riper and Forrester 2007).

Fowlpox virus (FWPV), which is the most studied virus amongst all APVs, causes a decrease in egg production and an increase in mortality in poultry (Tripathy and Reed 2008). Although its impact on wild birds is not as well recognised as on poultry, it is known to limit the survival of wild populations (Van Riper and Forrester 2007). In an island environment where the hosts, vectors, and APVs have not necessarily co-evolved, the disease may have significant implications for population decline and extinction (Atkinson

et al. 2005; Van Riper and Forrester 2007; Atkinson and LaPointe 2009). There is no record as to the origin and history of APV in New Zealand birds but it is believed to be endemic in New Zealand birds (Gartrell et al. 2003). A phylogenetic study revealed the presence of three different APV strains in New Zealand causing pathogenic APV infections in various species of birds (Ha et al. 2011; Chapter two). A considerable geographic overlap between different APV strains was observed in the areas where translocation of animals has been frequently carried out (Ha et al. 2011: Chapter two). Furthermore, APV outbreaks related to translocation of endangered bird species have been reported in New Zealand (Alley et al. 2010; Ha et al. 2011; Chapter two).

Global warming has resulted in an increase in the number and range of insect vectors for APV in New Zealand such as sandflies (family Simuliidae), introduced mosquitoes *Culex quinquefasicatus* and the native mosquito *Culex pervigilans*, posing greater threats to New Zealand birds (Tompkins and Gleeson 2006; Ha et al. 2011; Chapter two). As a result, APV is considered one of the 'diseases of concern' in conservation management in New Zealand and the presence of lesions suggestive of APV infection is monitored (Jakob-Hoff 2001; Biosecurity New Zealand. 2006). Serological tests to monitor the presence APV antibody were not available in New Zealand until recently. I recently confirmed a high seroprevalence of APV in a range of introduced passerines in New Zealand particularly in blackbirds (*Turdus merula)* and song thrushs (*Turdus philomelos*) (Ha et al. 2012a; Chapter four) but no information on the seroprevalence of APV in endemic birds is available. Consequently, this study was designed to provide baseline information on the seroprevalence of APV in an endemic bird species living on an offshore island in New Zealand.

5.3. MATERIAL AND METHODS

Blood samples from 15 North Island robins (*Petroica australis longipes*) from Kapiti Island, New Zealand (40° 52'S, 174° 54'E) were collected in March 2012 prior to translocation. Birds were captured using clap-traps and every captured bird was weighed and examined for feather condition, evidence of skin lesions, external injury and general body condition. After banding with numbered metal bands, blood samples were collected into heparinised capillary tubes using the brachial vein. The blood was immediately transferred into heparinised BD Microtainer® blood collection tubes (BD, Franklin Lakes, NJ, USA) and stored in the refrigerator overnight before being couriered in a chilled container to the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North. The samples were then centrifuged at 2,500 X g for 30 min and the separated plasma was kept at -20°C for later use in APV ELISA.

Fowlpox antigen and positive/negative control sera were prepared as described in Ha et al (2012a; Chapter four). Enzyme-linked immunosorbent assay (ELISA) assay was also performed as described in Ha et al. (2012a; Chapter four). ELISA assays were carried out only in duplicate per sample due to small sample amount. An $OD_{450} \geq 0.6$ was considered positive for APV antibodies and indicative that the bird had previously been exposed to APV and successfully mounted an immune response.

5.4. RESULTS

On clinical examination by a veterinarian one bird was found with tissue lesions suggestive of APV infection on the leg. No further diagnostic tests were carried out on this bird and it was released at the site of capture. All the remaining birds presented with good body condition and no lesions suggestive of APV or other signs of illness. The OD_{450} ranged between 0.045 and 3.15 (Mean = 0.94, SD = 0.63) by ELISA assay. Nine birds (60%, n = 15) were seropositive to APV. The one robin with APV suspected leg lesion was serologically negative with an OD_{450} of 0.46.

5.5. DISCUSSION

Accidental introduction of pathogens into a new environment or novel host species is of great concern in conservation management. Avipoxvirus, which is generally slow-spreading and self-limiting in mainland avifauna can spread rapidly when introduced to remote island ecosystems (Van Riper and Forrester 2007). For instance, 50% of short-toed larks (*Calandrella rufescens*) in the Canary Islands were infected by APV within a few years of its first report in 2000 (Smits et al. 2005). A recent study in Galapagos finches confirmed an increasing overall prevalence of APV over the last decade, with a variation between species and immune responses (Zylberberg et al. 2012). It is also known to be a major factor in population decline and extinction in Hawaiian bird species (Van Riper et al. 2002; Atkinson et al. 2005). Since its first report in New Zealand birds in 1953 (Westerskov 1953), it has been identified in a range of bird species including some threatened endemic species such as black robin, shore plover, wrybill (*Anarhynchus frontalis*) and brown kiwi (*Apteryx mantelli*) (Ha et al. 2011; Chapter two; Ha et al. 2012b; Chapter three). The

presence of at least three different APV strains in New Zealand has been confirmed (Ha et al. 2011; Chapter two).

A seroprevalence study of APV in introduced passerines revealed high seroprevalence (69.2%, n = 65) indicating that the virus is well-established among introduced avifauna in New Zealand (Ha et al. 2012a; Chapter four). This current study is the first to detect antibodies for APV in a native bird species in New Zealand. The ELISA assay was optimised to detect APV antibodies in poultry and introduced passerines in New Zealand and appeared to be effective for use in endemic passerines as well. Among 15 North Island robins tested eight (60%) were seropositive to APV by ELISA. Although the small sample size does not allow us to predict the seroprevalence of APV in a bigger geographic or population scale, it is likely that APV is wide spread among native bird species in New Zealand. At the very least, we are able to conclude that APV is an endemic disease on Kapiti Island as the robins are confined to the island, making exposure elsewhere unlikely. The route of introduction of the disease to the island could have been from previous translocations or more likely, the introduced species of birds that regularly commute between Kapiti Island and the mainland.

It is noteworthy that one bird with leg lesions was seronegative to APV (OD450 = 0.46). A diagnosis of APV was made by an experienced wildlife vet (K. McInnes[1] pers. obser.) although it was not possible to collect tissue samples to confirm by PCR. This disparity could be explained by an active APV infection as an elevation of antibodies could take up to two weeks post exposure to APV, following the development of skin lesions (Chapter six). Without a tissue sample it is impossible to confirm the infection, let alone the strain, though the A1 strain of APV had been previously isolated in a North Island robin from the

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nearby mainland (Ha et al. 2011; Chapter two). Future research to identify the strains of APV present on the island is warranted.

This study introduces the possibility of screening for APV antibodies prior to translocation of endangered native bird species. Avipoxvirus outbreaks related to translocation of endangered bird species have already been reported in New Zealand (Alley et al. 2010; Ha et al. 2011; Chapter two). The birds displayed no clinical abnormalities in quarantine prior to the translocations but were found with APV lesions post-translocation (Ha et al. 2011; Chapter two). Evidence suggests that there is overlap in the geographic distribution of APV strains within New Zealand and considerable geographic overlap between different APV strains was observed in the areas where translocations of animals have been frequently carried out (Ha et al. 2011; Chapter two). With many of New Zealand's endangered native species being managed through translocation programs, the risk of introducing disease to a naïve population is high, either by bringing the disease to a population or the population to the disease. Clarification of the geographic distribution of APV and host susceptibility to APV strains would allow for better conservation management decision making.

Further sampling of native bird species in New Zealand for the surveillance of APV infection or antibodies is required. This would provide information on the susceptibility and the immune response to APV and would be beneficial in estimating the risk and impact of APV infections in future conservation management programs.

CHAPTER SIX

Evaluation of the Pathogenicity of Avipoxvirus Strains Isolated from Wild Birds in New Zealand and the Efficacy of a Fowlpox Vaccine in Passerines

6.1. ABSTRACT

At least three different strains of Avipoxvirus (APV) have been identified in a range of bird species in New Zealand. The pathogenicity of two APV strains isolated from wild birds in New Zealand, representing fowl poxvirus A1 strain and canary poxvirus B1 strain were compared in zebra finches (*Taeniopygia guttata*). The efficacy of fowlpox (FWPV) vaccine at preventing clinical disease in passerines was also evaluated. Twenty-five zebra finches were divided into five groups (I-IV and a control group). Birds from Groups III and IV were vaccinated using FWPV vaccine prior to challenge. Subsequently two groups (I and III) were inoculated with a silvereye isolate (A1) which was propagated in chicken fibroblast cell culture and two groups (Group II and IV) were inoculated with a blackbird isolate (B1). Birds in the control group were inoculated with sterile PBS. Skin thickness at the inoculation sites was measured and the development of additional skin lesions was monitored. Antibody development was measured by ELISA pre- and post virus inoculation. Both APV strains caused either swelling or hyperplasia at the inoculation site of non-vaccinated birds (4/5 in Group I and 5/5 in Group II). The swelling was milder and no foot lesions were observed in vaccinated birds before or after challenge with the silvereye or blackbird APV strains. These findings indicated that the FWPV vaccine provided safe and appropriate protection for zebra finches exposured to the two wild APV strains and suggest that the vaccine has the potential to be used where APV threatens the captive management or translocation of native passerines in New Zealand.

