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Host-parasite interactions between *Plasmodium* species and New Zealand birds: prevalence, parasite load and pathology

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ABSTRACT

Avian malaria, caused by *Plasmodium* spp., is an emerging disease in New Zealand and has been reported as a cause of morbidity and mortality in New Zealand bird populations. This research was initiated after *P.* (Haemamoeba) *relictum* lineage GRW4, a suspected highly pathogenic lineage of *Plasmodium* spp. was detected in a North Island robin of the Waimarino Forest in 2011. Using nested PCR (nPCR), the prevalence of *Plasmodium* lineages in the Waimarino Forest was evaluated by testing 222 birds of 14 bird species. *Plasmodium* sp. lineage LINN1, *P.* (Huffia) e*longatum* lineage GRW06 and *P.* (Novyella) sp. lineage SYATO5 were detected; *Plasmodium relictum* lineage GRW4 was not found. A real-time PCR (qPCR) protocol to quantify the level of parasitaemia of *Plasmodium* spp. in different bird species was trialled. The qPCR had a sensitivity and specificity of 96.7% and 98% respectively when compared to nPCR, and proved more sensitive in detecting low parasitaemias compared to the nPCR. The mean parasite load was significantly higher in introduced bird species compared to native and endemic species. The data suggests that introduced bird species such as blackbirds have a higher tolerance for *Plasmodium* spp. infections than endemic and native species. The high prevalence of infection and high parasite load in introduced passerines confirmed that they are important reservoirs for avian malaria in the New Zealand. A clinical case of avian malaria in a captive wildlife setting was described for a little penguin (*Eudyptula minor)* at Wellington Zoo. Nested PCR results and DNA sequencing confirmed infection of the deceased penguin with *Plasmodium* (Huffia) *elongatum* GRW06. A retrospective analysis of little penguin cases in the Massey University post mortem database, combined with nested PCR for *Plasmodium* spp. on stored liver tissue samples and DNA sequencing, revealed three additional mortality cases due to *P. elongatum* lineage GRW06, *P*. *relictum* lineage SGS1 and *P.* sp. lineage LINN1 in one captive and two wild little penguins. Our results suggest that avian malaria causes sporadic mortality in New Zealand's little penguins, but there is no evidence of mass mortality events due to avian malaria in this species.

DECLARATION

The studies presented in this thesis were completed by the author whilst a postgraduate student at the Institute of Veterinary, Animal and Biomedical Sciences, Massey University. I officially state that this is my own work and the views presented are mine, and that the contents have not been submitted for any other degree. I certify that to the best of my knowledge any help received in preparing this thesis and all sources used have been acknowledged in the thesis.

Danielle Sijbranda

Supervisor

Brett Gartrell

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ETHICS APPROVAL AND CONSENTS

The handling of live birds during this research was approved by the Massey University Animal Ethics Committee (MUAEC protocol number 11/72), the New Zealand Department of Conservation (DOC Permit no. 34781-FAU) and the local land owners of the Waimarino Forest: Atihau-Whanganui Incorporation, Ngaporo-Waimarino Forest Trust, Pipiriki Incorporation and Ernslaw One Limited.

THESIS STRUCTURE AND FORMAT

This thesis comprises an introduction and literature review, three research chapters, and a general discussion chapter, followed by two sections containing references and appendices. The three research chapters were written as stand-alone papers with the intention of peer reviewed publication. Hence there is some replication in the introduction and discussion sections of these various chapters.

Chapter 1

The "introduction and literature review" provides a framework of background information regarding *Plasmodium* parasites and their life cycle, the related pathogenic effects in birds, diagnostic techniques for avian malaria and examples of the impact of avian malaria infections during wildlife management situations.

Chapter 2

"Avian malaria in introduced, native and endemic New Zealand bird species in a mixed ecosystem" describes a study evaluating the prevalence of various lineages of *Plasmodium* spp. in North Island robins and other bird species in the Waimarino Forest in order to evaluate this area as a source site for robin translocations. A modification of this chapter has been accepted as a research article by the New Zealand Journal of Ecology, and will be published in its 2016 edition 40(1). The article was first published on-line on the 3rd of September 2015 at http://newzealandecology.org/nzje/3241.

Chapter 3

"Use of a real-time PCR to explore disease dynamics of avian malaria in a mixed New Zealand ecosystem" describes the development of a real-time PCR protocol for *Plasmodium* spp. in order to quantify parasite load in individual birds.

Chapter 4

"Mortality of little penguins (*Eudyptula minor*) in New Zealand due to avian malaria" describes a clinical case of avian malaria in a blue penguin in a wildlife rehabilitation setting at Wellington zoo, and incorporates findings of a retrospective review of little penguin post mortem cases from the Massey University post mortem database.

Chapter 5

The "general discussion" reflects on the findings and implications for New Zealand birds of the aforementioned studies, and discusses ideas for further research.

References

To prevent duplication of references, all references have been grouped together after Chapter 5.

Appendices

Research data and laboratory protocols for nested and real-time PCR are added as appendices for reference.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Definition of avian malaria and nomenclature of *Plasmodium* lineages

Avian malaria is a mosquito-transmitted disease, caused by intracellular protozoal blood parasites of the order Haemosporidiae (Valkiunas, 2005). Haemosporidiae are single celled organisms with an apical complex, containing organelles which facilitate the invasion of host cells (Dubremetz et al., 1998; Yeh and DeRisi, 2011). Within the order Haemosporidiae parasites belonging to the families Plasmodiidae, Haemoproteidae, Leukozoidae and Garniidae have been identified in birds (Martinsen et al., 2008; Valkiunas, 2005). For the purpose of this thesis, avian malaria is defined as infection with parasites of the genus *Plasmodium*, belonging to the family Plasmodiidae. *Plasmodium* spp. have a worldwide distribution, and are known to infect most avian taxa (Valkiunas, 2005). Over 200 species of the genus *Plasmodium* have been described globally, of which 67 have been recognised in birds (Carlton et al., 2013). Various subgenera of the genus *Plasmodium*, comprising Haemamoeba, Bennettinia, Giovannolaia, Huffia, Novyella (Martinsen et al., 2007; Valkiunas, 2005) and a proposed sixth subgenus, Papernaia (Landau et al., 2010) have been distinguished based on morphological features. Subgenera often comprise various *Plasmodium* species, and multiple genetic lineages within these species, which are identifiable using molecular techniques. The current official used nomenclature is "*Genus* (Subgenus) *species* lineage name", e.g. *Plasmodium* (Huffia) *elongatum* lineage GRW06 (Bensch, 2009; Valkiunas, 2005).

1.2 Life cycle of *Plasmodium* spp.

Understanding the life cycle of *Plasmodium* spp. helps to understand the spread of avian malaria, the potential clinical signs in hosts and the possible impacts on wild bird populations. *Plasmodium* spp. need mosquito vectors as well as vertebrate hosts to complete their life cycle. Unless indicated, the life cycle information in the following paragraph is derived from Valkiunas (2005).

After a competent female mosquito (Diptera: Culicidae) ingests a blood meal from an infected bird, *Plasmodium* gametocytes escape the erythrocytes in the mosquito's digestive tract. Fertilisation between male and female gametocytes leads to the development of motile ookinetes, worm-like bodies of approximately 3x16 µm containing a nucleus, several vacuoles and pigment granules. Once ookinetes embed into the basal membrane of the midgut, they develop into 40 to 60 µm walled oocysts. Inside each individual oocyst hundreds of nucleated sporozoites with elongated bodies develop. When mature oocysts rupture, these sporozoites are transported to the mosquito's salivary glands where they persist for several weeks. The rate of sporogony in the mosquito is influenced by the characteristics of the mosquito species, the *Plasmodium* lineage and by abiotic factors such as temperature. For example, it takes approximately 6 days post-infection at 28^0C or 26 days at 17^0C for *P. relictum* sporozoites to appear in the mosquito's salivary glands, while sporogony was not observed below 17^0C (LaPointe et al., 2010).

In the avian host, asexual replication of *Plasmodium* spp. occurs in cells of mesodermal origin. After a mosquito injects sporozoites from its salivary glands into the avian blood stream, a first generation of primary exo-erythrocytic meronts (cryptozoites), usually of a diameter less than 30 µm, develops in reticular cells in organs throughout the bird's body. Merozoites developing inside these cryptozoites in turn infect macrophages, leading to the development of meta-cryptozoites, containing large numbers of merozoites. This process in macrophages is described as the second generation of primary exo-erythrocytic merogony. Some of these second generation merozoites again infect macrophages to induce a next generation of meta-cryptozoites, while others infect endothelial cells of capillaries or erythrocytes. The time during which these two generations of primary exo-erythrocytic merogony take place is defined as the prepatent period, and generally no clinical signs are seen during this phase of infection. For *Plasmodium relictum* lineages this prepatent period is normally completed within five days.

Once a merozoite has infected an erythrocyte, it develops into a trophozoite, which in some *Plasmodium* species takes on the characteristic ring shape. The parasite stage is

called a meront once the process of nuclear division starts, leading to a large number of intra-erythrocytic merozoites. Meronts contain pigment granules, which are a result of haemoglobin digestion. Some of the intra-erythrocytic merozoites again infect erythrocytes, leading either to the next cycle of merogony or to the formation of gametocytes inside these erythrocytes; other merozoites infect endothelial cells of capillaries of many organs, in which the secondary exo-erythrocytic merogony takes place. A sharp rise in parasitaemia normally coincides with the maturation of these intra-endothelial merozoites. This period of increased parasitaemia is when mortality and clinical signs are most commonly seen and is defined as the acute phase of infection. The acute phase can last from one week up to one or more months.

Birds that survive the acute phase of the infection progress to the chronic phase, during which a very low level of parasitaemia is seen. Intra-endothelial merozoites as well as the small numbers of erythrocytic meronts are responsible for maintaining a low parasitaemia during the chronic phase of infection. If stages of *Plasmodium* spp. become absent from the blood circulation, but are still present in organs, the infection is defined as latent. Birds surviving the infection will often stay infected for life, leading to a lifelong immune response, protecting these birds from reinfection with homologous isolates of the parasite (Atkinson et al., 2001a; Jarvi et al., 2002). Clinical signs during the chronic low parasitaemia phase of infection are more subtle. Sub-clinical effects on reproductive success, condition, behaviour and survival due to blood parasites have been reported (Cloutier et al., 2011; Lachish et al., 2011; Merino et al., 2000). Weakening of the immune system during the chronic or latent stages of infection can lead to recrudescence or relapse of parasitaemia (Valkiunas, 2005). Elevated parasitaemia levels have been reported during stressful events like rehabilitation of wild birds in captivity, or during the breeding season (Brossy, 1992; Knowles et al., 2009; Valkiunas et al., 2004).

1.3 Pathologic effects of *Plasmodium* infection in birds

1.3.1 Susceptibility to infection

A study by Palinauskas et al. (2008) showed a difference between various bird species in their susceptibility to infection with *P*. (Haemamoeba) *relictum* lineage SGS1, and also between individuals of the same species. When house sparrows (*Passer domesticus*) were inoculated with blood carrying this *Plasmodium* lineage only 50% developed a parasitaemia. However, in inoculated siskins (*Spinus spinus*), crossbills (*Loxia curvirostra*) and chaffinches (*Fringilla coelebs*) patent malarial infections developed in 100% of the birds, while starlings (*Sturnus vulgaris*) appeared to be resistant to infection. In addition, differences between bird species were recorded in the length of the prepatent period, the intensity of parasitaemia and parasite dynamics, effects on haematocrit values, and the severity of hepatosplenomegaly; these findings are likely to cause variation in clinical signs and mortality between wild bird species.

1.3.2 Clinical disease

Clinical signs of avian malaria are mostly seen during the acute phase of infection. They are predominantly caused by destruction of red blood cells and endothelial cells and include anaemia, lethargy and ruffled feathers, decreased activity and food consumption, weight loss, respiratory signs, vomiting, biliverdinuria, cerebral symptoms leading to impaired coordination and paralysis, and death (Atkinson et al., 2001a; Fix et al., 1988; Valkiunas, 2005; Williams, 2005; Yorinks and Atkinson, 2000). In addition, Valkiunas (2005) described how infected fledgling chaffinches during their peak of parasitaemia did not feed themselves, were less mobile and showed increased calling behaviour to attract attention of feeding personnel, while non-infected birds of the same age were fully weaned. Hayworth et al. (1987) demonstrated impaired thermoregulation and reduced abilities of oxygen consumption in canaries (*Serinus canaria* forma *domestica*). The severity of clinical signs can vary, not only between infections with different *Plasmodium* spp. or lineages, but also between different bird species infected with the same lineage. Infection of an birds with *P*. (Haemamoeba) *relictum* lineage GRW4 lead to a higher than

90% mortality in Iiwi (*Vestiaria coccinea*), while Nutmeg Mannikins (*Longura punctulata*) appeared to be completely refractory to infection (Atkinson et al., 1995). In addition, co-infections with multiple lineages of avian malaria occur frequently and can be more virulent than single infections (Palinauskas et al., 2011). The intensity of parasitaemia and the severity of clinical signs in bird species are influenced by innate and acquired immunity (Valkiunas, 2005). In Hawaiian Omao (*Myadestes obscurus*) a strong antibody response against *Plasmodium* resulted in an earlier, lower peak in parasitaemia followed by a more rapid decline to undetectable blood levels compared to other bird species, which coincided with a very low avian malaria related mortality in this species (Atkinson et al., 2001b). Acquired immunity is potentially life long and the high frequency of mixed infections in wild birds suggests that cross immunity doesn't develop (Valkiunas, 2005). However, evidence exists that immunity might extend to different but closely related parasite lineages, leading to lower levels of parasitaemia and significantly reduced clinical signs (Atkinson et al., 2001a; Cellier-Holzem et al., 2010).

1.3.3 Subclinical effects on condition, behaviour and reproduction

In an endemically infected area, the majority of wild birds infected with *Plasmodium* spp. are in the chronic, low parasitaemic phase of infection. Because clinical pathological signs and mortality are generally related to a high intensity of parasitaemia (Palinauskas et al., 2008), signs of infection in the chronic, low parasitaemic phase of infection are often subclinical. However, several studies have indicated that even sub-clinical infections with blood parasites can have a significant influence on wild bird population dynamics. For example, the body condition of female parent blue tits (*Parus caeruleus*) infected with *Plasmodium* spp. improved significantly when the total haemoparasite load was lowered with the anti-haematozoa drug primaquine (Merino et al., 2000). In addition, the proportion of nests with nestling mortality was significantly higher and the fledging success significantly lower when chicks were raised by untreated females with a higher parasitaemia (Merino et al., 2000). These effects can likely be explained by altered within-family differences, such as reduced inequality in hatching probability and fledging mass within broods reared by medicated females (Knowles et al., 2010a). In house martins (*Delichon urbica*) lowering the level of parasitaemia through primaquine treatment

improved the body condition of the infected birds and dramatically increased clutch size, hatching and fledging success (Marzal et al., 2005). These examples indicate that parental care is more efficient when there is no drain or re-allocation of energy towards parasite related pathological processes and immunological responses (Merino et al., 2000). Remarkably, reproductive pairs of the Hawai'i amakihi (*Hemignathus virens*) of which one or both parents were chronically infected with *Plasmodium* spp. had a higher reproductive success than non-infected parent birds (Kilpatrick et al., 2006). It was assumed that these chicks inherited a genetic make-up related to *Plasmodium* spp. resistance from their parents, leading to a better chick survival rate when infectious pressure for avian malaria is present. A negative effect of chronic infection with *Plasmodium* spp. on body condition might only be noticeable during times of high energy demand. A study of American kestrels (*Falco sparverius*), showed that birds positive for avian malaria exhibited a greater reduction in body condition during egg incubation compared to birds that were negative for avian malaria. This difference was not apparent in the pre-incubation period (Dawson and Bortolotti, 2000). In addition, there was a lower return rate of kestrels to the study and breeding site, associated with either an increased dispersal or reduced survival of birds that were positive for avian malaria.

There are indications that birds infected with haematozoa have higher rates of predation (Møller and Nielsen, 2007). A study by Møller (2008) showed that birds with a high number and prevalence of blood parasite species, including *Leucocytozoon, Haemoproteus, Plasmodium, and Trypanosoma*, responded with a shorter flight distance when approached by a predator. This difference in flight distance was still significant after correction for body condition, but it is not completely clear whether the change was solely caused by changes in the birds' risk taking behaviour, or also by a negative effect on fitness by physical factors. Similar avian behaviour was described by Palinauskas et al. (2008), when *Plasmodium* spp. infected siskins showed a reduced response to intrusion and would continue feeding when he entered the experiment room, while non-infected birds would take off to the safety of their roosts.

It is important to note that clinical signs of chronic infection can be different when multiple species of blood parasites are involved. House martins (*Delichon urbica*) with double infections of *Plasmodium* and/or *Haemoproteus* spp. had a lower body mass and laid larger clutches than birds with single or no blood parasite infection (Marzal et al., 2008).

1.3.4 Mortality and post-mortem signs

Mortality rates for avian malaria vary, due to differences in virulence between *Plasmodium* lineages and differences in susceptibility, tolerance and immunological response of different bird species (Valkiunas, 2005). Direct measurement of mortality rates in wild bird populations is extremely difficult and more reliable results regarding susceptibility to *Plasmodium* spp. can be gained by studies on wild birds under controlled conditions that mimic the natural infective situation (Atkinson et al., 1995). Controlled experiments in Hawaiian birds demonstrated that mortality can be as dramatic as 100% in susceptible and immunologically naive bird species, or as low as 0% in resistant and immunologically competent bird species that had an opportunity to co-evolve with their blood parasites (Atkinson et al., 1995; van RiperIII et al., 1986). An association between immune responses leading to survival of malaria and alleles of the major histocompatibility complex has been demonstrated (Jarvi et al., 2013a). In *Plasmodium* spp. infected great reed warblers (*Acrocephalus arundinaceus*) (Westerdahl, 2012) and house sparrows (Loiseau et al., 2011) this innate immunity, reflected in the genetic make-up of birds, decreased the rate of mortality through more efficient immunological responses. An increase in nest mortality due to altered behaviour and lower energy reserves of *Plasmodium* infected parents (Merino et al., 2000), as well as a decrease of mortality in chicks with infected parents, due to a higher inherited genetic related resistance to haematozoa (Kilpatrick et al., 2006) has been reported. Maternal-foetal antibody transfer, as was demonstrated in African black-footed penguin chicks (*Spheniscus-demersus*) in their early phase of life (Graczyk et al., 1994b), could also be a potential explanation for protection and decreased nest mortality when infectious pressure of *Plasmodium* spp. is present.

Post-mortem findings related to avian malaria are mainly associated with anaemia and destruction of cells of mesodermal origin, especially endothelial cells of organ capillaries (Alley et al., 2008; Krause et al., 2007). Gross post mortem findings include emaciation, watery blood, enlargement and discoloration (pale or dark) of spleen, liver and kidneys, hydro-pericardium, petechial haemorrhages, dark coloured, consolidated lungs and increased gall bladder size (Alley et al., 2008; Alley et al., 2010; Atkinson et al., 2000; Atkinson et al., 1995; Schoener et al., 2014; Williams, 2005).

Frequently reported post-mortem findings include hepatomegaly and splenomegaly combined with dark discoloration, as was described for crossbills that were experimentally infected with *P*. *relictum* lineage SGS1 (Palinauskas et al., 2008). In these crossbills the spleen was 11 times greater in infected crossbills compared to control birds. A detailed description of post-mortem findings in a blackbird reported a hydropericardium, friable liver and swollen spleen and kidneys (Schöner et al., 2009). Another frequently reported post-mortem sign in penguins is pulmonary oedema (Fix et al., 1988; Grim et al., 2003; Vanstreels et al., 2014).

Histological findings associated with avian malaria include: interstitial pneumonia; pulmonary oedema; extramedullary erythropoiesis and granulopoiesis in the hepatic parenchyma and in the medulla of the kidney and spleen; acute focal splenic necrosis; Kupffer cell hyperplasia; haemosiderin deposition in the spleen, liver and lungs; and exo-erythrocytic schizonts in endothelial cells of major organs (Alley et al., 2008; Alley et al., 2010; Atkinson et al., 1995; Schoener et al., 2014; Schöner et al., 2009; Williams, 2005).

1.4 Diagnostic techniques used to identify *Plasmodium* spp.

Signs of clinical disease and post-mortem findings associated with avian malaria are non-specific. Although they can be indicative of avian malaria, a positive diagnosis can only be reached through the use of various diagnostic techniques. Each of the following techniques has its own benefits and limitations.

1.4.1 Evaluation of blood smears

Historically, evaluation of blood smears was the only available technique for the ante-mortem diagnosis of avian malaria. The currently widely used Wright and Giemsa stains provide a cheap method with which all morphologically characteristic stages of *Plasmodium* parasites in the erythrocytes can be identified. Thus, this method allows for the identification of *Plasmodium* spp. up to the species level and for the identification of co-infections with multiple *Plasmodium* species (Valkiunas, 2005; Valkiunas et al., 2006). An estimate of parasite load can be made, and is defined as the number of infected blood cells per standard number of erythrocytes. The quality of blood smears and the experience of the observer significantly influence the sensitivity of this method; although evaluation of high quality blood smears by experienced ocular observers has shown comparable detection rates compared to modern molecular techniques (Krone et al., 2008; Okanga et al., 2013; Valkiunas et al., 2008a), a lower sensitivity has also been described repeatedly (Fallon et al., 2003; Jarvi et al., 2002). The prevalence of chronic infections with very low parasitaemia is likely to be underestimated based on blood smear screening only (Jarvi et al., 2002; Richard et al., 2002). This method can be very time consuming, especially if large numbers of blood smears are to be examined.

1.4.2 Nested Polymerase Chain Reaction (PCR)

Nested PCR is generally considered to be a more reliable, sensitive and faster method for large scale screening for *Plasmodium* spp. than blood smear analysis (Richard et al., 2002). However, this method can also underestimate the infection prevalence in birds with chronic and latent infections (Valkiunas et al., 2008a). The currently most widely used nested PCR protocol is one by Hellgren et al. (2004), which amplifies a 479 base pair fragment of the cytochrome b gene. Gene sequence analysis of nested PCR products allows for identification up to the species and species lineage level, and provides valuable information regarding the phylogenetic relationship between *Plasmodium* lineages (Hellgren et al., 2004; Martinsen et al., 2006; Valkiūnas et al., 2009). The use of these DNA sequencing techniques has helped to confirm the initial classification of *Plasmodium* sub-genera based on morphological studies (Martinsen et al., 2008). Molecular studies also

showed that *Plasmodium spp.* of birds and reptiles all fall within a single clade, with evidence for repeated host switching between birds and squamate hosts (Martinsen et al., 2008). Data acquired by nested PCR are less dependent on variables such as experience of the ocular observer and quality of blood smears. Variation in results can still be found due to variation in sample storage conditions, sample collection method and various PCR reagents used (Freed and Cann, 2006). Limitations of the nested PCR include the absence of information regarding morphological features of the parasites and the intensity of parasitaemia (Freed and Cann, 2006). This method also has a highly selective amplification of DNA which prevents the identification of co-infections with multiple lineages, which in some birds was estimated to be as high as 80% (Valkiunas et al., 2006). In addition, *Plasmodium* spp. have shown a large overall sequence variation, which can occasionally cause primers used for PCR to not be effective across all lineages (Richard et al., 2002; Valkiunas et al., 2008a). Besides false negative results in case of extremely low parasitaemias, false positive PCR results may also occur. To prevent the occurrence of false positive outcomes quality controls can be implemented, such as the use of negative controls and testing of multiple aliquots of each collected sample (Freed and Cann, 2006; Valkiunas et al., 2008a). To identify co-infections and minimise false negative PCR results, various authors advise the use of microscopic evaluation of blood smears in conjunction to molecular techniques (Martínez et al., 2009; Valkiunas et al., 2006).

1.4.3 Real-time (quantitative) PCR

Real-time PCR for *Plasmodium* spp. provides a quantitative measure of the intensity of parasitaemia (Friedl and Groscurth, 2012) which has shown to correlate with parasitaemia levels described by microscopic evaluation of blood smears, but is far more sensitive (Bentz et al., 2006). Combining prevalence data for avian malaria infection with quantitative data for parasite load intensity and parasite dynamics can give invaluable information regarding the virulence of *Plasmodium* lineages, and the susceptibility and resistance of various bird species (Westerdahl, 2012). Use of the real-time PCR enables to explore correlations between parasite load and parameters of interest, such as physical condition, reproduction or immune response (Friedl and Groscurth, 2012). The technique is less time consuming and more cost efficient than nested PCR. A protocol described by

Friedl and Groscurth (2012) targets an 85 bp fragment of the plastid-like large subunit ribosomal RNA (LSU-rRNA) gene of *Plasmodium* falciparum; due to a lack of variability in this region the resulting PCR products were not adequate to identify *Plasmodium* species or species lineages by DNA sequencing. However, the use of lineage specific primers that target sequences of just over 100 base pairs in the cytochrome b gene, which are more variable than the sequence described by Hellgren et al. (2004), have also been used successfully for qPCRs (Asghar et al., 2011; Zehtindjiev et al., 2008). In addition, targeting a 188 base pair region of the cytochrome b gene it was possible to differentiate between two *Plasmodium* spp. from divergent cytochrome b clades through variation in their qPCR product melting temperature (DNA dissociation temperature) (Knowles et al., 2011).

1.4.4 Serological assays

Serological responses to *Plasmodium* spp. infections can be identified and quantitated with the use of enzyme-linked immunosorbent assays (ELISA) (Graczyk et al., 1994b; Palmer et al., 2013) or immunoblotting methods (Atkinson et al., 2001a; Jarvi et al., 2002). Through identification of antibodies against *Plasmodium* spp. these methods identify birds which have mounted an immune response after being infected with *Plasmodium* spp., even when circulating parasites are absent or at such a low level that they are hard to detect by microscopy or PCR (Jarvi et al., 2002), as can be the case in chronic or latent infections. Anti-*Plasmodium* antibodies have been demonstrated from as early as 8 days post-infection and were clearly detectable 40 days post-infection; they were still present 3.5 years after infection in Hawai'i 'amakihi (Atkinson et al., 2001a).

While microscopy and PCR techniques demonstrate parasitaemia at one particular moment in time, serological test results reflect the acquired long lasting immunity of a bird that has been infected at some stage of its life (Atkinson et al., 2001a). Serological techniques are highly sensitive and give a more accurate measure of exposure of a bird population to *Plasmodium* spp. than molecular techniques or blood smear evaluation (Palmer et al., 2013). The ELISA is genus specific, which means that it can differentiate between antibodies against *Plasmodium* and *Haemoproteus* spp., but that it may be unable to distinguish between different *Plasmodium* species (Graczyk et al., 1994c).

1.4.5 Next generation sequencing

In contrast to older sequencing techniques, which mostly extend a single DNA fragment, next generation sequencing identifies bases of small DNA fragments in a massive parallel fashion across millions of reactions. This advance enables rapid sequencing of large stretches of DNA base pairs spanning entire genomes, with the latest instruments capable of producing hundreds of gigabases of data in a single sequencing run (Illumina, 2015). A study of avian malaria from Hawai'i 'amakihi and 'apapane (*Himatione sanguinea*) using next generation sequencing revealed that genetic diversity in sections of the mitochondrial cytochrome b gene of *P. relictum* lineage GRW4 is much higher than detected by direct sequencing (Jarvi et al., 2013b). This technique can be used to sequence entire *Plasmodium* spp. genomes or to identify fragments of parasite DNA with polymorphic sites, which would help to increase the understanding of genetic parasite diversity and evolution (Jarvi et al., 2013b). The high amount of host DNA in avian blood samples, due to nucleated red blood cells, has been an obstacle for genomic studies of avian haemosporidians. Palinauskas et al. (2013) described a method in which mature *Plasmodium* spp. gametocytes are induced to exflagellate in vitro without lysis of blood cells, to enable collection of pure parasite DNA material that can be used as a template for genetic analysis including next generation sequencing. However, a limitation in this method is that the separation of haemosporidians microgametes can only be efficiently initiated when the intensity of gametocytes in the blood sample is >2% (Palinauskas et al., 2013). Despite recent advancements, next generation sequencing is still a very costly technique, which prohibits its use in most studies.

1.5 Avian malaria in New Zealand

1.5.1 Detection of Plasmodium spp. infections in birds before 1950

The occurrence of blood stages of *Plasmodium* spp. parasites in peripheral blood smears from New Zealand birds was confirmed almost seven decades ago (Fantham and Porter, 1944a; Laird, 1950). In 1947 and 1948 Laird (1950) microscopically screened a total of 508 blood smears, comprising samples of 27 endemic or native and 6 introduced avian species.

Introduced species, like song thrush (*Turdus philomelos*) (3/8), blackbird (*Turdus merula*) (2/14) and house sparrow (*Passer domesticus*) (2/11), were found to be infected with *P. relictum*. However, no stages of *Plasmodium* spp. were found in the 120 samples of endemic or native bird species included in this study, keeping in mind that for many of these species, the number of tested individuals was extremely low. Laird confirmed the occurrence of avian malaria in introduced avian species, as was previously claimed by Dore (1920a). In addition, Dore described *P. relictum* in introduced skylark (*Alauda arvensis*) (Dore, 1921) and endemic pipit (*Anthus novaeseelandiae*) (Dore, 1920b), but the samples, stored in the Dominion Museum in Wellington, were too badly preserved for Laird to confirm these statements (Laird, 1950). An unidentified *Plasmodium* sp. was also reported in 1 out of 210 screened grey duck (*Anas superciliosa*) (Laird, 1950), and Fantham and Porter (1944) claim to have found *P. relictum* in one yellow eyed penguin (*Megadyptes antipodes*) from Foveaux Straight. The fact that endemic and native bird species appeared to be remarkably free of infection with *Plasmodium* spp. and that no indigenous species of avian haematozoa were found, suggests that the *Plasmodium* spp. described by Laird were likely introduced to New Zealand with their vertebrate hosts. For example, during the 1860s blackbirds, song thrushes (*Turdus philomelos*) and house sparrows were deliberately introduced from Europe, where avian malaria was present at that time (Duncan, 1997). Another possible route of introduction is through migration of infected birds; although data regarding infections in migrating shore birds are lacking, the shining Cuckoo (*Chrysococcyx lucidus*), a summer migrant to New Zealand, is a known host for *P*. *relictum* (Laird, 1960).

1.5.2 Plasmodium lineages detected since 2006

After more than 50 years without avian malaria studies in New Zealand, Tompkins and Gleeson (2006) published the first New Zealand molecular based avian malaria survey. To date, 17 lineages of *Plasmodium* spp., many of which are cosmopolitan, have been recorded in 35 endemic, native and introduced bird species in New Zealand (Schöner et al., 2014). A lack of phylogenetic affinity between these various *Plasmodium* species has been demonstrated, suggesting that multiple independent introduction events into New Zealand are likely (Ewen et al., 2012b). Globally common lineages such as *P*. sp. lineage

LINN1, *P*. (*Novyella*) sp. lineage SYATO5 and *P*. (Haemamoeba) *relictum* lineage SGS1 have a high prevalence in Europe, the origin of most of New Zealand's introduced bird species. However, these lineages have yet to be detected in Australia, from where many of New Zealand's native bird species originate, which strengthens the theory that they were imported with their European hosts (Bensch, 2015; Ewen et al., 2012b). Some *Plasmodium* lineages are more frequently seen in particular bird species in New Zealand, presumably due to a varying susceptibility of avian host species to the different *Plasmodium* lineages. *P.* (Huffia) *elongatum* lineage GRW6, which has been reported in 14 different bird species, is by far the most common and most generalist *Plasmodium* lineage detected in New Zealand; in descending order of frequency of detection and number of confirmed NZ avian host species, this is followed by *P*. sp. lineage LINN1 in five avian species, and *P*. (Haemamoeba) *relictum* lineage SGS1 in four avian species (Table 1.1). Other lineages have a more specific affinity for a particular avian host. Examples of high host-affinity include: the high incidence of *P*. (Haemamoeba) *cathemerium* lineage RBG1 in red billed gulls (*Larus novaehollandae*) (Cloutier et al., 2011); the unclassified *P.* sp. lineage BELL01 in bellbirds (Baillie and Brunton, 2011; Ewen et al., 2012b); P. *relictum* lineage GRW4 in house sparrows (Ewen et al., 2012b; Marzal et al., 2011); *P*. sp. lineage SYAT05 in blackbirds (Ewen et al., 2012b; Ha et al., 2013; Howe et al., 2012); and *P*. sp. lineages Kokako1&2 in North Island kokako (*Callaeas wilsoni*) (Ewen et al., 2012b; Howe et al., 2012) (Table 1.1).