Keywords: Avipoxvirus, New Zealand birds, zebra finch *Taeniopygia guttata*, Pathogenicity, Vaccination

Abbreviations: APV = avipoxvirus; FWPV = fowl poxvirus; CNPV = canary poxvirus; PCR = polymerase chain reaction; PBS = phosphate-buffer saline; CEF = chicken fibroblast; DMEM = Modified Eagle's Medium; CPE = cytopathic effect; $TCID_{50}$ = tissue culture infectious dose 50%; H & E = hematoxylin and eosin; ELISA = Enzyme-linked immunosorbent assay; TMB = tetramethylbenzidene; CAM = chorioallantoic membrane; PI = post inoculation

6.2. INTRODUCTION

Avipoxvirus (APV) is a well-known infectious disease in birds caused by a large DNA virus that belong to the genus *Avipoxvirus* within the subfamily *Chordopoxvirinae* in the family *Poxviridae* (Tripathy and Reed 2008). To date, more than 10 species have been reported in the genus *Avipoxvirus* including the well characterized fowl poxvirus (FWPV) (Tripathy and Reed 2008). Avipoxvirus is different from other DNA viruses as the virus replicates and matures in the cytoplasm of infected cells (Wagner et al. 2008). Antigenetic and immunological differences have also been reported between different species of APVs, however cross-relationships have been observed, limiting the identification of different strains as described by Tripathy and Reed (2008). Despite genetic differences in virus strains, the infection generally manifests in three different forms; the dry cutaneous, the wet diphtheritic, and the rare systemic form (Tripathy and Reed 2008). The cutaneous form is the most commonly observed and is usually characterized by the presence of nodular or tumor-like lesions on the skin (Tripathy and Reed 2008). Insects such as mosquitoes, mites, or flies play an important role in the transmission of avipoxvirus and may assist viral infection by compromising cutaneous defences when biting (Van Riper and Forrester 2007). The traditional diagnosis has been by gross examination or light microscopic examination of lesions for the presence of cytoplasmic inclusion bodies (Tripathy and Reed 2008). Recent development of polymerase chain reaction (PCR) assay to detect APV 4b core protein gene has made diagnosis much easier although PCR alone doesn't allow differentiation or characterization of the APV strain amplified (Lee and Lee 1997).

Fowl poxvirus is the most studied and understood of APVs due to the economic losses associated with the infection (Smits et al. 2005). While FWPV results in a decrease of egg production and growth rate and an increase of mortality in poultry, APV infection in wild birds has been a factor in limiting population growth, especially in species confined to islands (Van Riper et al. 2002; Atkinson and LaPointe 2009; Parker et al. 2011). Birds with the mild cutaneous form of APV may survive the infection and develop long-lasting immunity (Van Riper and Forrester 2007). However the infection can cause starvation or make birds more vulnerable to predation or trauma by causing impaired flight ability or limitations in vision (Van Riper and Forrester 2007). Immune-suppression due to APV infection often increases the risk of secondary bacterial or fungal infection which may increase mortality rates (Wang et al. 2006). To date, more than 200 bird species have been reported with APV infection worldwide and it is likely that more species are also susceptible (Bolte et al. 1999; Van Riper and Forrester 2007).

Avipoxvirus has been identified in a range of introduced, native, and endemic New Zealand bird species and includes critically endangered endemic species such as black robin (*Petroica traversi*), North Island saddleback (*Philesturnus carunculatus rufusater*), South Island saddleback (*Philesturnus carunculatus carunculatus*), wrybill (*Anarhynchus frontalis*), shore plover (*Thinornis novaeseelandiae*) and brown kiwi (*Apteryx mantelli*) (Westerskov 1953; Austin et al. 1973; Bolte et al. 1999; Alley 2002; Ha et al. 2011; Chapter two; Ha et al. 2012b; Chapter three). Moreover, we have previously confirmed the presence of at least three different strains of APV in New Zealand including A1, A3 and B1 with A1 and B1 strains responsible for the majority of infections (Ha et al. 2011; Chapter two). A study of introduced passerines in the southern North Island of New Zealand revealed a high seroprevalence (69.2%) of APV, suggesting APV is likely to be well-established in New

Zealand avifauna (Ha et al. 2012a; Chapter four). Despite the fact that APV causes mortality in at least two endangered species such as black robin and shore plover in New Zealand, there are no published reports on the pathogenicity of the New Zealand APV strains, their potential impact on New Zealand birds or the immune response after exposure. This study was designed to evaluate the pathogenicity of two APV strains isolated from wild birds; silvereye (*Zosterops lateralis)* A1 strain and blackbird (*Turdus merula*) B1 strain in New Zealand. In addition, we evaluated the efficacy of a commercial FWPV vaccine for the protection of zebra finches (*Taeniopygia guttata*) from APV to determine the vaccine's potential use in native species.

6.3. MATERIALS AND METHODS

6.3.1. Virus propagation

Avipoxvirus lesions from two wild birds were used as inocula for virus isolation. The first isolate (A1 strain) was from a silvereye (GenBank accession No. HQ701724) previously described in Ha et al. (2011; Chapter two) and the second isolate (B1 strain) was from a blackbird (GenBank accession No. JX683279). The lesions were on the right eye and on the left foot, respectively. Both birds were subjected to post mortem at the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, New Zealand and tissue lesions were excised and kept at – 20 °C. Avipoxvirus infection was confirmed by PCR as described in Ha et al. (2011; Chapter two).

The excised skin lesions were ground using sterile mortars and pestles in phosphate-buffer saline (PBS, pH 7.4) containing 1000 U/mL penicillin (Invitrogen, Carlsbad, CA, USA), 1

mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 0.4 µL/mL amphotericin B (as Fungizone) (Invitrogen, Carlsbad, CA, USA). The suspensions were incubated at 37 °C for 1 hr and clarified by centrifugation at 1500 x g for 5 min. The supernatant fluids were transferred to new sterile centrifuge tubes and refrigerated at 4 °C for 2 hr prior to inoculation of the chicken embryo fibroblast (CEF) cells (American Type Culture Collection, Rockville, MD, USA).

The CEF cultures were maintained according to the manufacturer's instruction with minor modifications. Advanced Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 1% GlutaMAX^{TM} (Invitrogen, Carlsbad, CA, USA), 1000 U/mL penicillin, 1 mg/mL streptomycin, and 2.5 µg/mL amphotericin B, and 2% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) was used as complete growth medium.

One mL of viral supernatant fluid from each isolate was added into a two-day old approximately 50% confluent T-75 flask of CEF cells containing 19 mL of the complete DMEM growth medium. The flasks were kept in a 37°C, 5% CO_2 humidified air incubator and cytopathic effect (CPE) was monitored for the next seven days. After seven days, the flasks were freeze-thawed and the cells were scraped off the surface. The collected lysates were vigorously vortexed for 2 min and then centrifuged at 800 x g for 30 min. The supernatant fluid was collected and refrigerated at 4°C for 2 hr and then passaged into fresh 50% confluent flasks of CEF cells. After five passages, media was removed and 2 mL of sterile PBS (pH 7.4) was added into each flask to facilitate cell lysis by one cycle of freeze-thawing. This was followed by manual scraping of the attached cells off the flasks and the lysates were transferred into new sterile centrifuge tubes. After vigorous vortexing, all the lysates were centrifuged at 800 x g for 30 min to remove cell debris. The resultant

supernatant fluids were used as inocula for this study. The isolates were re-confirmed as APV by PCR assay described by Ha et al. (2011; Chapter two).

6.3.2. Virus titration

The $TCID_{50}$ assay was carried out by described in Reed and Muench (1938) with slight modifications. Briefly, 100 µL of 1.0×10^5 CEF cells in the complete DMEM growth medium was added to each well of a flat-bottom 96 cell culture Microwell plate (NUNC A/S, Roskilde, Denmark) and placed in 37 °C, 5 % CO_2 humidified air incubator for 24 hours. Complete DMEM growth medium was added into each well of a separate V-bottom 96 Microwell plate (NUNC A/S, Roskilde, Denmark) for virus dilution. Viral supernatant (100 µL) was added into each well of the first column of the V-bottom 96 Microwell plate (NUNC A/S, Roskilde, Denmark) prepared with the complete DMEM growth medium. Progressively two-fold virus dilutions were performed. Diluted virus (100 µL) was then added to the previously prepared flat-bottom 96 well cell culture Microwell plate (NUNC A/S, Roskilde, Denmark). Plates were incubated at 37 °C, 5 % CO_2 and the cytopathic effect (CPE) was monitored for five days and the 50% end-point ($TCID_{50}$) calculated. The final concentration for both strains was determined to be 10^4 $TCID_{50}$/mL.

6.3.3. Experimental design

The use of live animals in this study was approved by Massey University Animal Ethics Committee (MUAEC 10/11). Twenty-five six-week-old zebra finches were divided randomly into five groups of five birds each; Group I, II, III, IV, and control. All five groups were kept in separate rooms to avoid cross contamination. Birds from Group III and IV were

vaccinated with a FWPV vaccine, Poxine (Duphar; Fort Dodge, IO, USA), on the right wing at the initiation of this study as described below. At two weeks post-vaccination of Group III and IV, birds from Group I and III were inoculated with the silvereye isolate (A1 strain) and birds from Group II and IV were infected with the blackbird isolate (B1 strain). Birds in the control group were inoculated with sterile PBS (pH 7.4). The virus or PBS inoculation was carried out on left wing and left foot in each bird. Due to the birds' size, the application of conventional wing-web method was not ideal and therefore, sterile 26-gauge syringe needles were used for vaccination and experimental infection. A sterile 26-gauge syringe needle was immersed in the virus suspension and used to prick the bird's wing web and foot pad, five times each. The birds were observed daily for the degree of activity and ability to fly. The development of pox-like lesions was monitored for 24 days and skin thickness at inoculation sites on the wings were measured three times/week using digital calliper (Tresna, Guangxi Province, China; accuracy = ±0.03).

6.3.4. Sample collection

Tissue samples were collected from all birds with visible foot lesions 2 weeks after virus inoculation. A small portion of each sample was subjected to DNA extraction and PCR to amplify APV 4b core protein gene as described by Ha et al. (2011; Chapter two). The remaining tissue samples were fixed in 10% buffered formalin, embedded in paraffin, cut at 5 µm, and stained with hematoxylin and eosin (H & E) for histological examination. Blood samples were collected from all the birds on a weekly basis throughout the study to measure the levels of humoral immune response. Less than 100 µL of blood from each bird was collected into heparinised capillary tubes using the brachial vein and the transferred into silicon coated BD Microtainer® blood collection tubes (BD, Franklin Lakes,

NJ, USA). The samples were centrifuged at 2,500 x g for 30 min and separated serum samples were kept at -20° for ELISA assay.

6.3.5. ELISA for examination of humoral immune responses

FWPV antigen was prepared and an ELISA assay was performed as described in Ha et al. (2012a; Chapter four). An $OD_{450} \geq 0.6$ was considered positive for APV antibodies.

6.3.6. Statistical tests used

Two-way ANOVA test was carried out using Minitab® Statistical Software (version 16. 1, Minitab Inc., State College, PA, USA) and all statistical analyses were considered significant when $p<0.05$.

6.4. RESULTS

6.4.1. Cytopathic effect of A1 and B1 APV strains

Both silvereye (A1 subclade) and blackbird (B1 subclade) APV isolates were successfully propagated in cell cultures. Cytopathic effects (CPE) including rounding and detachment of cells were obvious in the APV inoculated CEF cultures, but were not observed in the uninoculated control culture (Figure 6.1.). No obvious differences in CPE were observed between two different strains (Figure 6.1.).

6.4.2. Pathogenicity and immune response to FPV vaccination

The ten vaccinated birds in Group III (n = 5) and IV (n = 5) displayed no signs of illness or lesions suggestive of APV infection after FPV vaccination. All vaccinated birds developed humoral immune responses by two weeks post vaccination and exceeded the $OD_{450} \geq 0.6$ positive assay cutoff (Group III $OD_{450} = 0.865$ (SD = 0.062) and Group IV $OD_{450} = 0.861$ (SD = 0.161)).

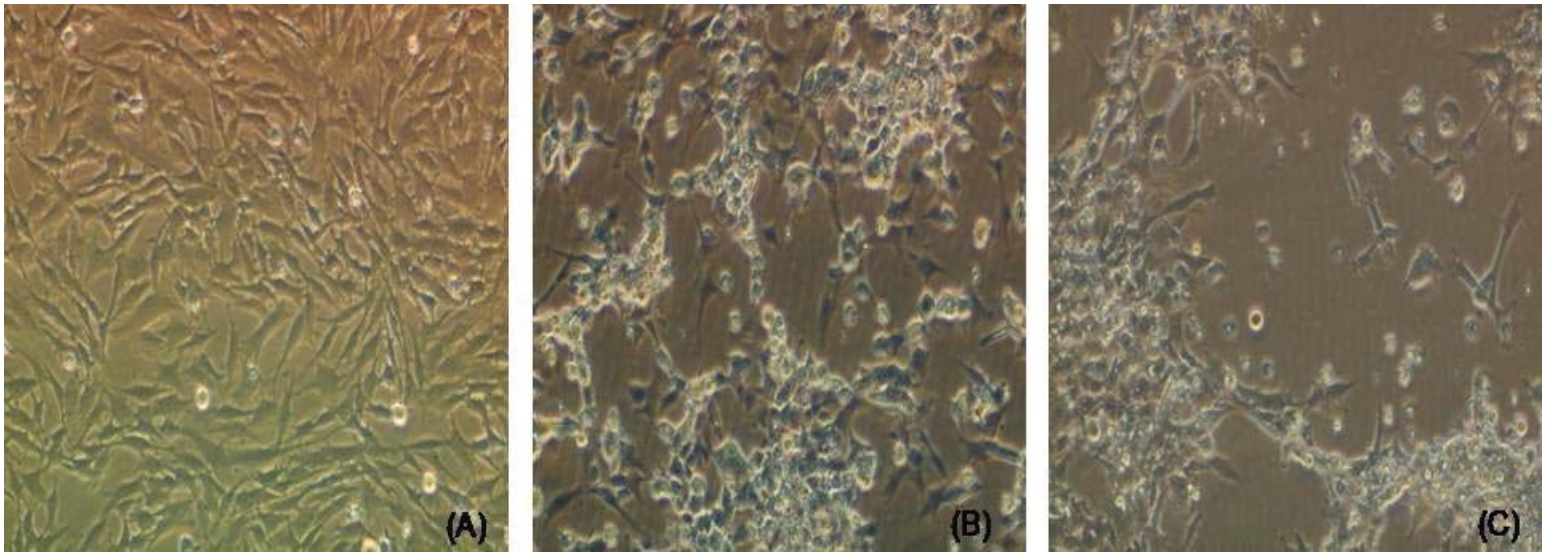

Figure 6.1. Cytopathic effect (CPE) observed at day seven after virus inoculation; (A), (400X magnification), inoculated with 1 mL of the complete DMEM growth medium; (B), (400X magnification): inoculated with 1 mL of the silvereye isolate (A1 strain); (C), (400X magnification), inoculated with 1 mL of the blackbird isolate (B1 strain). Rounding and detachment of cells can be observed in the APV inoculated CEF cultures (B & C), but not in the complete DMEM growth medium inoculated culture (A).

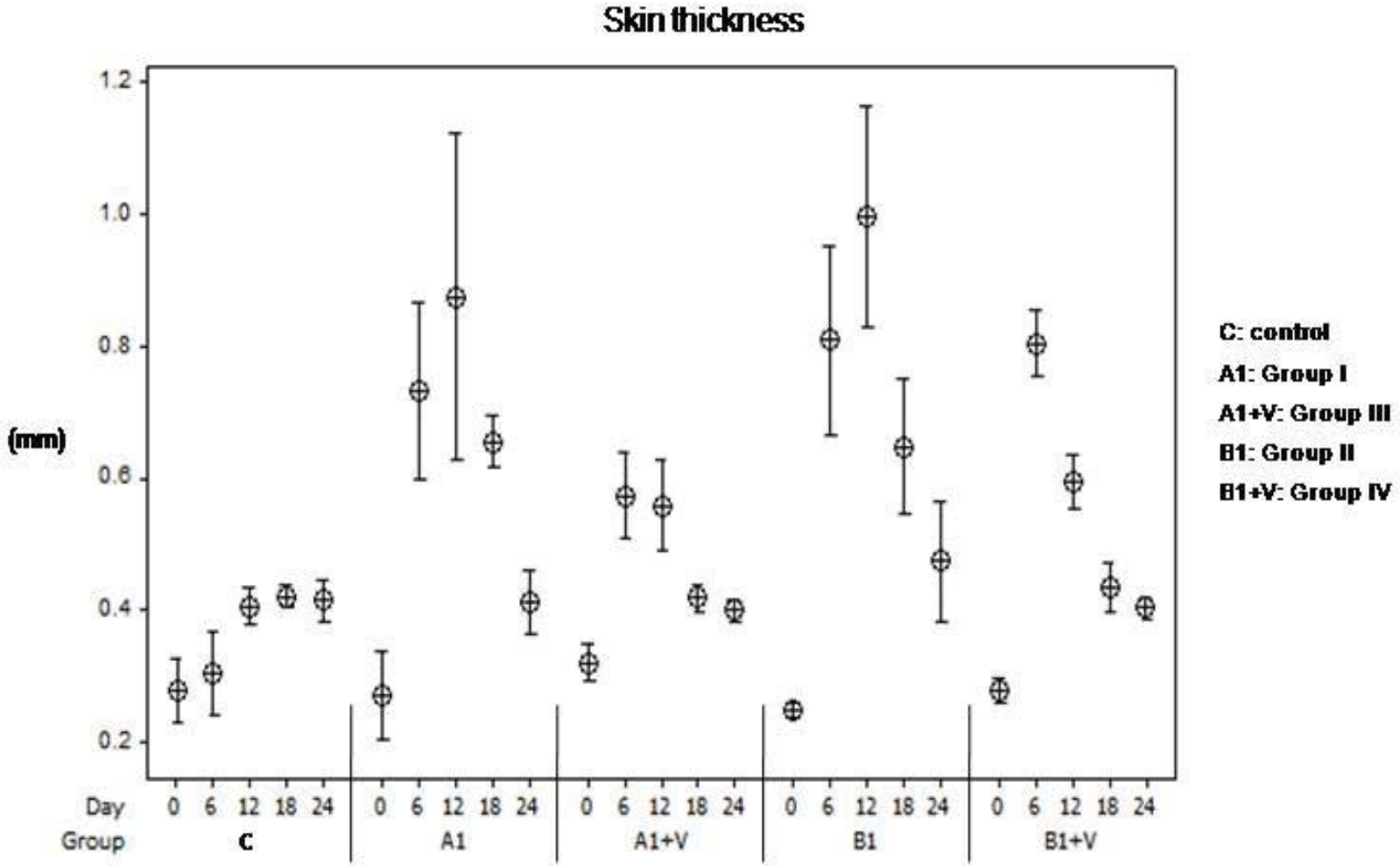
Skin thickness
(mm)
1.2
1.0
0.8
0.6
0.4
0.2
Day 0 6 12 18 24 0 6 12 18 24 0 6 12 18 24 0 6 12 18 24 0 6 12 18 24
Group C A1 A1+V B1 B1+V
C: control
A1: Group I
A1+V: Group III
B1: Group II
B1+V: Group IV

Figure 6.2. Evaluation of efficacy of fowlpox (FWPV) vaccine in zebra finches. Birds from Group I and II were inoculated with 10^4 $TCID_{50}$/mL of silvereye isolate (A1) and blackbird isolate (B1), respectively without prior FWPV vaccination. Birds from Group III and IV were inoculated with 10^4 $TCID_{50}$ of silvereye isolate (A1) and black bird isolate (B1), respectively with prior FWPV vaccination. Birds from the control group were inoculated with sterile PBS (pH 7.4). The skin thickness was measured at the inoculation site on the left wing web following virus inoculation and each bar displays mean ± SD from each group of birds.

6.4. 3. Pathogenicity after challenge with A1 and B1 APV strains

All birds (10/10) infected with silvereye (A1, Group I) and blackbird (B1, Group II) isolates without prior FWPV vaccination developed small circular lesions (less than 3 mm in diameter) on the wings. Lesions could be observed as early as three days post inoculation (PI) in all birds. However, no lesions were observed in the PBS inoculated control group. Skin thickness measured 0.27 mm (SD = 0.054) and 0.25 mm (SD = 0.010) in Group I and II, respectively, and peaked at 12 days PI (0.87 mm ± 0.199) and nine days PI (1.05 mm ± 0.163) in Group I and II, respectively (Figure 6.2.). The lesions became crusted starting from approximately ten days PI and regressed over the following 8-11 days. Birds in Group II displayed more severe skin lesions and slower regression (mean = 20 days) than those in Group I (mean = 18 days). Skin thickness in control and experimentally infected groups was significantly different ($P < 0.001$). The difference in skin thickness between Group I and Group II was not significant ($P > 0.1$).