It is plausible that some *Plasmodium* lineages are in fact endemic to New Zealand. Baillie and Brunton (2011) suggested that the modern-day *P.* sp. lineage BELLO1 may have originated in New Zealand earlier than 3 million years ago, independently of the evolutionary history of the bellbird. In addition, the kokako (*Callaeas cinerea*) isolates, *P*. sp. lineages Kokako1&2, (Ewen et al., 2012b; Howe et al., 2012) have to date not been found outside New Zealand.

Table 1.1. Incidence of *Plasmodium* spp. lineages reported in New Zealand bird species

Bird species	*P* .genus *P*. lineage	Huffia	AFTRU/LINN1		Haemamoeba					Novyella	Unclassified			Total
		GRW06	LINN1	AFTRU5	GRW4	SGS1	Kokako1	Kokako2	RBG1	SYAT05	BELL01	BELL02	PADOM02	*n*
Blackbird (I)		$4^{2,3,10}$	$8^{2,3,13b}$	2^{1}						$31^{1,2,3,13b}$				45
House sparrow (I)		$2^{3,7}$			$9^{3,7}$	$6^{3,7}$								17
Myna (I)						3^{11}								3
Song thrush (I)		$6^{3,10}$	$5^{3,13b}$	1^{1}										12
Starling (I)		3^{1}												3
Yellowhammer (I)		$1^{3,10}$				$2^{3,13a}$							$2^{3,12}$	5
Red Billed Gull (N)		1^{8}							18^{8}					19
Silvereye (N)		1^{2}												1
Bellbird (E)		13^{6}	$2^{3,13b}$							5^{6}	$79^{3,6}$	1^{6}		100
Brown Kiwi (E)		$18^{2,4}$												18
Great Spotted Kiwi (E)			1^{2}											1
Kokako (E)							$2^{3,13c}$	1^{2}						3
New Zealand Pigeon (E)										1^{2}				1
NI Robin (E)		1^{3}												1
NI Saddleback (E)		$18^{3,5,9,10}$	1^{5}			1^{5}					$1^{3,6}$			21
SI Robin (E)		1^{10}												1
SI Saddleback (E)		3^{9}												3
Stitchbird (E)					1^{2}									1
Tomtit (E)										$2^{3,13b}$				2
Tui (E)											$2^{3,6}$			2
Whitehead (E)		$4^{3,10}$												4
Total number		76	17	3	10	12	2	1	18	39	82	1	2	263
Number of bird species		**14**	**5**	**2**	**2**	**4**	**2**	**1**	**1**	**3**	**3**	**1**	**2**	

Superscript numbers in this table refer to the following references: 1. (Ha et al., 2013); 2. (Howe et al., 2012); 3. (Ewen et al., 2012b); 4. (Banda et al., 2013); 5. (Castro et al., 2011); 6. (Baillie and Brunton, 2011); 7. (Marzal et al., 2011); 8. (Cloutier et al., 2011); 9. (Alley et al., 2010); 10. (Pérez-Tris et al., 2007); 11. (Beadell et al., 2006); 12. (Bonneaud et al., 2006); 13. MalAvi database (Bensch, 2015) a. Waldenström et al 2002, b. Hellgren et al. unpubl.3, c. Ruth Brown unpubl. In case the MalAvi database was used as a reference and no amount of sequenced bird samples was specified, a value of 1 was used in this table. This was the case for BELLO1[6] in NI saddleback and tui, PADOM02[12] in yellowhammer, LINN1[13b] in bellbird, blackbird and songthrush, SYAT05[13b] in blackbird and Tomtit, GRW06[10] in blackbird, NI saddleback, songthrush, SI robin, whitehead and yellowhammer and Kokako01[13c] in kokako and SGS1[13a] in yellowhammer. Bird species are classified as (I) Introduced (deliberate human introduction), (N) Native (self-established, occur also in other countries) or (E) endemic (evolved and occur only in NZ).

1.5.3 Prevalence of Plasmodium spp. infections in New Zealand birds

Since 2006, nested PCR data regarding the prevalence of avian malaria in New Zealand bird species have become available. High avian malaria prevalence of over 30%, and in some cases up to 100%, are commonly found in introduced species like myna (*Acridotheres tristes*), blackbird, starling and songthrush (Ewen et al., 2012b; Ha et al., 2013; Howe et al., 2012; Ishtiaq et al., 2006; Tompkins and Gleeson, 2006), bird species that are thought to function as a reservoir for *Plasmodium* spp. To date, a lower prevalence is more often detected in endemic and native bird species, and *Plasmodium* spp. infections have not been detected in species such as Grey warbler, Kingfisher and Fantail (Armstrong and Ewen, 2002; Derraik et al., 2008; Ewen et al., 2012b; Howe et al., 2012). Some of the higher prevalence data reported for endemic bird species include 41% in red crowned parakeets (Ortiz-Catedral et al., 2011), 10% in stitchbirds (Ewen et al., 2012a), 13% in bellbirds (Baillie and Brunton, 2011), and 11% and 7% in South and North Island saddlebacks respectively (Castro et al., 2011; Ewen et al., 2012b). In a survey of avian malaria prevalence in 143 yellow eyed penguins from Otago peninsula during the summer of 2001/2002, no *Plasmodium* spp. DNA could be detected by nested PCR (Sturrock and Tompkins, 2007), even though previous serological studies found that the seroprevalence of avian malaria antibodies ranged from 23% to 100% (Graczyk et al., 1995a; Graczyk et al., 1995b). These findings suggest the exposure of a bird population to malarial parasites might be higher than nested PCR results indicate (Sturrock and Tompkins, 2007).

A decrease in the prevalence of *Plasmodium* spp. positive birds from northern to southern regions of New Zealand have been described, a pattern which closely matched the distribution of the mosquito *Culex quinquefasciatus* (Tompkins and Gleeson, 2006). Similarly, in bellbirds (*Anthornis melanura*) a higher prevalence of infection was reported in the northern half of the North Island compared to the southern parts or the South Island (Baillie and Brunton, 2011). This implies that, since potential vertebrate hosts were already abundant around the country, the increasing distribution of vectors, such as the introduced mosquito *Culex quinquefasciatus,* could be a driving factor in avian malaria emergence (Tompkins and Gleeson, 2006). In turn, varying temperatures in different

regions of New Zealand are likely driving the distribution of mosquito vectors (Leisnham et al., 2006; Mogi, 1992).

1.5.4 The threat of avian malaria to endemic and native New Zealand birds

The avian malaria risk posed to immunologically naive birds from isolated island archipelagos has been demonstrated in Hawai'i. After the mosquito *C. quinquefasciatus*, an important vector for *Plasmodium* spp., was first introduced in mosquito free Hawai'i in 1826, it spread rapidly over the islands (Hardy, 1960). During a study in the late 1930s, no avian malaria infections were detected in any native Hawaiian bird species, and *P. relictum* lineage GRW4 was detected in only 2 species of introduced birds (van RiperIII et al., 1986). It is highly likely that this *Plasmodium* lineage did not reach epizootic proportions until after the second or third decade of the 1900s, when many exotic avian host species, especially from Asia, were actively released on the Hawaiian Islands (van RiperIII et al., 1986). Infections with *P. relictum* lineage GRW4 contributed to a serious decline in native avifauna and are currently still one of the major factors affecting avian population patterns on the Hawaiian Islands (van Riper, 1991; van RiperIII et al., 1986). Selective pressure caused by the pathogenicity of *P. relictum* lineage GRW4 resulted in a restriction of native avifauna to high elevation forests, where mosquito-vectors were few or absent, and in an increasing density of introduced avian species with a higher resistance to *P. relictum* lineage GRW4 in the lower forest ranges (van Riper, 1991)

The impact of avian malaria on the New Zealand avifauna is not fully understood. The current loss of avian biodiversity has mainly been attributed to introduced mammal predators and habitat loss (Miskelly et al., 2008). However, even before predators such as weasels and stoats were introduced, bird species disappeared completely from certain areas after the introduction of exotic avian hosts for *Plasmodium* spp.; the occurrence of avian malaria epidemics offers a plausible explanation for these events, although this theory cannot be confirmed (Laird, 1950). The introduction of European passerines into New Zealand has been accurately documented and of 42 species, which were introduced from Britain between 1860 and 1885, 15 species successfully established in New Zealand (Duncan, 1997). During evolution in their native range these introduced bird species were

exposed to globally widespread *Plasmodium* lineages, which in turn managed to establish themselves in New Zealand (Ewen et al., 2012b). Between 1870 and 1877 Mynas were introduced from Asia, offering the possibility for introduction of *Plasmodium* spp. from another part of the northern hemisphere. Introduced bird species also include pet and exhibition birds like budgerigars and larger psittacine species, which have been imported from Australia to New Zealand until 1997 for almost 150 years (Christensen and Worthington, 2009). There is a lack of knowledge regarding the current susceptibility, resistance and tolerance of endemic and native New Zealand bird species towards avian malaria. It appears likely that endemic birds, which did not come into contact with the introduced *Plasmodium* lineages until after the human introduction of exotic bird species, are immunologically naive towards these parasites (Ewen et al., 2012b). However, in contrast to the Hawaiian situation, there are also indications that some New Zealand bird species carried endemic *Plasmodium* lineages that were present before human colonisation and exotic host introductions (Baillie and Brunton, 2011; Ewen et al., 2012b), implying that endemic species may have developed some resistance against *Plasmodium* spp. infections during their evolution. Differences in pathologic effects of avian malaria between exotic and native or endemic bird species may exist, based on *Plasmodium* related mortalities in wild New Zealand birds. Exotic bird species often function as asymptomatic carriers of *Plasmodium* spp., and mortality due to avian malaria has only been reported once in a blackbird (Schöner et al., 2009). In threatened endemic species, mortality has been reported in New Zealand dotterel (*Charadrius obscurus*) (Reed, 1997), South Island saddleback (*Philesturnus carunculatus carunculatus*) (Alley et al., 2010), yellowheads (*Mohoua ochrocephala*) (Alley et al., 2008), brown kiwi (*Apteryx mantelli*) (Banda et al., 2013), hihi (*Notiomystis cincta*) and great spotted kiwi (*Apteryx haastii*) (Howe et al., 2012).

1.6. Avian malaria and New Zealand wildlife management

1.6.1 Translocations

Translocations are important for wildlife management of many threatened New Zealand avian species, for both species conservation and regional biodiversity restoration (Armstrong, 2010). According to the IUCN guidelines, monitoring and management of disease should be standard practice when wildlife populations are translocated, to both maximise the health of translocated birds and minimise the risk of introducing new pathogens to a destination area (IUCN/SSC, 2013). Therefore, identification of previously unrecorded pathogens and spatial and temporal tracking of existing pathogens, such as *Plasmodium* spp., is beneficial for New Zealand conservation managers and provides a rational basis for disease risk management during translocations (Parker et al., 2006). The importance of this was highlighted when avian malaria was confirmed as the cause of death in five yellowheads that were translocated from the Blue Mountains in Otago to Orana Wildlife Park in Christchurch (Alley et al., 2008). At that time the prevalence of *Plasmodium* spp. infections in introduced bird species around Orana Wildlife Park, including blackbirds, house sparrows, starlings and song thrushes, was 53% based on PCR analysis; this prevalence was unusually high compared with the average of 9% for the Christchurch region (Tompkins and Gleeson, 2006). Later testing revealed that yellowheads in the Blue Mountain range appeared to be free of *Plasmodium* spp. infections (Derraik et al., 2008), making it likely that the translocated birds had no acquired immunity against avian malaria.

Another interesting case was the concurrent outbreak of avipox and avian malaria due to *P. elongatum* lineage GRW6 in 2002 on Motuara Island. Only 40% of a population of 150 South Island saddlebacks survived (Alley et al., 2010). The population made a full recovery over the following 3 years and was used as a source population for a translocation of 45 birds to neighbouring Long Island. The following year, clinical evidence of avian malaria infection was again seen in the translocated birds on Long Island, resulting in death and very poor body condition within the population. Repeated clinical signs of infection for both diseases were more severe than expected in the chronic phase of infection, and it is

feasible that these saddlebacks were more vulnerable to avian malaria infection because of immunosuppression due to co-infection with avipoxvirus and a low genetic diversity of this population after having passed through severe genetic bottlenecks (Alley et al., 2010; Taylor et al., 2007). These examples show that the exposure history of birds, and knowledge of the existing pathogens at the source and release sites, should be taken into consideration to improve the success of their translocation.

1.6.2 Captive wildlife management

Captive wildlife management in New Zealand includes strategies with direct conservation benefits, such as rearing and release, and strategies with indirect conservation benefits such as wildlife rehabilitation (Department of Conservation, 2003). Rearing and release is an important tool to increase numbers of threatened New Zealand bird species and to raise chicks until they reach an age at which they can evade or defend themselves against predators. Rehabilitation of wildlife has great value in endangered species with low numbers such as South Island Takahe (*Porphyrio hochstetteri*) and Kakapo (*Strigops habroptilus*), in which each rehabilitated individual can result in a significant contribution to the reproductive success of the species. Temporal and spatial analysis of clinical data from treated wildlife cases at rehabilitation centres can indicate trends in diseases or trauma of wildlife species. Risk factors for the spread of avian malaria in New Zealand's captive management facilities include a high density of susceptible juveniles (Banda et al., 2013), or the frequent integration of these facilities in zoos or wildlife parks, where vertebrate hosts in the form of introduced passerines and mosquito vectors for *Plasmodium* spp. aggregate in abundance (Tuten et al., 2012), resulting in an environment with increased possibilities for transmission of *Plasmodium* spp. (Derraik, 2004; Valkiunas, 2005). Although captive hatching and rearing is generally done indoors, access of mosquitoes to the facility can result in infection of the young birds. An example of this was seen at Operation Nest Egg site in Rotorua, where an outbreak of avian malaria caused by *P*. *elongatum* lineage GRW06 in captive brown kiwi resulted in the death of one juvenile brown kiwi and an overall prevalence of infection of 78% (Banda et al., 2013). These findings caused a serious disruption to the conservation management programme while an assessment of risk for the continued movement of kiwi juveniles was carried out

(Banda et al., 2013). In addition, avian malaria and avipoxvirus co-infection in New Zealand dotterel chicks caused mortality in at least one, and contributed to the death of 10 chicks in 1996 in captive rearing facilities at Auckland Zoological Park and Otorohanga Kiwi house (Reed, 1997). The increased chance of avian malaria transmission in wildlife rehabilitation settings is best illustrated in penguins, which appear to be highly susceptible to avian malaria in captivity (Graczyk et al., 1994a; Grim et al., 2003; Huijben et al., 2007). For example, although avian malaria in wild Magellanic penguins (*Spheniscus magellanicus*) is extremely rare, an outbreak occurred in this species in a rehabilitation centre in Brazil (Vanstreels et al., 2014). In a South African rehabilitation centre, five black-footed penguins (*Spheniscus demersus*) died of avian malaria (Grim et al., 2003). These examples indicate that in captive management institutions extra precautions should be taken to minimise the risk of exposure to *Plasmodium* spp. parasites.

1.7 Research hypothesis and aims

This study was initiated when an evaluation of the Waimarino Forest area as a source site for translocation of North Island robins (*Petroica longipes*) was requested by the New Zealand Department of Conservation and the Greater Wellington Regional Council, after one North Island robin from this area was found positive for the potentially high-pathogenic *P. relictum* lineage GRW4 in 2011. To evaluate this area as a source site for NI robin translocations, blood samples of multiple bird species were collected in this area for avian malaria screening; this offered opportunities to analyse different aspects of *Plasmodium* spp. infections in New Zealand bird species.

The overall hypothesis of this research is that endemic and native bird species have a lower resistance and tolerance towards infection with *Plasmodium* spp. than introduced bird species, resulting in an increased health risk of *Plasmodium* spp. infections to these endemic and native avian species. The specific aims of this research were to: 1. study the presence and prevalence of various lineages of *Plasmodium* spp. in North Island robins and other bird species in the Waimarino Forest area, in order to evaluate the suitability of the Waimarino forest as a source site for robin translocations (Chapter 2); 2. develop a

real-time PCR protocol for *Plasmodium* spp. in order to quantify parasite load in individual birds (Chapter 3); 3. study a clinical case of avian malaria in a wildlife rehabilitation setting (Chapter 4); and 4. review all main research findings in a wider New Zealand context (Chapter 5).