In contrast, the vaccinated birds in Group III and IV developed only mild swelling on their wings after virus challenge starting from two days PI (Figure 6.2.). In birds in Group III the swelling increased until nine days PI (0.64 mm ± 0.038) and decreased afterwards (Figure 6.2.). Likewise, after reaching a maximum at six days PI (0.80 mm ± 0.042) the swelling resolved in birds in Group IV (Figure 6.2.). The difference in skin thickness between the two vaccinated groups was significant ($P < 0.001$), however all vaccinated birds (Group III and Group IV) displayed less severe swelling on the skin and the regression was faster than that of non-vaccinated birds (Group I and Group II) (Figure 6.2.). The differences in skin thickness between birds with prior vaccination and birds without vaccination were significant ($P < 0.001$). No vaccinated-challenged birds in Group III and IV developed

cutaneous lesions on their foot by the end of this study.

In addition, APV 4b core protein gene was detected by the PCR in 4/5 birds in Group I and 5/5 birds in Group II which developed cutaneous lesions on their feet (Figure 6.3.). The development of foot lesions began approximately five to seven days PI in all birds (4/5 birds in Group I and 5/5 birds in Group II) and took longer to resolve than those on the wings (20-24 days). In most birds (3 birds in Group I and 2 birds in Group II) the foot lesions had resolved at the end of this study, but in one bird from Group I and three birds from Group II the lesions were still present.

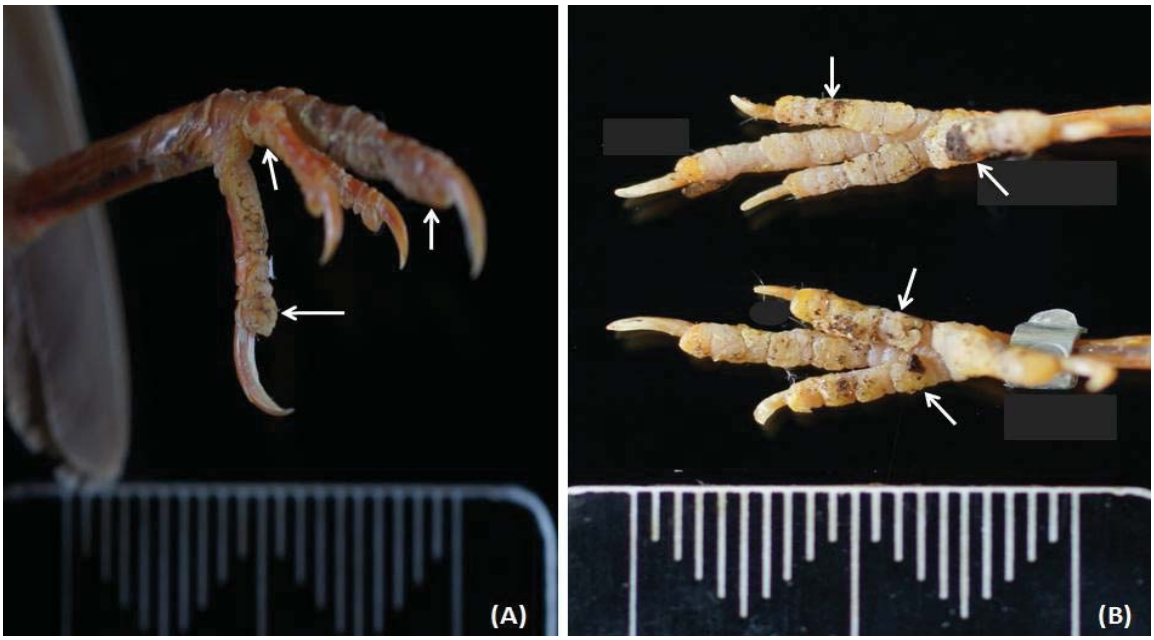

Figure 6.3. Zebra finches (*Taeniopygia guttata*) with proliferating poxvirus lesions on their feet (arrows). (A): a foot of a zebra finch inoculated with silvereye A1 strain, (B): feet of a zebra finch inoculated with blackbird B1 strain.

There was prominent multifocal dermal lymphoid nodule formation present in the dermis, particularly on the plantar surface on the foot (Figure 6.4A. & 4B.). Occasional fragments of pigmented seed material were embedded within the hyperkeratotic plaques (Figure 6.4A. & 4B.). Histopathology of a foot lesion from a bird inoculated with blackbird isolate (B1 strain) showed a marked thickening of the stratum spinosum (acanthosis), with patchy orthokeratotic hyperkeratosis, with evidence of vesicle and pustule formation in some areas (Figure 6.4C.). The basal and spiny-cell layers were disrupted by large numbers of intra-cytoplasmic inter-cellular clear vacuoles (Figure 6.4D.). Individual cells were shrunken and bright eosinophilic inclusion bodies were sometimes present (Figure 6.4D. & 4E. & 4F.). The dermal-epidermal junction was obscured by a mixed inflammatory cell infiltrate comprised mostly of lymphocytes and heterophils (Figure 6.4D.). Discrete lymphoid aggregates expanded the dermis and were composed of mature lymphocytes with fewer immature blast cells (Figure 6.4E.). They were typically situated in close proximity to dermal blood vessels (Figure 6.4E.). No birds from Group I and II died and all birds remained reasonably active by the termination of this study.

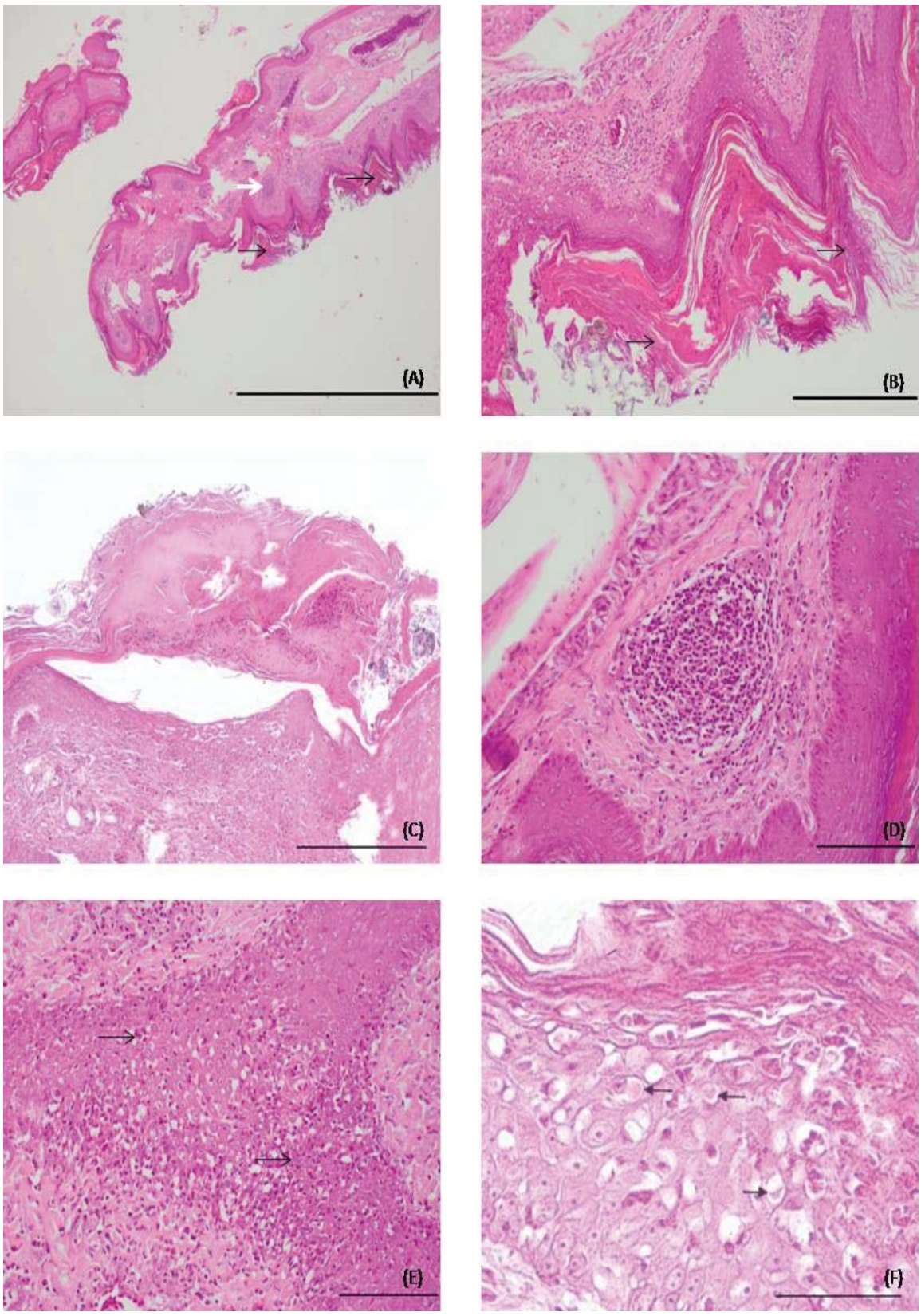
(A)
(B)
(C)
(D)
(E)
(F)

Figure 6.4. Light photomicrograph of a section of a foot from a bird inoculated with 10^4 $TCID_{50}$ of blackbird isolate (B1 strain). (A, B), Marked thickening of the stratum spinosum (acanthosis), with patchy orthokeratotic hyperkeratosis, occasional areas of spongy epidermal change (black arrows), and prominent multifocal dermal lymphoid nodule are present (white arrow) throughout the skin, particularly prominent on the plantar surface. Occasional fragments of pigmented seed material are embedded within the hyperkeratotic plaques. H & E, Scale bar = 2000 µm (A) and 200 µm (B); (C), A subcorneal pustule within the epidermis of a section of the toe from a bird inoculated with 10^4 $TCID_{50}$ of blackbird isolate. The underlying epidermis is disrupted and infiltrated with inflammatory cells. H & E, Scale bar = 200 µm; (D), High power view of the lymphoid nodules. The aggregates expand the dermis, are discrete, and composed of mature lymphocytes with fewer immature blast cells. They are typically situated in close proximity to dermal blood vessels. H & E, Scale bar = 200 µm; (E), Oblique section of a toe from a bird inoculated with 10^4 $TCID_{50}$ of blackbird isolate. The basal and spiny-cell layers are disrupted by large numbers of intra-cellular and inter-cellular clear vacuoles. Individual cells are shrunken and bright eosinophilic inclusion bodies are present (black arrows). The dermal-epidermal junction is obscured by an inflammatory infiltrate comprising mostly lymphocytes and heterophils. H & E, Scale bar = 200 µm; (F), High power view of the stratum spinosum of the toe from a bird inoculated with 10^4 $TCID_{50}$ of blackbird isolate showing eosinophilic intracytoplamic inclusions (black arrows). H & E, Scale bar = 20 µm.