CHAPTER 2

AVIAN MALARIA IN INTRODUCED, NATIVE AND ENDEMIC NEW ZEALAND BIRD SPECIES IN A MIXED ECOSYSTEM

CHAPTER 2: AVIAN MALARIA IN INTRODUCED, NATIVE AND ENDEMIC NEW ZEALAND BIRDS SPECIES IN A MIXED ECOSYSTEM

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2.1 Abstract

Avian malaria, caused by *Plasmodium* spp., has been reported as a cause of morbidity and mortality in New Zealand bird populations. The prevalence of *Plasmodium* lineages in the Waimarino Forest was evaluated in North Island robins (*Petroica longipes*), other passerines, blue ducks (*Hymenolaimus malacorhynchos*), and brown kiwi (*Apteryx mantelli*), using nested PCR. The presence of *P.* sp. lineage LINN1, *P.* (Huffia) e*longatum* lineage GRW06 and *P.* (Novyella) sp. lineage SYAT05 was demonstrated; *Plasmodium* (Haemamoeba) *relictum* lineage GRW4 was not found. The highest prevalence of infection was found in introduced European species (80.5%), followed by native (19%) and endemic species (3.5%), with a significant difference between these groups. All detected *Plasmodium* lineages have previously been identified in New Zealand and introduced species have been suggested as an important reservoir of infection. The results of this study will aid New Zealand conservation managers with disease risk management during bird translocations from the Waimarino forest.

2.2 Introduction

New Zealand's ecosystems are considered to be among the most extinction-prone in the world (Brooks et al., 2002; Myers et al., 2000). Due to their high proportion of bird-pollinated plants, New Zealand's terrestrial ecosystems are considered especially sensitive to losses in native bird biodiversity (Sekercioglu et al., 2004). Many New Zealand bird

populations are critically endangered (Hitchmough et al., 2007) and efficient wildlife management strategies have been employed (Craig et al., 2000) to safeguard the long-term viability of these bird species and sustain them as essential functional components of the terrestrial ecosystems. Ongoing predation and habitat changes since European settlement in New Zealand have contributed to population declines in many bird species (Bogich et al., 2012; O'Donnell, 1996) even in those still not considered at risk. For example, North Island robins (*Petroica longipes*) (henceforth NI robins) are currently considered to be 'not threatened' (Robertson et al. 2013), despite the IUCN reporting a decreasing population trend for this species (IUCN 2014). Moreover, the distribution pattern of NI robins has changed from being once widespread throughout the mainland of the North Island to a patchy distribution restricted to a band across the central North Island and colonies on Little Barrier and Kapiti Island (Heather and Robertson, 2005). Translocation in the form of assisted colonisation of a new area with suitable habitat is a successful management tool to secure NI robin numbers (Taylor et al., 2005). According to the IUCN guidelines, monitoring and management of disease should be standard practice when populations are translocated to both maximise the health of translocated birds and minimise the risk of introducing new pathogens to a destination area (IUCN/SSC, 2013). Therefore, identification of previously unrecorded pathogens and spatial and temporal tracking of existing pathogens is beneficial for New Zealand conservation managers and provides a rational basis for disease risk management during translocations (Parker et al., 2006).

Avian malaria, caused by various *Plasmodium* species, is an emerging disease in New Zealand (Schoener et al., 2014). A high mortality outbreak occurred after translocation of South Island saddlebacks (*Philesturnus carunculatus*) due to concurrent *Plasmodium* spp. and avipox virus infection (Alley et al., 2010). Furthermore, infection with *Plasmodium* spp. was the confirmed cause of death in five out of eight yellowheads (*Mohoua ochrocephala*) that died after being moved from an area with low *Plasmodium* spp. prevalence to a location with very high prevalence of both the parasite and the appropriate vector (Alley et al., 2008). Other wild bird species in which avian malaria-related mortalities have been documented are brown kiwi (*Apteryx mantelli*) (Banda et al.,

2013), great spotted kiwi (*Apteryx haastii*) (Howe et al., 2012), stitchbird (*Notiomystis cincta*) (Howe et al., 2012), and New Zealand dotterel (*Charadrius obscurus*) (Reed, 1997). Currently, 17 lineages of *Plasmodium* spp. have been reported in 35 New Zealand wild bird species, including introduced, native, and endemic species (Schoener et al., 2014). The most commonly detected lineages are *P.* (Huffia) e*longatum* lineage GRW06 and *P.* (Novyella) sp. lineage SYAT05, followed by *P.* (Haemamoeba) *relictum* (lineages GRW4 and SGS1) and *P.* sp. lineage LINN1, while *P.* (Novyella) lineage AFTRU08 and *P.* (Haemamoeba) *relictum* lineage LINOLI01 are rare (Schoener et al., 2014). All of these lineages are considered to be non-endemic to New Zealand and were likely introduced into this country with the importation of their avian hosts (Ewen et al., 2012b).

Pre-translocation health screening of 20 NI robins from the Waimarino Forest was undertaken by the Greater Wellington Regional Council in 2011. Health screens comprised physical checks for external lesions of avipoxvirus infection, cloacal swabs for bacterial culture of *Salmonella* and *Yersinia*, and blood sample collection for *Plasmodium* spp. PCR. The only detected pathogen of concern for translocation was *P. relictum* lineage GRW4, which was detected in one NI robin, while two NI robins were infected with *P.* e*longatum* lineage GRW06 (Nikki McArthur, Greater Wellington Regional Council, unpublished data). *Plasmodium* e*longatum* lineage GRW06 is widespread in New Zealand and has been found in a large variety of bird species (Alley et al., 2010; Baillie and Brunton, 2011; Banda et al., 2013; Castro et al., 2011; Ewen et al., 2012b; Howe et al., 2012; Marzal et al., 2011). Although sporadic deaths due to this lineage have been reported in wild birds in New Zealand (Banda et al., 2013; Howe et al., 2012), its pathogenicity in wild birds worldwide is generally considered to be low (Valkiunas, 2005). *Plasmodium relictum* lineage GRW4 is considered to be much more pathogenic. This lineage played an important role in the extinction of many endemic bird species in Hawai'i. The establishment of the *P. relictum* lineage GRW4 on the Hawaiian Islands, following the introduction of its mosquito-vector *Culex quinquefasciatus* and exotic avian hosts, caused a rapid spread of this lineage amongst the endemic avifauna with catastrophic results (van RiperIII et al., 1986). In Hawaiian bird species that survived, such as the apapane (*Himatione sanguinea*), high mortality is still a problem (Atkinson and Samuel, 2010). In

New Zealand, this lineage has been identified in wild birds in the northern part of the North Island, including one red-fronted parakeet (*Cyanoramphus novaezelandiae*) from Little Barrier Island (Ortiz-Catedral et al., 2011) and 17 house sparrows (*Passer domesticus*) from Drury, Tiritiri Matangi and Little Barrier Island (Ewen et al., 2012b; Marzal et al., 2011). The geographical spread of *P. relictum* lineage GRW4 and its impact on New Zealand's endemic and native bird species are currently not well understood. Therefore, the translocation of NI robins from the Waimarino Forest was halted until the risk of spreading Plasmodium lineages to new areas could be further evaluated.

The aim of this study was to evaluate the presence and prevalence of Plasmodium lineages in NI robins and other bird species in the Waimarino Forest area, as part of the assessment of the area's suitability as a mainland donor site for NI robin translocations. In addition, routine conservation management activity allowed for opportunistic surveying for Plasmodium spp. in brown kiwi and blue duck (Hymenolaimus malacorhynchos) living in the area

2.3 Materials and methods

2.3.1 Study site

Our study area, the Waimarino Forest, is located in the central North Island of New Zealand (39°26'17.66" S, 175°8'24.76" E). It is a privately owned area of 6937 ha, of which 3884 ha are planted in exotic pine (*Pinus radiata*) for logging. The remaining land is made up of indigenous forest, streams, riparian margins, landslip scars and farmland. It is bordered on the south side by the road from Raetihi to Pipiriki, on the north side by the Manganui o te Ao River, a conservation site for the nationally endangered blue duck, and on the west side by the Whanganui River. Its west side also borders the Whanganui National Park (39°34'59.88" S, 175°4'59.88" E), one of the largest remaining tracts of lowland forest with streams and rivers in the North Island (Figure 2.1). As a consequence, *Plasmodium* lineages found in the Waimarino Forest will also have an impact on birds in the Whanganui National Park. The mixed landscape within the Waimarino Forest results in

adjoining areas with various densities of endemic, native and introduced European bird species within the study site. Among the many endemic species, established breeding populations of NI robins and brown kiwi are present, which have both been used as source populations for translocations. In 1999, 40 NI robins were moved to Paengaroa Mainland Island and in 2001, 28 NI robins were moved to Bushy Park Reserve (Armstrong, 2010b). Although precise NI robin counts are lacking, the number in the area is estimated at ca. 1000 individuals, based on a 3-day survey by staff from the Greater Wellington Regional Council in 2010. The population is well established and expected to replenish its numbers easily after removal of up to 60 birds for translocation.

For the purpose of this study, the Waimarino Forest, bordering areas of the Whanganui National Park, and farmland surrounding Pipiriki were treated as one epidemiological unit. Therefore, we assumed that birds within this area were exposed to similar lineages of *Plasmodium* spp.

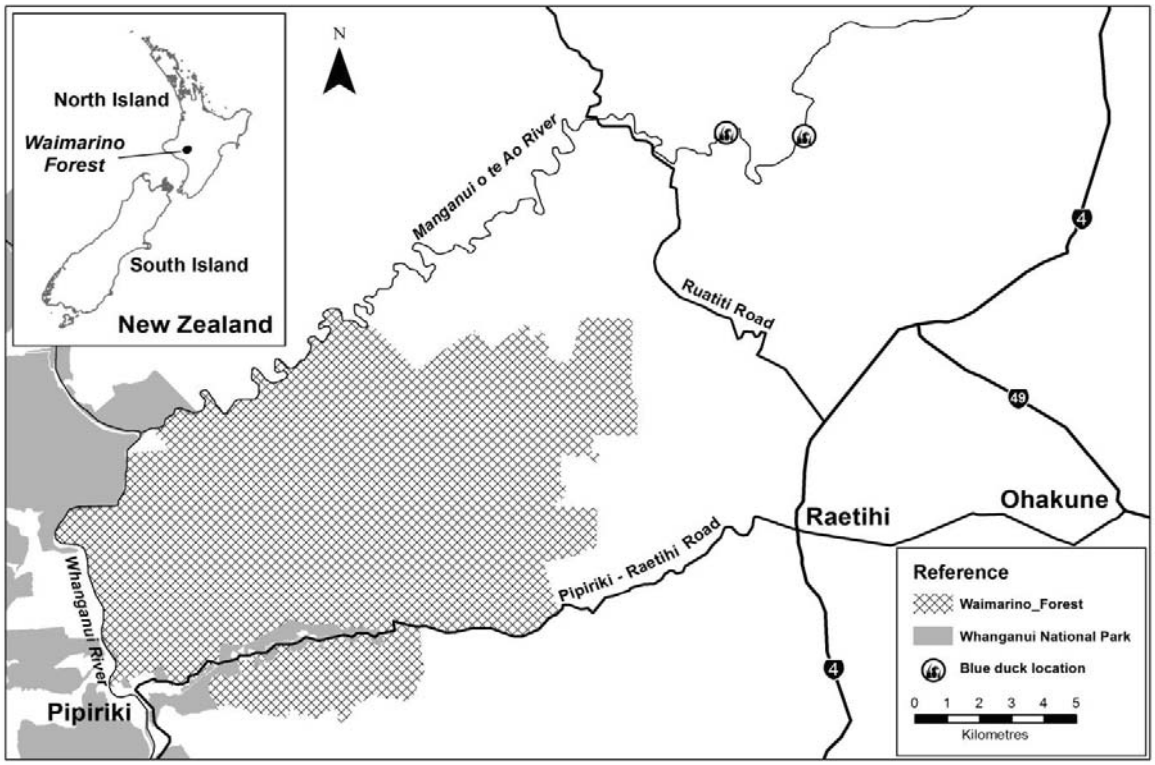


Figure 2.1 Location of the Waimarino Forest within New Zealand and its relation to surrounding geographical features

2.3.2 Blood sample collection and individual bird processing

One hundred NI robins were caught using clap-traps. Standard mist netting techniques were used to catch 88 passerines of various species (excluding robins) and 14 blue ducks. During spring sessions in the first week of October 2012, NI robins and other passerines were caught in the western half of the forest. During summer sessions in the last week of February 2013, NI robins were caught in the eastern and northern parts of the forest, while other passerines were caught close to the south-west border of the forest around the village of Pipiriki and neighbouring farmland. Logging activities inside the Waimarino Forest determined which areas were available to the fieldwork team. The area near Pipiriki was chosen for mist netting during the second fieldwork week because of the observed higher density of exotic passerine birds in this area.

All passerines were processed at their capture site and identified with uniquely numbered metal leg bands. We collected blood samples, ranging in volume from 0.05 to 0.25 ml and equalling less than 1% of each bird's body mass, using heparinised capillary tubes after puncturing the brachial vein with a sterile 27 gauge needle. Body mass (to nearest 0.5 g), tarsometatarsal length (to nearest 0.1 mm) and wing chord (to nearest 0.5 mm) were measured, and a physical health check was performed, before the birds were released. When possible, birds were classified as juvenile or adult, and as male or female according to their plumage characteristics. Individuals recaptured in mist nets were released immediately. Blue duck blood samples of up to 1 ml per bird were collected opportunistically during general health screens with the New Zealand Department of Conservation (DOC) in 2013 at the Manganui o te Ao River during mid-February (39°18'42.900" S, 175°16'48.301" E) and mid-May (39°18'49.814" S, 175°15'12.306" E) (Figure 2.1). Blood samples were collected using a sterile 27-gauge needle and 1-ml syringe and transferred to BD Microtainer® tubes with Lithium Heparin additive.

Packed cell volume (PCV) was determined for each blood sample on the day of blood collection, using a portable ZIPocrit haematocrit centrifuge (LW Scientific, Georgia, Australia) at 10 000 rpm for 5 min, after which the samples were stored in BD Microtainer® tubes with Lithium Heparin additive at –20°C until further processing.

To calculate a quantitative body condition index (BCI) for individual blackbirds (*Turdus merula*), silvereyes (*Zosterops lateralis*) and NI robins, the following formula was used:

$$\mathrm{BCI} = \frac{\text{body weight (g)}}{\text{tarsometatarsal length (mm)}}$$

As part of an ongoing brown kiwi translocation project between the Waimarino Forest and Maungatautari Ecological Reserve (38°01'00" S, 175°34'00" E), 20 blood samples for pre-translocation health screens were collected from brown kiwi in the Waimarino Forest during 2012, 2013 and 2014. Results for *Plasmodium* spp. PCR were made available to us by Maungatautari Ecological Island Trust (MEIT).

2.3.3 Molecular biology

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia CA, USA), following the manufacturer's instructions for nucleated whole blood. Extracted DNA samples were stored at –20°C until used for molecular analysis. The presence of the cytochrome b gene of *Plasmodium* spp. was identified using a nested PCR using the primer sets HaemNF1/HaemNR3 and HaemF/HaemR2 as described by Hellgren et al. (2004). A known *Plasmodium* positive blood sample, confirmed through sequencing as *P.* sp. lineage LINN1, was used as a positive control, while nuclease-free water was included as a negative control. To confirm successful amplification, the final PCR products were run on a 1.5% agarose gel (Invitrogen, Carlsbad, CA, USA) containing ethidium bromide for 1 hour at 100 V.

Positive amplicons were purified using a PureLink PCR purification kit (Invitrogen, Auckland, New Zealand) and subjected to automatic dye-terminator cycle sequencing with the BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) to confirm the genomic sequences, using both the forward and reverse primers. Chromatograms were aligned using Geneious™ (Biomatters, Auckland, New Zealand) and examined for conspicuous overlapping peaks suggestive of *Plasmodium* spp. co-infection. The *Plasmodium* isolate sequences obtained were compared to other published sequences

available from GenBank (Benson et al., 2014) using NCBI Blast and from the MalAvi database (Bensch et al., 2009).

2.3.4 Statistics

The apparent prevalence for *Plasmodium* spp. and the 95% confidence interval was determined for various groups and species of birds using the Epitools software, following the Wilson binomial approximation (Brown et al., 2001). Chi-square tests (Preacher, 2001) were used to analyse whether prevalence of *Plasmodium* spp. differed significantly between various groups and species of birds. If the frequency in one or more bird categories was below five, a Fisher's exact test was used (Preacher and Briggs, 2001). Because data for BCI and PCV in the various bird species were not normally distributed, a non-parametric analysis (Mann-Whitney U tests, SPSS, version 22, IBM Statistics) was used to determine whether there was a significant difference in these parameters between *Plasmodium* spp. negative and positive birds.