6.4.4. Development of immune response after A1 and B1 APV challenge

The unvaccinated birds in Group I and II developed humoral immune responses after experimental infection. The level of antibodies increased and exceeded the OD_{450} > 0.6 positive assay cutoff by one week PI (Group I OD_{450} = 0.539 ± 0.125; Group II OD_{450} = 0.938 ± 0.421) (Figure 6.5.). In both Groups I and II, the level of antibody reached its maximum at week two PI (Figure 6.5.). The elevation of antibody titre was not observed in birds in the control group with similar negative antibody levels at day 0 and two weeks PI (OD_{450} = 0.407 ± 0.090 and OD_{450} = 0.477 ± 0.061 respectively) (Figure 6.5.). While the differences in immune response between control group and Groups I and II were significant ($P < 0.001$), the differences between Group I and II were not ($P > 0.2$).

An increase in the level of antibody was also observed after virus challenge in the previously vaccinated birds in Groups III and IV (Figure 6.5.). The elevation of antibody level was slower in Group III than Group IV showing the highest peak at two weeks PI (OD_{450} = 1.004 ± 0.210) (Figure 6.5.). Birds in Group IV presented the highest level of antibody at one week PI (OD_{450} = 1.018 ± 0.168) (Figure 6.5.). However, the differences between Group III and Group IV were not significant ($P > 0.2$). Minor decreases in the antibody levels were observed in both vaccinated groups after two weeks PI (Figure 6.5.). When compared with non-vaccinated groups, vaccinated groups maintained higher antibody levels at the completion of the study (Figure 6.5.). The differences in antibody levels between APV A1 strain challenged non-vaccinated Group I and vaccinated Group III were significant ($P < 0.001$) as were the differences in antibody levels between APV B1 strain challenged non-vaccinated Group II and vaccinated Group IV ($P < 0.02$).

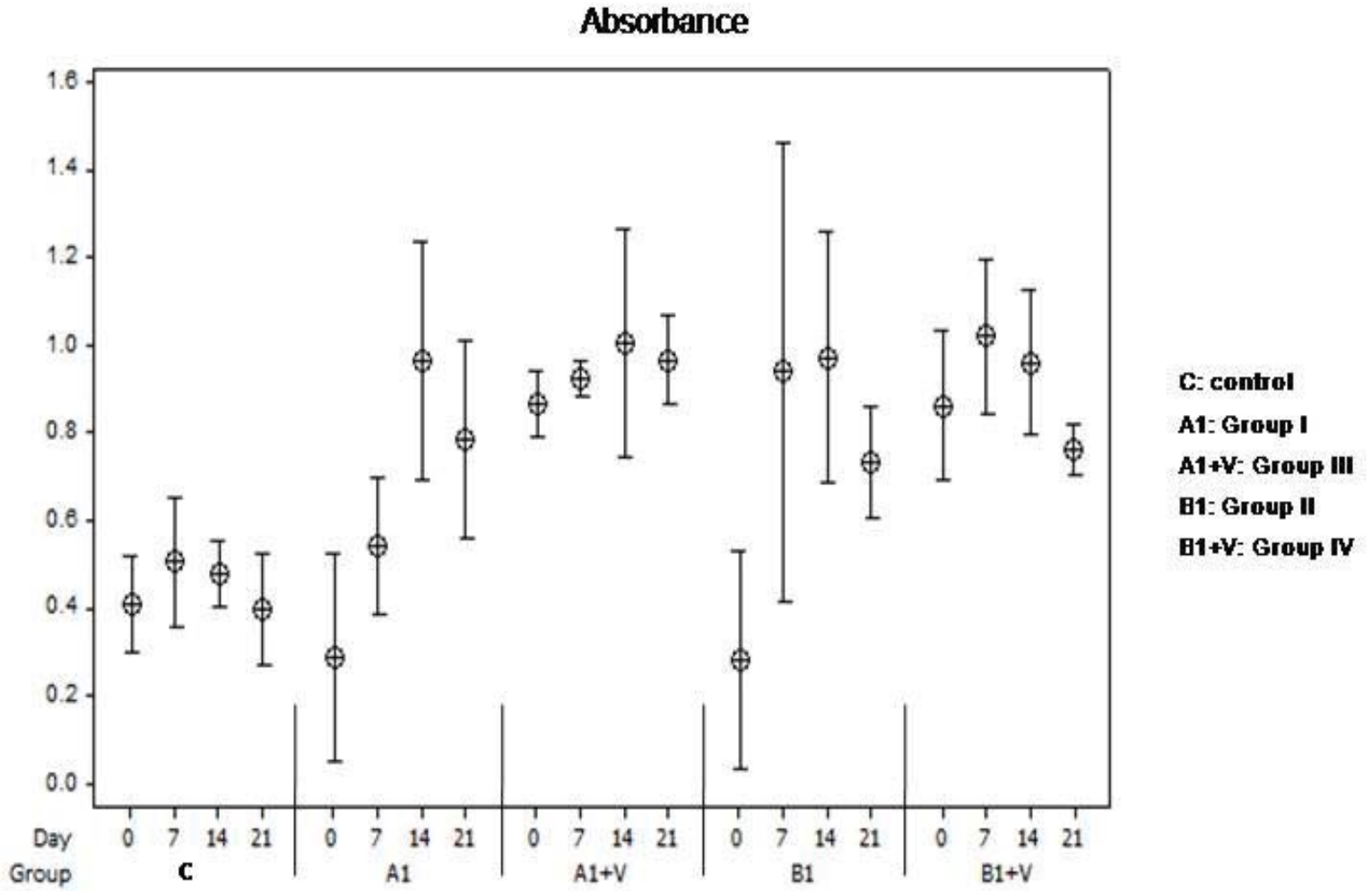
Absorbance
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Day
Group
0 7 14 21
C
A1
A1+V
B1
B1+V
C: control
A1: Group I
A1+V: Group III
B1: Group II
B1+V: Group IV

Figure 6.5. Evaluation of efficacy of fowlpox (FWPV) vaccine in zebra finches. Birds from Group I and II were inoculated with 10^4 $TCID_{50}$/mL of silvereye isolate (A1) and blackbird isolate (B1), respectively without prior FWPV vaccination. Birds from Group III and IV were inoculated with 10^4 $TCID_{50}$ of silvereye isolate (A1) and black bird isolate (B1), respectively with prior FWPV vaccination. Birds from the control group were inoculated with sterile PBS (pH 7.4). Absorbance was measured by ELISA reader at 450 nm. Each bar displays mean ± SD from triplicates of birds from Group I, II, III, and IV from day 0 to day 21 post inoculation.

6.5. DISCUSSION

The isolation of APV in cell culture, especially isolates from wild birds often fails as some strains have been found to grow only on chorioallantoic membrane (CAM) of chicken embryos while other strains prefer cell culture (Tripathy et al. 2000; Kim and Tripathy 2006a). Both the silvereye and blackbird APV isolates used in this study were successfully isolated and propagated in CEF cell culture. Both these isolates caused mild local lesions at the inoculation sites in the model species. Traditionally, APVs were believed to be reasonably host specific or only infect closely related host species. However, recent findings indicate that they are capable of infecting multiple species residing in a similar geographic range (Adams et al. 2005). The two strains of APV used in this study have been identified in various species of birds in New Zealand including ratites, waders, plovers, and passerines (Ha et al. 2011; Chapter two; Ha et al. 2012b: Chapter three). This suggests these viruses are wide spread throughout New Zealand. A seroprevalence study carried out by the authors also suggests that APV is well-established in New Zealand birds (Ha et al. 2012a; Chapter four). To date, it appears that island bird species are more susceptible to APV than continental species (Atkinson et al. 2005; Van Riper and Forrester 2007) and for this reason it is likely that a wide range of New Zealand birds are susceptible to the infection.

Both strains used in the study were from clinical cases however the B1 strain, collectively known as ‘canarypox virus’ strain, appeared to be more pathogenic in the model species. Birds challenged with B1 strains developed more severe swelling at the inoculation sites than those challenged with A1 strains. Although both APV strains presented reasonably mild pathogenicity in the model species, the fact that the birds were kept in an

environment where strict hygiene protocols were applied should be taken into consideration as this may have reduced the risk of secondary bacterial or fungal infection. Poxviruses are known to suppress the immune response by encoding proteins which work as inhibitors for a range of inflammatory mediators or by compromising the integrity of T-lymphocytes (Moss 2007). Immune suppression caused by poxvirus infection can make the infected individuals more susceptible to secondary infection and also increase the pathogenicity of other pathogens (Wang et al. 2006). Moreover, a combination of pathogens can result in severe infection even to the point of altering avirulent organisms into virulent ones (Thomas et al. 2003).

Avipoxvirus infection is understood to be a contributing factor for the decline of island bird species (Wikelski et al. 2004; Lapointe 2008; Atkinson and LaPointe 2009; Parker et al. 2011). The relationship between the prevalence of APV and the abundance of insect vectors has been well-documented in the Hawaiian islands (Van Riper et al. 2002; Aruch et al. 2007; Lapointe 2008). The number and range of insect vector populations in New Zealand are believed to be increasing due to the climate change (Tompkins and Gleeson 2006). Of the two mosquito species that are already well-established throughout New Zealand; *Culex quinquefasciatus* and *Ochlerotatus notosriptus* (Derraik 2004), *C. quinquefasciatus* is known to be a vector for APV in Hawaiian islands (Van Riper et al. 2002). A close link between the abundance of vector species and the prevalence of APV has been observed in these regions (Van Riper et al. 2002; Aruch et al. 2007). New Zealand mosquito fauna represents markedly narrow diversity, leaving concerns about the invasion of exotic mosquitoes (Derraik 2004). *Aedes albopictus*, another mosquito species reportedly known as a vector for APV in Hawaiian islands (Van Riper et al. 2002), has been identified in New Zealand but fortunately a viable population has not been established in

this country (Derraik 2004). Sandflies (family *Simuliidae*), the introduced mosquito *C. quinquefasicatus*, and the native mosquito *C. pervigilans* are the alleged vectors for APV infection in New Zealand (Gartrell et al. 2003; Ha et al. 2011; Chapter two). However, the relationship between the presence of insect vectors and APV in New Zealand birds is yet to be investigated.