2.4 Results

Blood samples were collected from 15 species in three different avian orders, of which 5 species are classified as introduced, 2 as native, and 8 as endemic to New Zealand (Table 2.1). All birds showed bright and alert behaviour and were in good physical condition. Overall, 45/222 (20.3%) birds tested positive for the presence of *Plasmodium* DNA using nested PCR. The *Plasmodium* spp. prevalence for introduced bird species was 80.5% (33/41; 95% CI 0.66–0.90), for native species 19% (7/37; 95% CI 0.10–0.34) and for endemic New Zealand bird species 3.5% (5/144; 95% CI 0.02–0.08). The difference in prevalence of avian malaria infection between these three groups was significant (χ^2 = 60.04, d.f. = 2, $P < 0.001$).

DNA sequencing was successful for 30 out of 44 *Plasmodium*-positive passerine blood samples. For the remaining 15 samples sequencing failed due to the PCR product being too weak to sequence, or due to the presence of overlapping peaks throughout the sequence. In all 30 samples, nucleotide sequences of amplified DNA showed >99%

similarity with known sequences from GenBank as determined by NCBI BLAST. *Plasmodium* sp. lineage LINN1 (GenBank GQ471953) was found in blackbirds (12/21), silvereyes (2/2), song thrushes (*Turdus philomelos)* (1/2), NI robins (1/4), and brown kiwi (1/20). *Plasmodium* e*longatum* lineage GRW06 (GenBank DQ368381) was found in blackbirds (5/21), song thrushes (1/2), and NI robins (3/4), while *P.* sp. lineage SYAT05 (GenBank DQ847271) was found only in blackbirds (4/21) (Table 2.1). *Plasmodium relictum* lineage GRW4 (GenBank AY099041) was not found in any of the sequenced PCR products and thus has a maximum true prevalence range within the research area of 0–0.04 in NI robins, and of 0–0.02 in all sampled avian species combined, at the 95% confidence level. For the three detected lineages, the overall true prevalence at the 95% confidence level was 0.05–0.13 for *P.* sp. lineage LINN1, 0.02–0.05 for *P.* e*longatum* lineage GRW06 and 0.01–0.05 for *P.* sp. lineage SYAT05. The difference in prevalence between these lineages was significant ($\chi^2 = 9.04$, d.f. = 2, $P = 0.01$).

Clinical data were further evaluated for bird species for which *Plasmodium* spp. positive as well as negative samples were found, comprising blackbirds, silvereyes and NI robins. No significant differences in values for PCV and BCI were found between *Plasmodium* spp. negative and positive birds (Table 2.2), although in silvereyes a trend towards a better BCI in *Plasmodium* spp. positive birds was seen ($n = 33$, U = 120.000, $P = 0.072$).

For the three bird species for which results were further analysed, accurate sex determination by visual inspection of plumage could only be performed for blackbirds, while accurate classification as juvenile or adult was possible for blackbirds and silvereyes. No significant difference in avian malaria prevalence was detected between male and female blackbirds (Fisher's exact, $P = 0.51$), or between juvenile and adult blackbirds (Fisher's exact, $P = 0.30$) or silvereyes (Fisher's exact, $P = 1.00$).

Table 2.1 The prevalence of avian malaria in birds in the Waimarino Forest area and the *Plasmodium* lineages identified

Order	Family	Species*	Pos/*n*	Prev	95% CI	Plasmodium Lineages†
Passeriformes	Turdidae	Song Thrush *Turdus philomelos* (*I*)	4/4	1.00	0.51–1.00	LINN1(1), Elongatum(1)
	Prunellidae	Dunnock *Prunella modularis* (*I*)	1/1	1.00	0.21–1.00	
	Turdidae	Blackbird *Turdus merula* (*I*)	28/34	0.82	0.67–0.92	LINN1(12), Elongatum(5), SYAT05(4)
	Fringillidae	Chaffinch *Fringilla coeleps* (*I*)	0/1	0.00	0.00–0.79	
	Fringillidae	Goldfinch *Carduelis carduelis* (*I*)	0/1	0.00	0.00–0.79	
	Zosteropidae	Silvereye *Zosterops lateralis* (*N*)	7/33	0.21	0.11–0.38	LINN1(2)
	Rhipiduridae	Fantail *Rhipidura fuliginosa* (*N*)	0/4	0.00	0.00–0.49	
	Petroicidae	New Zealand Robin *Petroica longipes* (*E*)	4/100	0.04	0.02–0.10	LINN1(1), Elongatum (3)
	Petroicidae	Tomtit *Petroica macrocephala toitoi* (*E*)	0/3	0.00	0.00–0.56	
	Mohouidae	Whitehead *Mohoua albicilla* (*E*)	0/3	0.00	0.00–0.56	
	Acanthizidae	Grey Warbler *Gerygone igata* (*E*)	0/2	0.00	0.00–0.66	
	Meliphagidae	Bellbird *Anthornis melanura* (*E*)	0/1	0.00	0.00–0.79	
	Meliphagidae	Tui *Prosthemadera novaeseelandiae* (*E*)	0/1	0.00	0.00–0.79	
		Total for all Passeriformes combined	44/18	0.23	0.18–0.30	LINN1(16), Elongatum(9), SYAT05(4)
Anseriformes	Anatidae	Blue Duck *Hymenolaimus malacorhynchos* (*E*)	0/14	0.00	0.00–0.22	
Apterygiformes	Apterygidae	Brown kiwi *Apteryx mantelli* (*E*)	1/20	0.05	0.01–0.24	LINN1(1)

* whether a species is introduced (*I*), native (*N*), or endemic (*E*) to New Zealand is shown in parenthesis behind the scientific name
Pos/*n* stands for the number of *Plasmodium* positive birds per total number of tested birds of this species
Prev. stand for prevalence of infection
† the number of times that *Plasmodium* lineages were demonstrated for an avian species is shown in parentheses

Table 2.2. Packed cell volume (PCV) and body condition index (BCI) with standard errors (SE) for species with Plasmodium negative (neg) and positive (pos) birds

Species*	PCV% (SE)†		BCI‡ (SE)†	
	neg birds	pos birds	Neg birds	Pos birds
Blackbird *Turdus merula* (*I*)	44.3 (2.3)	43.9 (0.9)	2.72 (0.06)	2.65 (0.06)
Silvereye *Zosterops lateralis* (*N*)	46.0 (0.7)	44.8 (1.8)	0.63 (0.02)	0.73 (0.05)
New Zealand Robin *Petroica longipes* (*E*)	46.4 (1.6)	51.7 (2.3)	0.83 (0.01)	0.82 (0.01)

* whether an avian species is introduced (*I*), native (*N*), or endemic (*E*) to New Zealand is shown in parenthesis behind the scientific name

† standard error (SE) is shown in parenthesis behind the PCV and BCI values

‡ BCI is defined as bodyweight in grams divided by tarsus length in millimetres

2.5 Discussion

The aim of this study was to evaluate the presence and prevalence of avian malaria lineages in avian species within the Waimarino Forest area to aid assessment of the suitability of this location as a source site for NI robin translocations. We identified three of the 17 *Plasmodium* lineages currently known in New Zealand, *P.* sp. lineage LINN1, *P.* e*longatum* lineage GRW06, and *P.* sp. lineage SYAT05. The establishment of avian *Plasmodium* lineages in an ecosystem and their consequent prevalence of infection in various bird species depend on multiple factors. Susceptibility and tolerance to infection of the avian hosts, presence and competence (transmission efficiency) of arthropod vectors, virulence of the *Plasmodium* spp., spatial and temporal distribution of host and vector, as well as climate, each play an important role (Benning et al., 2002; Bensch and Åkesson, 2003; Samuel et al., 2011; Westerdahl, 2012). The severity of pathologic effects due to avian malaria infections differs between *Plasmodium* species (Lachish et al., 2011), but also between avian species (Palinauskas et al., 2008). Morbidity and mortality with malarial infections can be severe, especially in immunologically naïve and susceptible hosts, or when co-infections with other infectious agents, such as avipoxvirus or even intestinal parasites, are present (Alley et al., 2008; Alley et al., 2010; Atkinson and Samuel, 2010; Graham et al., 2005). Decreased activity and food consumption, pale mucous membranes, respiratory signs, vomiting and behavioural changes can be seen, signs which are caused by destruction of erythrocytes, inflammatory reactions predominantly in

spleen, liver and lungs, and infiltration of parasites into the brain (Dunn et al., 2011; Valkiunas, 2005; Yorinks and Atkinson, 2000).

For the *Plasmodium* lineages detected in the Waimarino Forest, information regarding their virulence and pathogenicity in New Zealand's wild bird species is limited. All three lineages detected in this study are common in exotic passerines, in which they are expected to have a relatively low pathogenicity, based on the high prevalence of chronic infections in apparently healthy birds (Tompkins and Gleeson, 2006). The lower prevalence in native and endemic bird species, combined with reported mortality cases, strengthens the belief that the impact on these New Zealand species is different (Ewen et al., 2012b; Howe et al., 2012; Tompkins and Gleeson, 2006). One explanation may be that many endemic and native species, which did not evolve with the introduced *Plasmodium* spp., have less immune-competence to infection with these parasites, resulting in a lower percentage of infected birds surviving until the chronic phase of infection.

Plasmodium sp. lineage LINN1 (GenBank GQ471953) was detected most frequently in the widest range of bird species. To our knowledge, this is the first study to show the presence of *P.* sp. lineage LINN1 infection in native silvereyes and endemic NI robins. *Plasmodium* sp. lineage LINN1 is a cosmopolitan generalist parasite and has been isolated from a wide range of avian species and mosquitoes throughout Europe, Asia and America (Bentz et al., 2006; Cosgrove et al., 2008; Ferraguti et al., 2013; Hellgren et al., 2011; Kimura et al., 2010; Szoellosi et al., 2011; Wood et al., 2007). *Plasmodium* sp. lineage LINN1 forms a cluster with the 99% genetically similar lineages *P.* sp. lineages AFTRU5 (GenBank DQ847263) and WA39 (GenBank EU810610), which have also been reported in New Zealand (Howe et al., 2012). Within New Zealand, the lineage LINN1 has been previously reported in blackbirds, great spotted kiwi, song thrush and bellbird (*Anthornis melanura*) (Ewen et al., 2012b; Howe et al., 2012). *Plasmodium* e*longatum* lineage GRW06 was the second most common lineage in our study and appears to be the most prevalent *Plasmodium* lineage in NI robins from the Waimarino area. It is a cosmopolitan parasite with a wide host range, and has been detected in over 60 avian species of at least nine orders, including waterfowl (Anseriformes), raptors (Falconiformes), owls (Strigiformes) and particularly passeriformes, which are considered to act as reservoirs (Valkiunas, 2005;

Valkiunas et al., 2008b). Within New Zealand, *P. elongatum* lineage GRW06 is considered the most common malarial parasite with the widest host range (Schoener et al., 2014). It is present throughout New Zealand (Baillie and Brunton, 2011) and has been described in the North Island saddleback (*Philesturnus rufusater*), South Island saddleback (*P. carunculatus*), silvereye, brown kiwi, blackbird, house sparrow, songthrush, bellbird, yellowhammer (*Emberiza citronella*), whitehead (*Mohoua albicilla*), and NI robin (Alley et al., 2010; Baillie and Brunton, 2011; Banda et al., 2013; Castro et al., 2011; Ewen et al., 2012b; Howe et al., 2012; Marzal et al., 2011). In a captive rearing program of brown kiwi, an outbreak resulted in the death of one kiwi and parasitaemia in 25 out of 32 (78%) of the concurrent captive population (Banda et al., 2013). Infection with this lineage has contributed to a high level of mortality in translocated South Island saddlebacks with concurrent avipox infection (Alley et al., 2010). This indicates that although the pathogenicity of the *P. elongatum* lineage GRW06 in exotic passerines appears to be moderate to low, its impact on endemic species can be severe. *Plasmodium* sp. lineage SYAT05 was found only in blackbirds. This lineage is very closely related to the African lineages W38 (GenBank EU810633, MalAvi AFTRU08) and W37 (GenBank EU810632, MalAvi AFTRU08) (Beadell et al., 2009). Hellgren et al. (2007) have reported the presence of *P.* sp. lineage SYAT05 in multiple passerine species from Africa and Europe, and in 13 species of intercontinental migrants. European blackbirds have a high prevalence of *P.* sp. lineage SYAT05, while lower prevalences were found in other European and American passerine species (Bentz et al., 2006; Dimitrov et al., 2010; Hellgren et al., 2011; Santiago-Alarcon et al., 2011). Within New Zealand, *P.* spp. lineage SYAT05 is a common lineage present in European blackbirds in the North Island; however, data regarding its geological spread in the South Island are lacking (Howe et al., 2012). Additionally, the *P.* sp. lineage SYAT05 has been isolated from kereru (*Hemiphaga novaeseelandiae*), tomtits (*Petroica macrocephala*), and bellbirds (Baillie and Brunton, 2011; Ewen et al., 2012b; Howe et al., 2012). This lineage is considered to have low pathogenicity in exotic passerines, but it is currently unclear what effect *P.* sp. lineage SYAT05 has on the unique range of New Zealand's native and endemic species.

To evaluate the impact of *Plasmodium* infection on the birds' physical parameters, BCIs and PCVs were compared between *Plasmodium* spp. positive and negative blackbirds, silvereyes, and NI robins, but no significant differences were found. Changes in PCV and BCI are more likely seen in the acute, high parasitaemia phase of infection, coinciding with an increased destruction of erythrocytes and reduced food intake (Moller and Nielsen, 2007). The *Plasmodium* positive birds we screened were likely in the chronic phase of infection, as studies suggest that acutely infected birds move around less, are more likely to be caught by predators and less likely to be captured in mist nets (Moller and Nielsen, 2007; Yorinks and Atkinson, 2000). Interestingly, in silvereyes we found a non-significant trend towards higher BCIs for *Plasmodium* infected birds compared with non-infected birds. It is plausible that in silvereyes a higher pre-infection weight to size ratio coincided with a more efficient immune response and a higher survival rate during the acute phase of infection (Atkinson et al., 1995; Moller and Saino, 2004). An initial deterioration in BCI and PCV could have occurred in the acute phase of infection, followed by a return to the pre-infection condition once surviving birds reached a chronic, low parasitaemia phase. Nevertheless, as long as there are no existing data regarding the empirical validation of a relationship between BCI and survival of avian malaria in silvereyes, speculations regarding this association have to be made with extreme caution (Barnett et al., 2015). To confirm this theory, a higher number of birds would need to be tested (Power = 0.637 in our analysis. G3Power 3.1.7), and a quantitative analysis of parasite load would need to be performed. The fact that all birds with positive PCR results for avian malaria were also in good physical condition suggests that they were either in the chronic or latent phase of infection, or that the infection was non-pathogenic.

Plasmodium relictum lineage GRW4, the lineage that raised concerns after it was identified in a NI robin in 2011, was not found during this study. Because the geographical spread of *P. relictum* lineage GRW4 and its impact on New Zealand's endemic and native bird species are currently not well understood, bird translocations from areas where this lineage is confirmed to be established to areas that are potentially free of the linage GRW4 should be avoided. The absence of *Plasmodium relictum* lineage GRW4 in the 100 NI robins and in the 144 birds of other species sampled during this study suggests that this

lineage is either not established within the Waimarino Forest or that its prevalence in NI robins in the area is less than 4%.

In summary, the three *Plasmodium* lineages that we detected during this study are widespread throughout New Zealand, with a high prevalence in introduced bird species (Baillie and Brunton, 2011; Ewen et al., 2012b; Howe et al., 2012), and *P. relictum* lineage GRW4 was not found in any of the tested birds. Based on our data, we contend that there is little chance of introducing *Plasmodium* lineages from the Waimarino Forest to new areas during NI robin translocations. The high incidence of introduced bird species co-inhabiting with native and endemic species appears to create a melting pot for *Plasmodium* spp., an aspect of the Waimarino Forest that may be reflected in other ecosystems across New Zealand. Therefore, further research is needed to monitor the appearance of new pathogens in this area and to clarify the impact of *Plasmodium* spp. on endemic and native NZ bird species.