The FWPV vaccine used in this study has been widely used in the poultry industry in New Zealand, especially in the northern region (PacificvetLimited 2007). Of the two commercially available attenuated FWPV vaccines in New Zealand, the one used in this study is recommended for chickens six weeks of age or older (PacificvetLimited 2007). Despite the fact that vaccine safety data for use in wild bird species is limited, this vaccine was shown to be safe in the model species tested. Other studies have found that commercial vaccines have not always produced adequate protection across "variant" strains (Fatunmbi and Reed 1996; Singh et al. 2000) but this has not been found for the two isolates used in this study. All vaccinated birds remained active and healthy in daily observation, displaying no signs of illness after challenge. In comparison, non-vaccinated birds appeared as active as vaccinated birds when observed but the majority of non-vaccinated birds developed local lesions on their feet. This was regardless of the challenge strain suggesting that cross-protection between different strains of APV is possible. Outside of the clean laboratory situation the presence or absence of foot lesions and resulting secondary infections could have significant influence on mortality. Nevertheless our results suggest that the vaccine used in this study is likely to be safe in some species of wild passerines and at the same time effective in preventing clinical disease due to more than one strain of APV.

Unfortunately, this experiment could not be carried out in endemic or native New Zealand bird species of which little is known about the potential impact of APV infection. Not only is the pathogenicity of APV infection in wild birds poorly understood but also the effects of APV can vary in different species of birds (Van Riper and Forrester 2007). This complicates the prediction as to the expected pathogenicity of APV strains in new hosts such as endangered New Zealand birds in which APV infection has never been reported. Without long-term data on the prevalence and seroprevalence of APV, it is difficult to extrapolate experimental data to estimate risk of infection to wild or captive populations of these birds in New Zealand. However, our results suggest that FWPV vaccine has the potential to be used in the captive management of native passerines, such as shore plovers to prevent clinical disease caused by APV.

Vaccination could also play a role in the conservation management of other endangered passerine species that are significantly affected by APV disease. In particular, this is of relevance to the endangered passerines of island ecosystems where APV is a relatively recent introduction, such as Hawaii (Aruch et al. 2007) and New Zealand (Ha et al. 2011; Chapter two). In our study, the commercial fowlpox virus provided good cross protection against the New Zealand field strains, however the diversity of APV strains present in a population (Ha et al., 2011; Chapter two) must be considered when selecting an appropriate vaccine.

Avipoxvirus outbreaks in New Zealand are generally associated with wildlife translocations (Alley 2002; Alley et al. 2010; Ha et al. 2011; Chapter two). Considering the amount of time, cost and effort required during translocation processes, mortalities following

translocations can be costly for the agencies involved, as well as for the animals being translocated. Translocation itself induces significant physical and environmental stress to the animals and this increases their susceptibility to pathogens that might exist at both source and destination sites. The outcome of translocations could therefore be disastrous if there is introduction of new pathogens into naïve populations. The presence of skin lesions suggestive of APV infection is routinely monitored during quarantine for the translocation of birds in New Zealand. The results of this study suggest that antibody screening for APV should be used routinely as part of assessment for the suitability of individuals prior to translocation especially where there is a risk of introducing APV into naïve populations. Where naïve individuals are at risk of exposure in a new location then vaccination would be strongly encouraged. Given that up to 14 days were required for vaccinated birds to achieve protective antibody levels and the stress caused by translocation on transported animals, vaccination would have to be carried out in advance of any translocation of naïve birds. This would reduce the likelihood of mortalities caused by APV following translocations.

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

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*.

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Evaluation of the pathogenicity of avipoxvirus strains isolated from wild birds in New Zealand and the efficacy of a fowlpox vaccine in passerines

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CHAPTER SEVEN

General Discussion

There were four main objectives for this study. The first objective was to examine the genetic diversity of avipoxviruses (APVs) affecting native and introduced bird species in New Zealand. Secondly, I aimed to determine how widespread APV is in some selected bird populations in New Zealand. The third objective was to identify the pathogenicity of New Zealand APV strains. Lastly, the safety and efficacy of a commercially available fowlpox (FWPV) vaccine in New Zealand were investigated. The study was successful in meeting all above objectives and the main findings can be divided into six broad points.

7.1. AVIPOXVIRUS STRAINS IN NEW ZEALAND AND THE DIFFERENCES BETWEEN NEW ZEALAND AND OVERSEAS APV

Avipoxvirus 4b core gene was detected by PCR from samples taken from lesions typical of APV infection in 27 birds in New Zealand. All of the 25 APV isolates sequenced fully or partially in this study belonged to the three subclades of APVs previously described overseas; A1, A3 and B1 (Jarmin et al. 2006; Manarolla et al. 2010). Subclades A1 and B1 were the major APV groups existing in New Zealand. Avipoxvirus isolates identified in New Zealand were closely related to European isolates (Luschow et al. 2004; Manarolla et al. 2010) and some Hawaiian isolates (Jarmin et al. 2006), but distinct from American isolates (Adams et al. 2005). A lack of heterogeneity and host specificity was apparent within the New Zealand isolates. This may be due to a small clonal population of APV that has spread through various avian species in a reasonably short time frame. Alternatively, it may be due to the small sample size of 48 birds taken over the relatively short time span of this study. Investigation of APV from more diverse and larger bird populations over an extended period of time is recommended as this would provide more reliable information about nationally distributed APV strains.

The A1 subclade, which represents the majority of APV infections in New Zealand, is highly related to APVs reported in other parts of the world, mainly in the poultry industry and in several free-ranging birds (Luschow et al. 2004; Jarmin et al. 2006; Manarolla et al. 2010). This current study is unprecedented in detecting sixteen isolates of APV in the same clade as FWPV, from ten different species of bird. It is therefore likely that this strain of APV has been well established throughout New Zealand. Interestingly, the New Zealand APV isolates belonging to the subclade A1 displayed 100% genetic homology with the attenuated FWPV vaccine (Poxine) used by the poultry industry in New Zealand (Pacificvet Limited 2007). These results encourage speculation that FWPV may have been introduced to New Zealand by poultry and become endemic among New Zealand birds. The findings from the vaccination study further support this line of speculation as Poxine provided complete clinical protection in the model passerine species against challenge with the A1 strain of APV isolated from a clinical case seen in a New Zealand passerine. Yet, the 4b core genes compared in this study are only parts of the entire APV genomes and the virulence of FWPV in various New Zealand birds has not been investigated. Further work is required to explore the role of poultry or poultry vaccination in the spread of the FWPV throughout New Zealand avifauna.

The other New Zealand APV isolates were found to be closely related to overseas isolates, in particular European isolates (A3 & B1 subclades). For instance, a kereru (HQ701713), a kiwi isolate and a paradise shelduck isolate presented genetic similarities with albatrosspox ABPV (AM050392) which has been previously identified in passerines in Europe (A3 subclade). This suggests that some APV strains might have been introduced to New Zealand from Europe with the introduction of European bird species.

A South Island saddleback isolate (HQ701715), two shore plover isolates (HQ701714, HQ711991) and a blackbird isolate (JX683279) belong to the subclade B1, along with canarypox CNPV 1445 (AM050375), APV isolate PM33/2007 (EF634350) and APV Hawaiian strain HAAM 22.10H8 (EF568395). This suggests there may have been an introduction of these APV strains from overseas along with the introduction of exotic bird species such as passerines. It is noteworthy that New Zealand APV isolates presented great genetic similarities with APV Hawaiian isolate (EF568395). This may be because they are from a source common to both island groups or, more likely, they were introduced from Hawaii to New Zealand with the introduced mosquito *Culex quinquefasicatus*.

Our study therefore supports the hypothesis that there have been previous introductions of exotic strains of APV to New Zealand avifauna, and that these were probably concurrent with the deliberate introduction of northern hemisphere birds. While the introduction of mammalian predators is without doubt the key factor in the decline of New Zealand's avifauna, what remains unresolved is what impact these exotic APV strains have had on the dramatic decline of native biodiversity. The current work raises the possibility that APV may have played a previously unrecognized role in the loss of species diversity, although the true extent of this may never be fully determined as the mammalian predators and the introduced bird species arrived at more or less the same time.

7. 2. SUSCEPTIBILITY OF NEW ZEALAND BIRDS TO APV INFECTION

The results of the current study confirm that many New Zealand native bird species are susceptible to pathogenic strains of APV infection. The birds tested positive for APV infection were from 16 different avian species including 10 endemic, one native and five

introduced species. Given the difficulty of monitoring lesions consistent with APV infection in free-ranging birds and the lack of sampling for disease surveillance in free-living birds in New Zealand, it is likely that an even greater range of bird species is susceptible to pathogenic APV infection.

Of the 27 APV positive birds, 21 birds (78%) were either native or endemic species in which APV infection has never been or only infrequently reported previously, which supports the idea that most avian species are susceptible to at least one strain of APV (Van Riper and Forrester 2007). The high incidence of APV in native and endemic New Zealand birds may reflect more extensive monitoring and sampling in these species than introduced birds however the possibility that they are more susceptible to APV infection cannot be overlooked. Bird species endemic to isolated islands, and confined to restricted ranges are known to have diminished natural immunity to introduced diseases (Kleindorfer and Dudaniec 2006). Two likely causes of this are firstly, that island species are less likely to be exposed to a high diversity of pathogens, which in turn makes them more vulnerable to the introduction of new pathogens (Kleindorfer and Dudaniec 2006) and secondly, that they have reduced genetic diversity, particularly at the MHC locus which can result in increased susceptibility to a range of pathogens as proposed in endemic bird species in New Zealand (Hale and Briskie 2009). Comprehensive understanding of the susceptibility of many endangered New Zealand birds would require studies involving experimental infection using different strains of APV and this is unlikely to be possible due to their conservation status.

Of particular interest, was the finding of APV in an endemic kiwi population and although there are reports of several bacterial or parasitic diseases in the species (Alley 2002), no viral disease had been documented prior to this study. While both kiwi infected by APV recovered from the infection; one was infected by A3 strain and the other by B1 strain, the virulence of the virus in kiwi still remains unclear. In 2012, several juvenile kiwi were found with suspected but unconfirmed APV lesions (pers. comm. I. Castro[1]). Although kiwi are known to share burrows (Potter 1989; I. Castro pers. obs.) they are mainly solitary so the occasional appearance of clinical cases may be due to high kiwi and vector population densities as was the case with these birds. Given that all the confirmed or suspected APV cases were observed only in juvenile kiwi, there is a possibility that APV compromises their survival.

The best case scenario would be that APV infection poses no significant threat to already threatened New Zealand birds including kiwi, however this would be a boldly optimistic view as immune suppression caused by poxvirus infection is a well established phenomenon and often results in a secondary bacterial or fungal infection (Wang et al. 2006).

7.3. GEOGRAPHIC DISTRIBUTION

The geographic distribution of APV isolates in New Zealand suggests that at least one strain of APV is well established. Other strains present more fragmented distributions which may be suggestive of their spread by population translocation. Alternatively this may be merely an artifact of the sampling procedure as discussed previously and further sampling in these areas will give a clearer picture.

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Subclade A1 was the most widely distributed throughout New Zealand including the Chatham Islands, which are more than 850 km east of the New Zealand mainland. Subclade A3 and subclade B1 both displayed similar geographic distributions within the northern part of the North Island and on the offshore islands between the North and South Island. It is noteworthy that regions where there was geographic overlap between different strains also represent regions where frequent animal movements such as translocations have occurred in the past.

Avipoxvirus infection associated with translocation of threatened species has been identified in this study, and includes South Island saddlebacks (HQ701715, HQ701718) and shore plovers (HQ701714, HQ711991). Each of the affected host birds had a history of translocation from the New Zealand mainland to an offshore island prior to the development of clinical signs consistent with APV infection. These birds displayed no clinical abnormalities during the quarantine procedure carried out prior to translocation. It is probable that the two shore plovers became infected by APV after being released from captivity. The supporting evidence for this conclusion is that: 1) they presented no abnormalities before translocation, 2) they developed similar clinical signs 4-15 days after translocation, and 3) the strain of APV isolated from them differed from APV isolated from the source population. In contrast, APVs isolated from South Island saddleback 1 (HQ701718) and 2 (HQ701715) were different strains despite the fact that their origins and translocation sites were the same. This indicates that there may be overlap in the geographic distribution of APV strains in New Zealand within a single species as observed in previous studies overseas (Luschow et al. 2004; Weli et al. 2004; Manarolla et al. 2010).

Variation between APV isolates within a single species was also observed in the two brown kiwi despite their same geographic origin. The kiwi in this case were introduced to the island from various source populations 47 years ago, which would have allowed the accidental introduction of different strains of APV. Alternatively, and probably more likely, different strains of APV might have been introduced by poultry, passerine or migratory birds and maintained on the island. The existence of two strains raises the question of whether other additional strains exist on this and other islands. More intensive monitoring of APV in kiwi populations is required in order to provide useful information on the virulence, prevalence and the potential impacts of APV on New Zealand's endangered kiwi. A study of the seroprevalence of APV in this and other kiwi populations is necessary to clarify the degree of exposure and immune response and allow a more informed approach to risk management of the disease in free-ranging and captive populations.

7.4. SEROPREVALENCE OF APV IN NEW ZEALAND BIRD POPULATIONS

The present study is the first to detect APV antibodies in introduced and native birds in New Zealand. A remarkably high seroprevalence (69.2%) was observed within introduced passerines in the southern part of the North Island and the study of a small group of native birds found that more than half were seropositive. The high seroprevalence in introduced birds reinforces the hypothesis that APV is well established in wild birds. Although the small sample size in native species does not allow extrapolation of the seroprevalence data to a population scale, it is likely that APV is widespread among native bird species also, as native and introduced species often display considerable overlap of territory in the wild (Chambers 2009). Nevertheless, without an extensive seroprevalence survey in native species, it will be impossible to determine how widespread APV is. It is not

yet clear whether the virus has already spread through the native populations and become endemic or if New Zealand's native wild bird populations still face the potential crisis to biodiversity associated with APV.

This study introduces the possibility of screening for APV antibodies prior to translocation of endangered native bird species. While it would be ideal to establish seroprevalence information for all native populations in the country, it may be more practical to establish blood testing prior to translocation as a routine measure. This would produce a steadily increasing volume of information about APV seroprevalence in species of interest. While this information alone is useful, it may also be possible to detect populations that are so far unexposed and apply appropriate protections for them, for example by limiting translocations into these populations.

In addition to the seroprevalence study, the degree of association with *Plasmodium* spp. infection in free-ranging introduced bird species was investigated. Interestingly, all the eleven blackbirds and one song thrush which tested positive to *Plasmodium* spp. also showed a high antibody titre to APV. Sixty seven per cent of *Plasmodium* positive starlings were also seropositive to APV. It is possible that these birds were infected by both APV and *Plasmodium* spp. via a common vector over time or possibly even at the same time. This high commonality of seroprevalence of APV and *Plasmodium* spp. infection in blackbirds and starlings exceeded the degree of co-infection reported in other island bird populations (Atkinson et al. 2005) and suggests that in New Zealand these birds have a high natural exposure and recovery from these pathogens. This raises the possibility that introduced birds may be a reservoir of infection for both organisms. The possibility that blackbirds might play a role as reservoirs of *Plasmodium* spp. because of the high prevalence of

infection in the population, has already been raised in several other New Zealand studies (Tompkins and Gleeson 2006; Baillie and Brunton 2011; Howe et al. 2011).

7.5. PATHOGENICITY OF MAJOR APV STRAINS IN NEW ZEALAND

The two strains of APV used as inoculants in this study have been identified in various species of birds in New Zealand including ratites, waders, plovers and passerines. Both strains used were from clinical cases, however the B1 strain, collectively known as 'canarypox virus' strain, was more pathogenic in our model species. Birds challenged with B1 strains developed more severe swelling at the inoculation sites than those challenged with A1 strains. On the whole, clinical signs were reasonably mild in the model species however the birds were kept in an environment where strict hygiene protocols were applied. This may have enabled the birds to survive the infection without further complications by reducing the risk of secondary bacterial or fungal infection. Poxviruses are known to suppress the immune response by encoding proteins which work as inhibitors for a range of inflammatory mediators or by compromising the integrity of T-lymphocytes (Moss 2007). Immune suppression caused by poxvirus infection can make the infected individuals more susceptible to secondary infection and also increase the virulence of other pathogens (Wang et al. 2006). Moreover, combination of pathogens can result in severe infection even to the point of altering avirulent pathogens into virulent ones (Thomas et al. 2003).

The use of a model species for the study was a practical necessity. However, Zylberberg et al. (2012) claims that there is a variation in the susceptibility and severity to APV between even closely related bird species and this is an important consideration in extrapolating

the results of this experiment. Zebra finches (*Taeniopygia guttata*) are a continental species and the susceptibility of island versus continental bird species has been well-described (Van Riper and Forrester 2007).

7.6. SAFETY AND EFFICACY OF A COMMERCIAL FOWLPOX VACCINE

The fowlpox vaccine used in this study has been widely used in the poultry industry in New Zealand, especially in the northern region (Pacificvet Limited 2007). Of the two commercially available attenuated FWPV vaccines in New Zealand, the one used in this study is recommended for chickens six weeks of age or older (Pacificvet Limited 2007). Despite the fact that vaccine safety data for use in free-ranging bird species is limited, this vaccine appeared to be safe in zebra finch model. Other studies have found that commercial vaccines have not always produced adequate protection across "variant" strains (Fatunmbi and Reed 1996; Singh et al. 2000) but this was not found with the two isolates used in this study. All vaccinated birds remained active and healthy, displaying no signs of illness after challenge. Contrary to non-vaccinated birds, none of the birds vaccinated developed local lesions on their feet regardless of challenge strain, indicating that cross-protection between different strains of APV is possible. Under field conditions the presence or absence of foot lesions and resulting secondary infections could have significant influence on mortality. These results suggest that the vaccine used in this study is likely to be safe for use in some species of free-ranging passerines and also effective in preventing clinical disease due to a range of APV strains.

Birds, either infected or vaccinated, generally develop long-lasting immunity to the same strain of APV and some APV vaccines can present a broad range of protection (Tripathy

and Reed 2008). Further investigation of the use of commercial APV vaccines in native species is encouraged as the APV strain (FWPV) used for vaccination in this study has been identified in various native species.

7.7. IMPLICATIONS

Introduced infectious diseases have the potential to be major threats to New Zealand's unique native avifauna but little work has been done to evaluate or identify these diseases. The role of exotic APV strains in the decline of native biodiversity still remains unclear. However, this current study raises three major concerns regarding APV infection in New Zealand. Firstly, APV may limit the success of translocation of threatened New Zealand birds. Secondly, an increasingly widespread insect vector may spread APV to a greater degree than has occurred previously. Lastly, concurrent infection by APV and *Plasmodium* spp. may be more common and pose a greater risk to New Zealand birds than was initially believed.

7.7.1. Avipoxvirus and animal movement

In New Zealand, re-introduction of endangered species has been increasingly used as an important management tool to forestall extinctions of most of the endangered avifauna (Armstrong et al. 2007). A number of New Zealand endemic bird species have been successfully managed through offshore island or mainland island translocation programs (Department of Conservation 1994, 2001; Holzapfel et al. 2008) and disease risk control is an important component of translocation planning. Undetected asymptomatic carriers of APV may pose a threat via translocations to naïve translocated or destination populations,

not only by introducing new strains of virus but also by increased shedding and disease expression, enhanced in turn by induced immune suppression and stress associated with the translocation (Cunningham 1996; Alley et al. 2010). The key risks associated with APV for wildlife translocations is either the unintentional transfer of APV into new geographic areas with the movement of the host species, or the movement of susceptible host species into an area with existing pathogenic strains of APV (Cunningham 1996). If these risks are to be managed, then clarification of the geographic distribution of APV and host susceptibility to APV strains should be taken into account in the conservation management of endangered bird species.