2.6 Acknowledgements

The handling of live birds during this research was approved by the Massey University Animal Ethics Committee (MUAEC protocol number 11/72) and the New Zealand Department of Conservation (DOC Permit no. 34781-FAU). Approval and support for this research project was given by the local land owners: Atihau-Whanganui Incorporation, Ngaporo Waimarino Forest Trust, Pipiriki Incorporation and Ernslaw One Limited. Various field teams worked under different NZ banding office permits (nos 2007022, 2010011, 2012007, 2012009, 2013003, 2013004, and 2013005). Financial support was received from The New Zealand Department of Conservation (DOC) and the Pacificvet Avian Health Research Fund of the Institute of Veterinary, Animal and Biomedical Sciences (IVABS). Permission to include blue duck data in this study was granted by DOC. Nested PCR results for *Plasmodium* spp. for 20 kiwi samples were made available to us by Maungatautari Ecological Island Trust (MEIT). The final version of Figure 2.1 was created by Peter Lock (DOC, Whanganui District.

CHAPTER 3

USE OF A REAL-TIME PCR TO EXPLORE DISEASE DYNAMICS OF AVIAN MALARIA IN A MIXED NEW ZEALAND ECOSYSTEM

CHAPTER 3: USE OF A REAL-TIME PCR TO EXPLORE DISEASE DYNAMICS OF AVIAN MALARIA IN A MIXED NEW ZEALAND ECOSYSTEM

3.1 Abstract

Avian malaria is an emerging disease in New Zealand. During this study, a new real-time PCR (qPCR) protocol to detect and quantify *Plasmodium* spp. infection in New Zealand birds was developed and compared to the established nested PCR (nPCR) assay. Two hundred and two blood samples from 14 bird species with known nested PCR (nPCR) results were used. The qPCR prevalences for introduced, native and endemic species groups were 70% (0.54-0.82), 11% (0.04-0.25) and 21% (0.15-0.29) respectively, with a qPCR sensitivity and specificity of 96.7% and 98% when compared to nPCR. The qPCR appeared to be more sensitive in detecting low parasitaemias compared to the nPCR. The mean parasite load was significantly higher in introduced bird species (mean = 2,245 parasites per 10,000 erythrocytes, s.e. ± 700) compared to endemic species (mean = 31.5 parasites per 10,000 erythrocytes, s.e. ± 18.4) (t = 2.74, d.f. = 52, P = 0.01). In New Zealand robins a significantly lower PCV was found in *Plasmodium* positive compared to negative birds (n = 98, U = 534.000, P = 0.029). Our data suggest that introduced bird species, such as blackbirds, have a higher tolerance for circulating parasite stages of *Plasmodium* spp. than endemic and native species. These results reinforce results from previous studies that have suggested that introduced species are an important avian malaria reservoir due to a high infection prevalence and parasite load in New Zealand. Use of this qPCR protocol in future studies will improve the understanding of *Plasmodium* parasite dynamics in bird species in New Zealand.

3.2 Introduction

Avian malaria, caused by *Plasmodium* spp., is an emerging disease in New Zealand. To date, 17 distinct genetic lineages have been described in the country and infections have

resulted in morbidity and mortality in several of New Zealand's bird species (Schoener et al., 2013). Various studies have explored the prevalence of infection in New Zealand bird populations, the *Plasmodium* lineages involved, and the geographical spread of the disease (Baillie and Brunton, 2011; Castro et al., 2011; Ewen et al., 2012b; Howe et al., 2012; Marzal et al., 2011). However, no quantitative molecular analysis of parasite load in individual New Zealand birds has been done to study the infection intensity for *Plasmodium* spp. infections.

When birds become infected with *Plasmodium* spp., an acute phase with high levels of parasitaemia is followed by a chronic phase of infection where parasites are either absent or present in low numbers in the peripheral circulation. The parasite load during the acute or chronic phase of infection tends to vary between avian host species and between different *Plasmodium* lineages, consequently leading to a varying severity of pathological signs. High acute phase infection intensities have been associated with high mortality (Asghar et al., 2011; Zehtindjiev et al., 2008), while the low levels of parasitaemia during the chronic phase of infection have been shown to have more subtle effects on individual host fitness, such as influencing reproductive success or life span (Asghar et al., 2011; Asghar et al., 2015). Parasite dynamics and disease expression are influenced by environmental factors, parasite virulence, and host factors such as a bird's susceptibility, and its levels of resistance and tolerance towards *Plasmodium* spp. infection.

Adding results for infection intensity to infection prevalence studies will lead to a deeper understanding of the pathogen dynamics of various *Plasmodium* lineages in different avian species. This is of special interest in New Zealand, where introduced passerines and endemic bird species inhabit the same ecological systems. Introduced European species like blackbirds (*Turdus merula*) and song thrushes (*Turdus philomelos*) have, over a long period of time, co-evolved in their native range with the *Plasmodium* lineages that are now frequently found in New Zealand. Higher survival rates and higher parasite loads in surviving birds can be present in bird species that had time to evolve towards a higher tolerance to *Plasmodium* parasites (Atkinson et al., 2013). However, New Zealand's endemic bird species likely weren't exposed to these parasites prior to the late 19th

century, when European passerines were introduced to New Zealand (Duncan, 1997; Laird, 1950). Thus, New Zealand's endemic bird species and the introduced *Plasmodium* parasites have had a relatively short time to genetically adapt to each other (Ewen et al., 2012b), potentially leading to a lower tolerance and survival following infection (Atkinson et al., 2013). Chronic infection amongst healthy-looking introduced blackbirds, song thrushes and starlings (*Sturnus vulgaris*) is common, with prevalence ranging from 50 to over 90% described in birds from the North Island (Ewen et al., 2012b; Howe et al., 2012; Sijbranda et al., 2016; Tompkins and Gleeson, 2006). In contrast, studies report a low to moderate prevalence of avian malaria in endemic bird species; a few examples include a prevalence of 4% in North Island robins (*Petroica longipes*) (Sijbranda et al., 2016), 6% and 11% in North and South Island Saddlebacks (*Philesturnus carunculatus*) (Armstrong and Ewen, 2002; Castro et al., 2011), 7% in whiteheads (*Mohoua albicilla*) (Ewen et al., 2012b), 13% in bellbirds (*Anthornis melanura*) (Baillie and Brunton, 2011), but also a high 41% in red crowned parakeets (*Cyanoramphus novaezelandiae*) (Ortiz-Catedral et al., 2011). Sporadic mortality in endemic species has been demonstrated in brown kiwi (*Apteryx mantelli*), great spotted kiwi (*Apteryx haastii*), hihi (*Notiomystis cincta*), yellowheads (*Mohoua ochrocephala*), and saddlebacks (Howe et al., 2012), indicating that *Plasmodium* lineages that are assumed to be of relatively low pathogenicity in exotic species, could have serious implications for endemic species.

This study describes the development and validation of a real-time PCR (qPCR) protocol to detect and assess infection intensity with *Plasmodium* spp. in New Zealand birds. The effect of avian host species on the level of parasitaemia was examined, and physiological correlates of infection were evaluated by assessing packed cell volume (PCV) and body condition index (BCI).

3.3 Methods

3.3.1 Samples included in this study

The samples included in this study were collected from fourteen bird species during a previous avian malaria research project in and around the Waimarino forest of New Zealand (39° 26' 17.66" S, 175° 8' 24.76" E), as described in Chapter 2. DNA samples with known nPCR results of 100 New Zealand robins, 88 birds belonging to other passerine species and 14 blue duck, which had been stored at -20^{0}C (Chapter 2) were analysed by qPCR.

3.3.2 Real-Time PCR (qPCR)

To calculate the quantity of *Plasmodium* parasites in blood samples from the real-time PCR (qPCR) assay, standard curves were generated, using samples with known quantities of a fragment of the plastid-like large subunit ribosomal-RNA (LSU-rRNA) gene (Figure 3.1). A 595 bp fragment of the LSU-rRNA gene from a positive blood sample was amplified using the forward and reverse primers L1 and L2, as described by Tan et al. (1997). These fragments were ligated into vectors using the pGEM – T Easy Vector System (Promega, Madison, USA) according to the manufacturer's instructions and *E.coli* JM109 high efficiency competent cells (Promega, Madison, USA) were transformed (Appendix 2). Transformed colonies were selected using blue/white selection and grown overnight at 37^{0}C in 1 ml Luria-Bertani (LB) broth containing 100µg/ml Ampicillin sodium salt (Gibco®, Life technologies, New York, USA). DNA was extracted using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen, Life technologies, Carlsbad USA) according to the manufacturer's instructions. Resulting plasmid DNA was quantified using a Nanodrop 2000 (Thermo-Fisher, Wilmington, USA). To prepare standard samples with known quantities of vector DNA copies, purified vector DNA was diluted in Tris-EDTA (TE) buffer to a concentration of 1ng/µl. Of this 1ng/µl solution, seven ten-fold standard dilutions were made in TE buffer ranging from 1ng/µl (2.61 x 10^8 copies of the target sequence/µl) to 1 x 10^{-7}ng/µl (26 copies of the target sequence/µl). Made up standard dilutions were stored at 4^{0}C and consequently used within a day of preparation.

To detect *Plasmodium* sp. DNA in samples and standards, an 85 bp product of the LSU-rRNA gene was amplified using the primers Plasmo474for and Plasmo558rev as described by Friedl and Groscurth (2011) with the following modifications. Each 20 µl qPCR reaction comprised 10µl of 2x Perfecta SYBR Green Fastmix® (Quanta biosciences, Gaithersburg, USA), 0.25 µM of the primers Plasmo474for and Plasmo558rev and 100 ng of sample DNA or 1µl of a standard DNA template. The qPCR included initial steps of 2 minutes at 50^0C and 10 minutes at 95^0C, followed by 45 thermo-cycling rounds of 95^0C for 15 s and 60^0C for 1 min, followed by a pre-melt conditioning at 95°C for 15 s and a 0.3 degree incremental melt curve over a 55-95°C temperature range with a 5 second hold, and was performed on the Eco™ Real-Time PCR system (Eco™ Software V4.0.07; Illumina Inc., San Diego, CA, USA) (Appendix 2). All seven standard dilutions were run with each round of qPCR and had Cq-values between 7.4 and 35.0 thermo-cycles, using a threshold of 0.020 (Figure 3.1). All standard curves were highly reproducible and had R^2-values of >99%. The concentration of *Plasmodium* DNA in each test sample was calculated from the standard curves. It was assumed that each *Plasmodium* parasite contains one copy of the LSU-rRNA gene, so that the amount of copies of the target sequence in an unknown sample correlates directly with the amount of *Plasmodium* parasites in that sample (Wilson et al., 1996).

Parasite load was defined as the number of *Plasmodium* parasites per 10,000 avian blood cells. For the purpose of calculation, it was assumed that the average genome size of passerines is 2.8pg (Tiersch and Wachtel, 1991), so that each ng of total DNA in unknown samples reflected 357 cells. Thus, the amount of avian blood cells in each reaction was calculated by multiplying the total amount of DNA in ng by 357.

Due to a lack of variability in the qPCR target region, the resulting PCR products from this assay are not adequate to identify different *Plasmodium* species or lineages by DNA sequencing or by dissociation temperature. Extrapolation of previously acquired DNA sequencing results for nPCR products (Chapter 2), which reflect only one *Plasmodium* lineage in case of coinfection with multiple lineages in a single host, to qPCR results, which reflect the total parasite load in a single host, is not appropriate.

3.3.3 Statistics

The apparent *Plasmodium* prevalence and 95% confidence interval for various groups and species of birds was determined using the Epitools software following the Wilson binomial approximation (Brown et al., 2001). Chi-square tests (Preacher, 2001) were used to analyse differences in *Plasmodium* prevalence between various groups and species of birds. In analyses where frequencies were below 5, Fisher's exact tests were used (Preacher and Briggs, 2001).

To generate a quantitative BCI, body weight in grams was divided by tarsus length in mm. The BCI was assessed for blackbirds, silvereyes and New Zealand robins. Because data for BCI and PCV in the various bird species were not normally distributed a non-parametric analysis (Mann-Whitney U tests, SPSS, version 22, IBM Statistics) was used to determine whether there was a significant difference in these parameters between *Plasmodium* spp. negative and positive birds. The correlation between parasite load and the physical parameters PCV and BCI was explored using linear regression (Microsoft Excel 2010).

Analyses of the effect of bird species group on quantitative parasite load were made. The parasite load data were not normally distributed so a log transformation of the data was applied before evaluating differences in parasite load between introduced, native and endemic birds groups using a one-way analysis of variance (one-way ANOVA), followed by *posthoc* Bonferroni comparisons.

3.3.4 Latent class analysis to estimate sensitivity and specificity of qPCR and nPCR

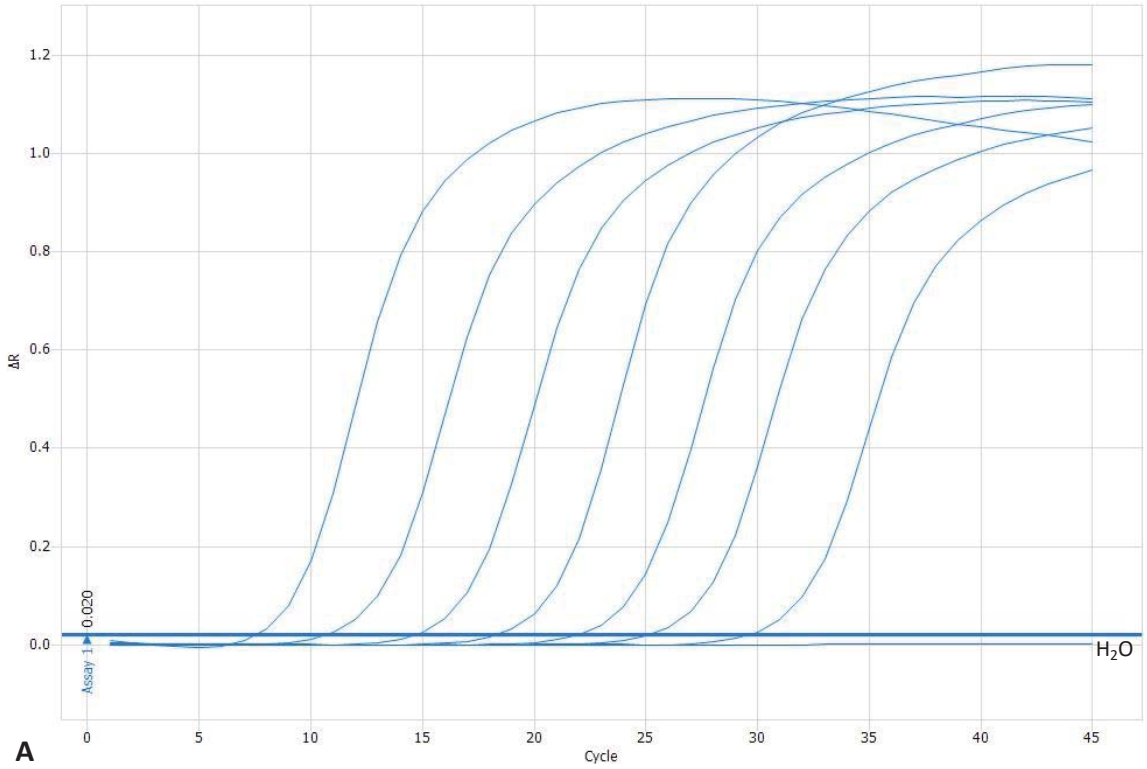
Markov chain Monte Carlo latent class analysis (LCA) was conducted in R software (R Core Team 2013) to generate estimates of the true prevalence and determine the sensitivity and specificity of the nPCR and qPCR for the groups of introduced, native and endemic species, assuming the tests were conditionally independent. The LCA methods were based on those created for analysis of imperfect diagnostic tests (Branscum et al., 2005). This routine allows posterior testing for differences in prevalence estimations between populations. The apparent prevalence (AP) estimates of *Plasmodium* spp. infection in the

3 bird groups (introduced, native and endemic) were used for input into the LCA model. Prior estimates of test sensitivity and specificity and of infection prevalence were derived from expert opinion and were specified using beta distributions calculated in Betabuster software (http://betabuster.software.informer.com/). Stability of each model was tested using uninformative priors (beta[1,1]), and posterior distributions were compared with models containing informative priors. Pairwise tests for differences in prevalence of *Plasmodium* spp. infection among the 3 bird groups were performed using a Bayesian statistical probability for estimating differences in prevalence between populations, where values close to 0 and 1 indicate potential significant differences. Plots of true and AP estimates were created in the ggplot2 package (Wickham 2009).