Importation of birds into New Zealand is currently banned, partly because of the risks to biosecurity. Nevertheless, there is a demand for importation of racing pigeons, exotic passerines and psittacine bird species for aviculture and zoo birds, which could potentially introduce exotic pathogens. The present study revealed that A2 (turkeypox virus), A4, B2 (starlingpox virus) and C (psittacinepox virus) strains described overseas are likely to be exotic in New Zealand avifauna (Jarmin et al. 2006). Although a record of incursion by psittacinepox virus in New Zealand exists (Gartrell et al. 2003), this strain of APV was not identified in our study. There is always a risk of introduction of pathogens with the importation of animals, especially with asymptomatic carriers. Therefore, APV should be considered a disease of concern if further importation of birds is undertaken in the future.

7.7.2. Avipoxvirus and its vectors in New Zealand

The relationship between the prevalence of APV and the abundance of insect vectors has been well-documented in the Hawaiian islands (Van Riper et al. 2002; Aruch et al. 2007; Lapointe 2008). Two mosquito species including *Aedes albopictus* and *C. quinquefasciatus* are known to be vectors for APV in the Hawaiian islands (Van Riper et al. 2002). The greatest prevalence of APV at 1,000 – 1,200 m and below 600 m in elevation may be attributed to *C. quinquefasciatus*, which is abundant at elevations of less than 1,500 m above sea level (Van Riper et al. 2002; Ahumada et al. 2004; Aruch et al. 2007).

In New Zealand, sandflies (*Simuliidae* spp.) are thought to be the major vectors for APV (Gartrell et al. 2003). Recent global warming has resulted in an increase in the number and range of insect vectors for APV in New Zealand including the native mosquito *C. pervigilans* and the introduced mosquito *C. quinquefascicatus* (Derraik 2004; Tompkins and Gleeson 2006). There are 16 species of mosquito present in New Zealand; 12 endemic and four introduced (Derraik 2004). The mosquito *C. quinquefasciatus* was introduced into northern New Zealand about 30 years ago and has extended its distribution throughout the country since then (Tompkins and Gleeson 2006). On the other hand, the forest day mosquito *A. albopictus* is yet to become established in New Zealand although this species has been detected during biosecurity control (Derraik 2004). Avipoxviruses are unable to penetrate intact skin and the most common form of transmission is through insect bites (Van Riper and Forrester 2007). Acting as mechanical transmitters, they can infect multiple individuals following a single bite of an infected individual (Van Riper and Forrester 2007). The genetic similarities between recent APV isolates in New Zealand and the APV Hawaiian strain suggests possible introduction or maintenance and transmission of new strains of APV by

insect vectors to or within New Zealand. These introductions of invasive mosquitoes are of particular concern as they could pose a further threat to already endangered endemic New Zealand birds. The relationship between the presence of insect vectors and APV in New Zealand birds is yet to be investigated.

7.7.3. Avipoxvirus and *Plasmodium* spp.

Given the potential for immune-compromise by APV (Tripathy and Reed 2008), concurrent infections with *Plasmodium* spp. are reported to result in amplified severity of acute avian malarial infections. This can lead to the possibility of increased virulence and spread of both pathogens with the potential of relapsing or chronic infections, population decreases or even extinctions (Van Riper et al. 2002; Atkinson et al. 2005). For example, concurrent infection by APV and *Plasmodium* spp. was believed to be responsible for the decline of 60% of the local saddleback population in two disease outbreaks in the Marlborough Sounds during the summer of 2002 and 2007 (Alley et al. 2010).

Unexpectedly, *Plasmodium* lineages AFTRU5/LINN1, which are little known in New Zealand, were confirmed in several introduced bird species in this study. Lineages within this group have typically demonstrated a high level of specificity at the avian host family level (Beadell et al. 2009) and a high level of host sharing between the *Culex* vectors (Kimura et al. 2009). In New Zealand, death has been reported in a blackbird and an endemic great spotted kiwi, suggesting that this lineage can be pathogenic and its prevalence should be closely monitored (Howe et al. 2011).

To understand the epidemiology of AVP and *Plasmodium* spp. and the role of introduced avian species in their transmission, continuous sampling of both native and introduced species, in multiple regions, and at different times of the year is necessary. Comprehensive knowledge of the abundance of the vectors and pathogens would allow the reduction in potential population loss which can be caused by these infectious diseases.

7. 8. FUTURE RESEARCH AVENUES

This study has provided valuable information on the phylogenetic relationships between different strains of APV and their geographic distribution in New Zealand. In addition, the introduction and optimization of an ELISA assay to detect exposure to APV adds further options to the conservation management of many threatened New Zealand birds. Accessibility and sample sizes are always an issue when working with endangered species but nonetheless, the introduction of serological tests into the quarantine process prior to translocation of many threatened New Zealand birds would be of value. The results of this study also present useful baseline information on the seroprevalence of APV, the pathogenicity of New Zealand APV isolates and the safety and efficacy of a commercial FWPV vaccine in New Zealand birds. Further research involving regular and continuous monitoring and sample collection in native and endemic New Zealand birds is urgently required. If we are to obtain a more comprehensive understanding of APV in New Zealand birds, I would propose these following areas for future research:

7.8.1. Prevalence of APV

Island bird species display a high susceptibility to APV, resulting in a greater prevalence (Van Riper and Forrester 2007). For example, within a few years of its first report in short-toed larks (*Calandrella rufescens*) in the Canary Islands prevalence of APV in the population had reached 50% (Smits et al. 2005). In general this increased prevalence has seasonal, regional and species variations (Van Riper et al. 2002; Aruch et al. 2007; Zylberberg et al. 2012). There is no reliable information on the prevalence of APV in New Zealand birds whether introduced, native or endemic species. Monitoring and sampling in various species of birds in different regions at different times of the year would shed light not only on the prevalence of APV but also the susceptibility, severity or pathogenicity of APV infection in various bird species as well as the long-term trends in infection dynamics.

Monitoring of bird populations for lesions suggestive of APV allows early detection of infected birds. Introducing a universal lesion scoring system and standardised sample collection method similar to those used overseas (Kleindorfer and Dudaniec 2006; Van Riper and Forrester 2007; Zylberberg et al. 2012), which could be utilised by conservation workers throughout the country, would be beneficial in preventing confusion between different observers and also establishing accurate long-term data. In addition, the employment of a standardised reporting protocol would be ideal. Currently it is difficult to acquire tissue samples from birds with clinical signs unless the birds are dead. These difficulties are usually administrative or ethical requirements and due to lack of disease awareness rather than technical difficulties. If routine sample collection and report protocols were facilitated, valuable information on the susceptibility of NZ birds to APV, the presence of exotic strains of APV in NZ and the prevalence of APV could be

accumulated.

7.8.2. Vectors

It is important to note and record the presence of insect vectors during the monitoring and sampling process. The potential role of insect vectors in the transmission of APV is well-known (Van Riper et al. 2002; Vander Werf et al. 2006) yet the relationship between insect vectors and the prevalence of APV in New Zealand has not been investigated. Vectors will also have effects on the spread of *Plasmodium* spp. (Tompkins and Gleeson 2006; Aruch et al. 2007; Lapointe 2008; Kimura et al. 2009). A long-term study as to the relationship between vector density, host densities and the prevalence of APV would provide vital information on the importance of vector transmission for APV dynamics in New Zealand.

If vectors are found to greatly contribute to the spread of APV in New Zealand, vector control should be taken into account in conservation management. In captivity, this can be done by prohibiting the contact between vectors and birds using insect screens (Van Riper and Forrester 2007). The reduction of adult insect vectors as well as control of breeding areas using biocontrol agents could be useful.

7.8.3. Seroprevalence of APV

Further seroprevalence studies focused on native and endemic bird species would be beneficial to better understand disease dynamics and immune function to APV in those species. A recent study demonstrates a variation in immune function even in closely related bird species (Zylberberg et al. 2012). Other than the seroprevalence studies

presented in this dissertation no information regarding the seroprevalence of APV in New Zealand birds exist. Whether native and endemic bird species around New Zealand would present similar high seroprevalences identified in the several introduced bird species investigated to date remains unclear.

Establishing seroprevalence data on a larger population and geographic scale would provide information on the degree of exposure of wild bird populations to APV which is currently lacking. This information would then be able to facilitate conservation managers in making decisions on destination and source populations for translocation events. Quantitative data would be useful as cross-protection between different strains of APV could occur, and individuals with elevated antibody to APV might present a mild form of disease when exposed to exotic strains of APV.

7.8.4. Vaccination

While different strains of APV vaccines including FWPV vaccine, canarypox vaccine and pigeonpox vaccine are available internationally, only FWPV vaccines are available in New Zealand. The results of the vaccine study highlight the potential value of commercial APV vaccines for the prevention of clinical diseases caused by different strains of APV in species of free-ranging passerines. Treatment of APV infection generally involves application of iodine-glycerine to lesions to promote healing and the use of broad-spectrum antibiotics to prevent secondary bacterial infection (Van Riper and Forrester 2007). Regardless of the treatment strategies, the disease usually runs its course which makes prevention the most desirable option (Van Riper and Forrester 2007). Insect control is usually of limited values and vaccination is the most efficient and safe way to control the disease, especially where

bird species conservation is increasingly dependent on translocations for success. It is likely that vaccination will become an integral part of conservation management in the future, especially in captive management and translocation. With this goal in mind further investigation into the safety and efficacy of available vaccines is required.

The ideal candidate for vaccination is an APV strain which causes only a mild local lesion at the inoculation site (Van Riper and Forrester 2007). Potential candidates which can provide a long-lasting or life-time immunity as well as cross-protection across various strains of APV need to be carefully examined. Any evidence of vaccine strains reverting in pathogenicity in the vaccinated populations should be monitored. The safety and efficacy of vaccine strains should be thoroughly examined in a wide range of native and introduced bird species before application to any endangered New Zealand birds as they may have diminished genetic diversity and increased susceptibility to exotic pathogens.

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