3.4 Results

3.4.1 Infection prevalence based on qPCR

Blood samples of 14 bird species from 2 orders, including 5 introduced, 2 native and 7 endemic species, were tested by qPCR during this study (Table 3.1). If qPCR results revealed dissociation temperatures within the range of 76.6-78.4°C (to account for *Plasmodium* spp. variation), samples were accepted as positive for the presence of *Plasmodium*-specific DNA (Figure 3.2). The qPCR showed an overall prevalence of *Plasmodium* infection for all tested birds of 29% (58/202); the prevalence for introduced, native and endemic species groups was 70% (0.55-0.82), 11% (0.04-0.25) and 21% (0.11-0.29), respectively (Table 3.1). The difference in prevalence between native and endemic species was not significant, while introduced species showed a significantly higher prevalence compared to native (χ^2=27.7, d.f. = 1, $P < 0.001$) or endemic species (χ^2=33.7, d.f. = 1, $P < 0.001$) (Table 3.1).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1.0
0.8
0.6
0.4
0.2
0.0
ΔR
0.020
Assay 1
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0
5
10
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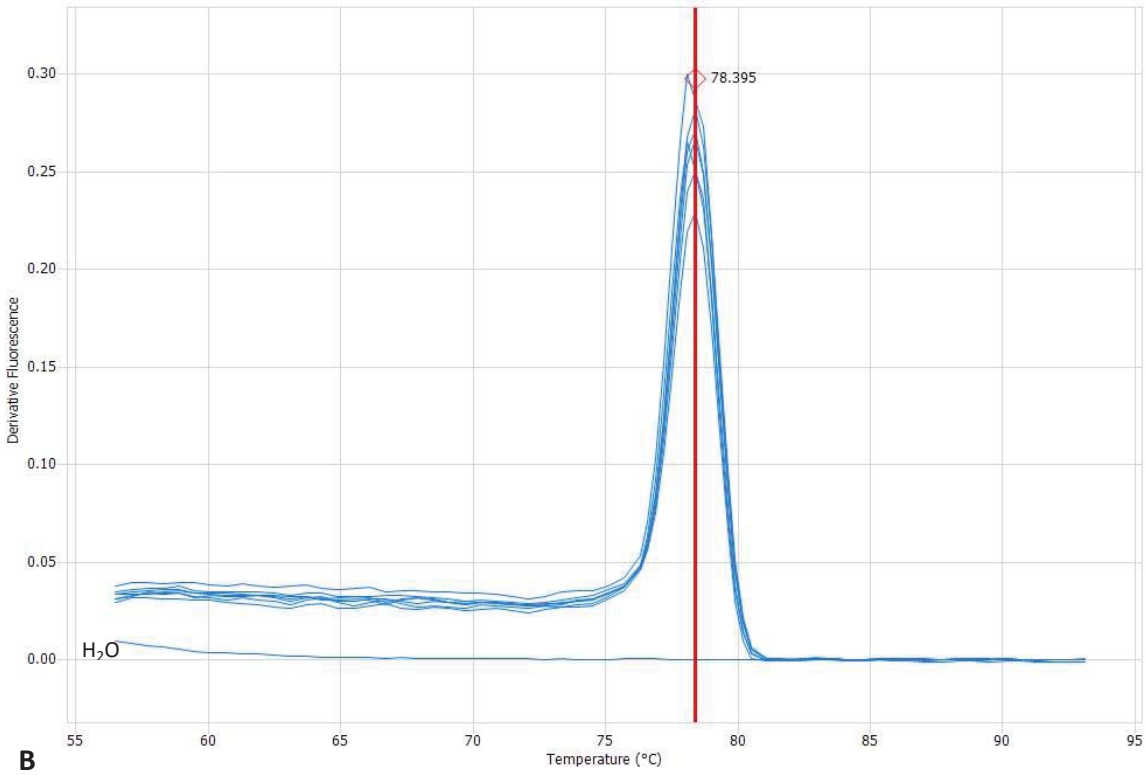
Cycle

A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0.25
0.20
0.15
0.10
0.05
0.00
Derivative Fluorescence
78.395
H2O
55
60
65
70
75
80
85
90
95
Temperature (°C)

B

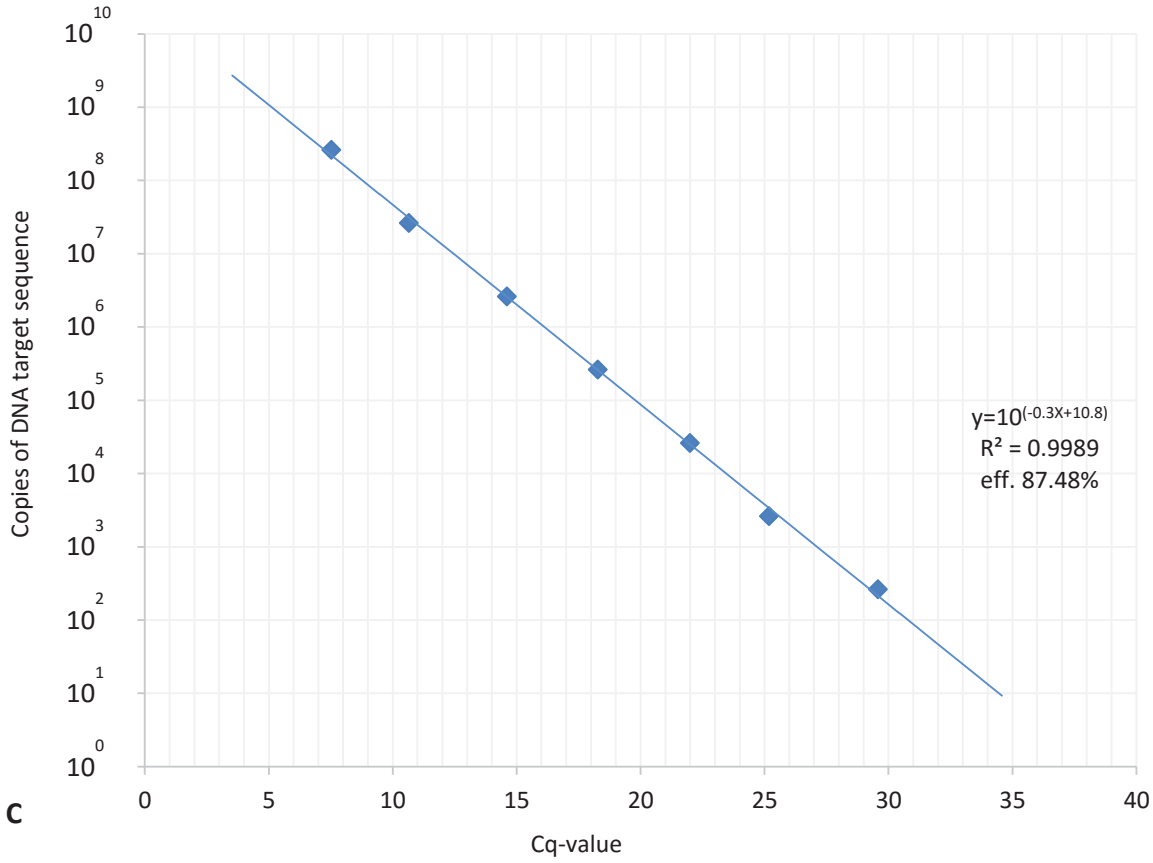


Figure 3.1. Real-time PCR amplification curves (A), derivative melt curves (B) and standard curve (C) of 7 standard samples with a dilution factor of 10 and a starting quantity of 2.61 x 10^8 copies of the qPCR target DNA sequence (Friedl and Groscurth, 2012). Nuclease free H_2O was used as a negative control.

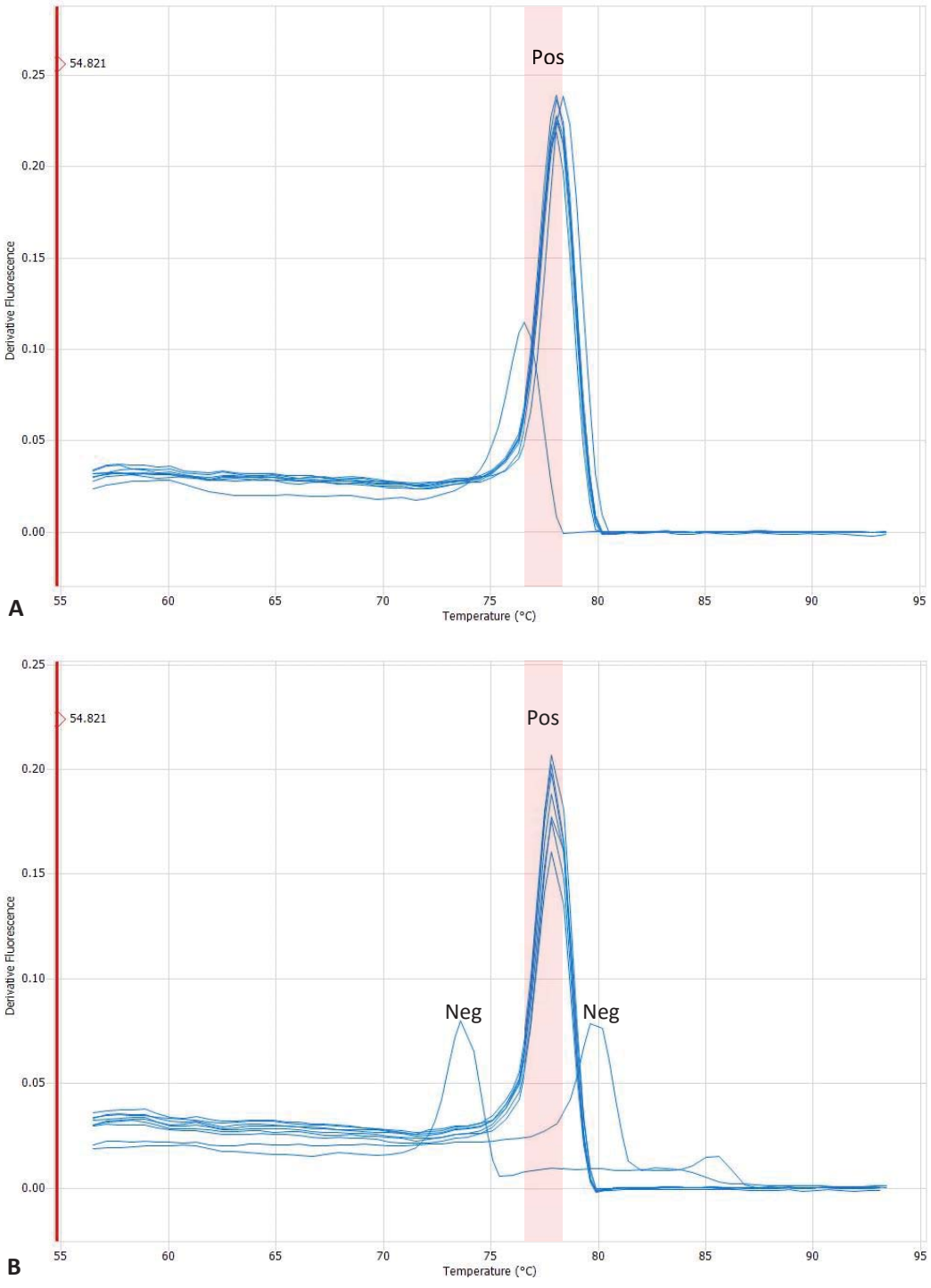


Figure 3.2. Meltcurves for samples positive for *Plasmodium* specific DNA (A), and samples negative for *Plasmodium* specific DNA, but revealing the presence of non-specific DNA amplicons (B). The red shading indicates the temperature range of dissociation temperatures for *Plasmodium* spp.

Table 3.1. The estimated true prevalence of *Plasmodium* spp. infection based on real-time PCR (qPCR) results including 95% confidence intervals (CI)

Order	Family	Species	NZ status	$n^{\dagger}$	Prevalence	95% CI
Passeriformes	Turdidae	Blackbird *Turdus merula*	I	34	0.77	0.60-0.88
	Turdidae	Song Thrush *Turdus philomelos*	I	3	0.67	0.21-0.04
	Fringillidae	Chaffinch *Fringilla coeleps*	I	1	0.00	0.00-0.79
	Prunellidae	Dunnock *Prunella modularis*	I	1	0.00	0.00-0.79
	Fringillidae	Goldfinch *Carduelis carduelis*	I	1	0.00	0.00-0.79
		Total for all introduced species	I	40	0.70	0.55-0.82
	Zosteropidae	Silvereye *Zosterops lateralis*	N	33	0.12	0.05-0.27
	Rhipiduridae	Fantail *Rhipidura fuliginosa*	N	4	0.00	0.00-0.49
		Total for all native species	N	37	0.11	0.04-0.25
	Petroicidae	New Zealand Robin *Petroica longipes*	E	102	0.22	0.15-0.31
	Petroicidae	Tomtit *Petroica macrocephalatoitoi*	E	3	0.67	0.21-0.04
	Mohouidae	Whitehead *Mohoua albicilla*	E	3	0.00	0.00-0.56
	Acanthizidae	Grey Warbler *Gerygone igata*	E	2	0.00	0.00-0.66
	Meliphagidae	Bellbird *Anthornis melanura*	E	1	0.00	0.00-0.79
	Meliphagidae	Tui *Prosthemadera novaeseelandiae*	E	1	0.00	0.00-0.79
Anseriformes	Anatidae	Blue Duck *Hymenolaimus malacorhynchos*	E	14	0.14	0.04-0.40
		Total for all endemic species	E	126	0.21	0.15-0.29
		Total for all sampled birds (*I*+*N*+*E*)	I+N+E	203	0.29	0.23-0.35

[*] NZ status for each bird species is indicated as introduced (*I*), native (*N*), or endemic (*E*) to New Zealand
[†] *n* is the total number of bird captured of a particular species

3.4.2 Latent class analysis

Discrepancies in infection prevalence were found between qPCR and the previously performed nPCR. For example, in New Zealand robins, the results of the two assays differed significantly, with the qPCR showing a higher prevalence of infection when compared to the nPCR (χ^2=14.28, d.f. = 1, P < 0.001). To estimate the difference in sensitivity and/or specificity of these two tests a latent class analysis (LCA) was performed. The resulting overall estimated true prevalence for nPCR was 22.3% (95% CI 17.1–28.5%) and for qPCR 28.7% (95% CI 22.9-35.3%). A higher sensitivity and specificity were found for the qPCR (96.7% and 98.0%) than for the nPCR (80.9% and 85.4%). Pairwise comparison found significant differences in prevalence of *Plasmodium* spp. infections between introduced, native and endemic species. The infection prevalence in introduced species differed significantly from native (P < 0.001) and endemic species (P < 0.001). The difference between native and endemic species was also significant (P = 0.005).

3.4.3 Infection intensity

The infection intensity (parasite load) in qPCR positive samples ranged from 0.08 to 1.61 x 10^4 *Plasmodium* DNA copies per 10,000 avian blood cells. For those samples that were also positive on nPCR, parasite loads ranged from 0.3 to 1.61x10^4 *Plasmodium* DNA copies per 10,000 avian blood cells (Appendix 3). Therefore, the lower detection limit for the qPCR was close to one *Plasmodium* DNA copy per 100,000 cells, compared to three DNA copies per 1,000 avian blood cells for the nPCR. This shows that the qPCR is better at detecting low levels of parasitaemia than the nPCR, which explains the higher sensitivity of qPCR compared the nPCR, as was indicated by LCA. Consequently the qPCR detected low parasitaemias in twenty-two samples of endemic bird species with negative nPCR results.

The mean infection intensity was determined for the groups of introduced, native and endemic bird species (Table 3.2). A significant difference in parasite load was present between these groups (df = 2, F = 33.7, $P < 0.001$). The mean parasite load was significantly higher in introduced bird species compared to endemic species ($P < 0.001$) and native species ($P < 0.001$). There was no significant difference in infection intensity between native and endemic species (P = 1.0).

Table 3.2. Estimated true prevalence with 95% confidence interval (95% CI) and mean parasite load with standard error of mean (SEM) for *Plasmodium* spp. infections in introduced, native and endemic species, defined as number of *Plasmodium* DNA copies per 10,000 avian cells

Bird group	Prevalence	95% CI	Mean Parasite load	SEM
Introduced	0.68	0.53-0.80	2,245	700
Native	0.11	0.04-0.25	2.8	1.7
Endemic	0.20	0.14-0.28	31.5	18.4
All birds	0.29	0.23-0.35	1156	385

3.4.4 Effect of infection status and parasite load on BCI and PCV

Mean PCV and BCI values were determined for introduced, native and endemic birds (Table 3.3) Based on qPCR results, robins infected with *Plasmodium spp.* showed a significantly lower PCV than robins that were uninfected (n = 98, U = 534, $P = 0.029$). In addition, no significant relationship was found between total parasite load and BCI or PCV in blackbirds, silvereyes, or NZ robins using linear regression.

Table 3.3. Mean packed cell volume (PCV) and body condition index (BCI) $^{+}/_{-}$ standard errors of means (SEM) for *Plasmodium* spp. positive and negative birds based on qPCR results

Species	PCV		BCI	
	Pos birds	Neg birds	Pos birds	Neg birds
Blackbird	43.65 $^{+}/_{-}$ 0.89	44.88 $^{+}/_{-}$ 1.70	2.57 $^{+}/_{-}$ 0.11	2.70 $^{+}/_{-}$ 0.05
Silvereye	51.00 $^{+}/_{-}$ 3.18	47.07 $^{+}/_{-}$ 1.48	0.75 $^{+}/_{-}$ 0.09	0.63 $^{+}/_{-}$ 0.01
Robin	43.79 $^{+}/_{-}$ 0.89	46.74 $^{+}/_{-}$ 0.58	0.85 $^{+}/_{-}$ 0.01	0.83 $^{+}/_{-}$ 0.01

3.5 Discussion

Using a new qPCR protocol for the detection and quantification of *Plasmodium* spp. in New Zealand birds, this study determined levels of parasitaemia in qPCR positive samples between 0.08 and 1.61×10^{4} *Plasmodium* DNA copies per 10,000 avian blood cells, implying a lower detection limit for the qPCR of close to one DNA copy per 100,000 cells. This confirmed a higher sensitivity of the qPCR to detect low level parasitaemias compared to the nPCR, for which a lower detection limit of three DNA copies per 1,000 avian blood cells was found using the qPCR data. Therefore, compared to the qPCR, the nPCR resulted in more false negative results for samples with a low parasitaemia. On the other hand, six samples that were positive following the nPCR were negative in the qPCR. Due to poor amplification of the nPCR target sequence, these six samples were not positively confirmed as *Plasmodium* spp. by DNA sequencing, meaning that the nPCR results were potentially false positive. False positive nPCR results would contribute to a lower specificity of the nPCR. LCA confirmed a higher sensitivity and specificity of the qPCR compared to the nPCR. To increase the reliability of these results duplicate samples should be tested; due to money and time restraints this was not done in this initial trial. In addition, the use of an internal extraction control, such as an avian housekeeping gene, should be used in future experiments to confirm DNA quality and whether the DNA can be successfully amplified using qPCR techniques (Nolan et al., 2015).

Interestingly, the prevalence as well as the parasite load of *Plasmodium* spp. was significantly higher in introduced passerines than in native and endemic species. This finding further supports previous studies that have suggested that introduced species like blackbirds and song thrushes function as an important reservoir of infection and are able to sustain *Plasmodium* lineages within an ecological unit or to introduce them to other sites (Ewen et al., 2012b; Schoener et al., 2013; Tompkins and Gleeson, 2006).

To gauge the potential impact of avian malaria on the fitness of different bird species, PCV and BCI were compared between *Plasmodium* infected and non-infected blackbirds, silvereyes and New Zealand robins. As the evaluation of BCI within bird groups containing more than one species is not appropriate, evaluation of these parameters within the overall groups of introduced, native and endemic bird species was avoided, (Labocha and Hayes, 2012). Based on qPCR results, *Plasmodium* positive NZ robins had a significantly lower PCV than *Plasmodium* negative ones (Table 3.3). This implies that in NZ robins, chronic avian malaria may have a negative effect on the concentration of red blood cells. Although during the acute phase of infection anaemia and impairment of BCI are common (Atkinson et al., 2000; Valkiunas, 2005), birds in this phase tend to be less active and either succumb to predation or simply don't get caught in nets (Moller and Nielsen, 2007; Yorinks and Atkinson, 2000). Therefore, it is possible that birds caught in nets were most likely in the chronic, low parasitaemic phase of infection. Previous studies have reported recovery to the original PCV and BCI during this phase (Motta et al., 2013; Paulman and McAllister, 2005). Repeat studies would be needed to confirm the finding of reduced PCV values in *Plasmodium* positive NZ robins, especially since no significant relationship was found between total parasite load and PCV using linear regression. The reduction in red blood cells did not appear to have a clinical impact on the overall fitness of NZ robins, as no decrease in BCI was found in any of the tested bird species.

The chronic effect of *Plasmodium* infections on reproduction, or on physical parameters during times of high energy demand, reduced food intake or co-infection with multiple

Plasmodium spp., has not been evaluated in wild NZ bird species. Multiple examples from abroad illustrate the importance of including these factors in future NZ studies. For example, a negative correlation between parasitaemia and BCI was previously demonstrated in incubating female Kestrels (Dawson and Bortolotti, 2000). In addition, Marzal et al. (2008) found that chronic infection with a single *Plasmodium* lineage didn't have a significant impact on body mass or PCV, while infection with multiple strains did. A negative correlation between the level of parasitaemia and the number of fledged offspring has also been demonstrated (Asghar et al., 2011; Marzal et al., 2005). Futhermore, a reduced lifespan combined with a reduced number and quality of offspring was reported in chronically infected great reed warblers (Asghar et al., 2015). Potential explanations for the fact that in our study no significant impact of infection on PCV or BCI was seen in most of the bird groups are that the tested birds were likely in the chronic state of infection and breeding females were excluded. Alternatively, the birds in our study had reached a balance between tolerance and immune response to the parasite that allowed normal physiological function.

Based on the findings, speculation regarding disease dynamics of avian malaria in various New Zealand bird species and groups can be made. While *Plasmodium* prevalence can be widely influenced by environmental factors, such as temporal and spatial distribution of the parasite and host, infection intensity is largely influenced by the genetic make-up of host and parasite (Westerdahl, 2012). Immuno-alleles in the genetic make-up of birds can either lead to qualitative or quantitative resistance against *Plasmodium*, or result in a higher susceptibility to infection (Westerdahl, 2012). Additionally, over time host-parasite relationships can evolved towards a higher tolerance and moderated immune response of the host against the micro-organism, avoiding damaging inappropriate inflammatory reactions and allowing hosts to carry parasites without severe pathological signs (Atkinson et al., 2013; Rook, 2009). These two theories are likely to explain some of the differences that were found between bird species or groups during this study. Differences in resistance, immunity and tolerance towards *Plasmodium* lineages between various bird

species are easier to demonstrate when both parasite load and prevalence of infection are evaluated in these species. These traits are likely to vary between introduced and endemic bird species due to the evolutionary time difference during which these species had the chance to genetically adapt to the parasites (Ewen et al., 2012b). The combined high prevalence and high parasitaemia seen in introduced birds with a healthy appearance suggests an adaptation of these birds to avian malaria through increased tolerance (Atkinson et al., 2013). To survive infection, susceptibility and tolerance must go hand in hand with a quantitative resistance at a level sufficient to maintain a healthy condition in most of these birds. The fact that high parasitaemia values were not found in endemic or native species, could mean that these species have a lower load tolerance towards *Plasmodium* spp., meaning that in case of high parasitaemia, a cascade of inflammatory and pathological reactions to fight the infection causes too much damage to the birds to survive (Atkinson et al., 2013). Another explanation could be that endemic and native birds are less susceptible to infection or are able to lower parasite numbers more than introduced passerines through more efficient immunological responses (higher quantitative resistance) (Westerdahl, 2012). The fact that the prevalence of infection in endemic species is often significantly lower than in introduced species supports the first explanation over the second. If endemic species were well equipped with an immune response to lower parasite numbers, many birds would survive the acute phase of infection and higher prevalence of chronically infected birds with low-parasitaemia would be expected in these species (Westerdahl, 2012). Based on this theory, *Plasmodium* infections are potentially more pathogenic in endemic bird species; however, the presence of a low numbers of chronically infected individuals with a low parasitaemia also shows that a certain level of quantitative resistance is present in endemic birds. Higher sample sizes are needed to further clarify the effect of the differences in prevalence and parasite loads between bird species.

Also, there is limited knowledge regarding immuno-alleles in New Zealand bird species, or how they correlate to our findings. To prove Westerdahl's (2012) theory regarding

quantitative resistance due to immuno-alleles, these genetic features have to be studied. For a complete understanding of disease dynamics, serological data demonstrating which birds were infected in the past and have recovered from infection, as well as controlled ongoing studies into parasite loads in birds of various ages, sexes and in various seasons would be needed. Exploration of the use of lineage specific qPCR primers for *Plasmodium* sp. will be useful for ongoing studies (Asghar et al., 2011; Perandin et al., 2004; Zehtindjiev et al., 2008). With a fast increasing prevalence of avian malaria in New Zealand over the last 15 years, and shifts in the geographical spread of avian malaria vectors, studying disease dynamics in our New Zealand birds is of utmost importance to plan efficient future wildlife management strategies for our endemic bird species.

3.6 Acknowledgements

This research was approved by the Massey University Animal Ethics Committee (MUAEC protocol number 11/72) and the New Zealand Department of Conservation (DOC Permit no. 34781-FAU). Approval and support for this research project was also given by the local land owners: Atihau-Whanganui Incorporation, Ngaporo-Waimarino Forest Trust, Pipiriki Incorporation and Ernslaw One Limited. Financial support was received from The New Zealand Department of Conservation (DOC) and the Pacificvet Avian Health Research Fund of the Institute of Veterinary, Animal and Biomedical Sciences (IVABS). Permission to include blue duck data in this study was granted by DOC.

CHAPTER 4

MORTALITY OF LITTLE PENGUINS (EUDYPTULA MINOR) IN NEW ZEALAND DUE TO AVIAN MALARIA

Chapter 4: Mortality of little penguins (*Eudyptula minor*) in New Zealand due to avian malaria

4.1 Abstract

Case history

A captive little penguin (*Eudyptula minor*) of wild origin at Wellington zoo became inappetant and lethargic in March 2013. Despite supportive care in the zoo's wildlife hospital the bird died within 24 hours.

Clinical findings

Weight loss, dehydration, pale mucous membranes, weakness, increased respiratory effort and biliverdinuria were apparent on physical examination. Microscopic evaluation of blood smears revealed intra-erythrocytic stages of *Plasmodium* spp. and a regenerative reticulocytosis in the absence of anaemia.

Pathological findings

Post mortem findings included reduced body condition, dehydration, pulmonary congestion and oedema, hepatomegaly, splenomegaly, hydropericardium and subcutaneous oedema. Histology revealed parasite stages in lung, liver and spleen. A marked, diffuse, sub-acute interstitial histiocytic pneumonia was present. Accumulation of haemosiderin was noticed in the liver's Kupffer cells and in histiocytic type cells in the spleen.

Molecular biology

DNA was extracted from frozen portions of the liver. Nested PCR results and DNA sequencing confirmed infection of the deceased penguin with *Plasmodium* (Huffia) *elongatum* lineage GRW06.

Diagnosis

Avian malaria due to *Plasmodium* (Huffia) *elongatum* GRW06

Retrospective analysis of the Massey wildlife post-mortem database

A retrospective analysis of little penguin cases in the Massey University post mortem database was combined with retrospective analyses of archived tissues using a nested PCR for *Plasmodium* spp. Nested PCR and DNA sequencing revealed the previous mortality of a little penguin from Auckland Zoo infected with *P. elongatum* GRW06 and two wild little penguins infected with *P. relictum* SGS1 and *P.* LINN1. The overall frequency of avian malaria as a confirmed cause of death in little penguins in the Massey University post mortem database was 1.36% (4/295).

Clinical relevance

Our results suggest that avian malaria causes sporadic mortality in New Zealand's little penguins both in the wild and in captivity, but there is no evidence of mass mortality events due to *Plasmodium* spp. infection.

4.2 Introduction

Avian malaria, caused by infection with *Plasmodium* spp., is a cosmopolitan mosquito-borne disease infecting hosts of nearly all avian taxa (Valkiunas, 2005). In New Zealand, 17 *Plasmodium* lineages belonging to at least 4 subgenera have been detected (Schoener et al., 2013). These lineages are mostly considered non-endemic and have likely been co-introduced into New Zealand with their introduced avian hosts, in which they generally have a high prevalence (Ewen et al., 2012b). While some lineages show high prevalence and low pathogenicity in their introduced avian hosts, they may cause significant morbidity and mortality in endemic and native New Zealand bird species (Alley et al., 2008; Banda et al., 2013). Clinical signs of avian malarial infection can include anaemia, decreased activity and food consumption, pale mucous membranes, respiratory signs,

vomiting, behavioural changes and mortality (Atkinson et al., 2001b; Møller, 2008; Valkiunas, 2005; Williams, 2005).

Internationally, penguin species are considered highly susceptible to infection with *Plasmodium* spp. when kept in open air exhibits and rehabilitation centers, and in these settings avian malaria is an important cause of penguin mortality. Published infection prevalences for avian malaria in captive settings include 6% to 43% in magellanic penguins (*Spheniscus magellanicus*) in Brazil (Vanstreels et al., 2014; Vanstreels et al., 2015), up to 35% in African penguins (*Spheniscus demersus)* in South Africa (Parsons and Underhill, 2005), 24% to 50% in African penguins in Baltimore Zoo (Beier and Stoskopf, 1980; Stoskopf and Beier, 1979), and over 58% in magellanic penguins during an outbreak in Blank Park Zoo, Des Moines, Iowa (Fix et al., 1988). In Baltimore Zoo 62% of non-infected young penguins acquired avian malaria naturally after entering an enclosure that was accessible for mosquitoes (Cranfield et al., 1994). High mortality rates of 83% (Fix et al., 1988) and 32% (Beier and Stoskopf, 1980) have been reported during avian malaria outbreaks.

In wild penguins however, the detection of *Plasmodium* spp. is extremely rare. Incidental cases with a strong suspicion of *Plasmodium* spp. infections, based on microscopic examination of blood smears, were reported in two yellow eyed penguins (*Megadyptes antipodes*) from Foveaux Straight and Campbell Island (Fantham and Porter, 1944; Laird, 1950), three Fiordland crested penguins (*Eudyptes pachyrhynchus*) from the Snares islands (Laird, 1950), one African penguin (Brossy, 1992a), and one juvenile yellow eyed penguin (Alley, 2001). However, infections with *Plasmodium* spp. were not detected in African penguins (Brossy, 1992; Parsons and Underhill, 2005), yellow eyed penguins (Sturrock and Tompkins, 2007), magellanic and humboldt penguins (*Spheniscus humboldti*) (Sallaberry-Pincheira et al., 2014) in their natural habitat, using nPCR. Therefore, a theory was proposed that wild penguins only became infected with *Plasmodium* spp. after they were housed in captivity (Brossy, 1992). However, this theory lost credibility after the discovery that up to 35% of African penguins at a rehabilitation centre had parasitaemias within their first 5 days after admission (Parsons and Underhill, 2005); with a reported prepatent

period of 5 days for *Plasmodium relictum* (Valkiunas, 2005), it appears likely that these penguins had already been infected in the wild. This may imply that latent avian malaria infections occur in wild penguins, and that recrudescence of *Plasmodium* spp. occurs when corticosterone levels become elevated during stressful events, such as rehabilitation (Cranfield et al., 1994). The theory of latent and subclinical infections is supported by the detection of high seroprevalences for antibodies against *Plasmodium* spp. in wild penguins, indicating high levels of exposure to these parasites. Seroprevalences were found of 23% to 98% in yellow eyed penguins (Graczyk *et al*. 1995a; Sturrock and Tompkins 2007), 33% in gentoo penguins (*Pygoscelis papua*), 52% in African penguins, 58% in king penguins (*Aptenodytes patagonicus*) and 100% in yellow eyed penguins (Graczyk 1995b). Antarctic adelie penguins (*Pygoscelis adeliae*), which were not in contact with mosquito vectors for avian malaria in their natural habitat, had no detectable antibodies (Graczyk et al., 1995b). More recently, a close to 100% seroprevalence was found in Galapagos penguins (*Spheniscus mendiculus*), and surprisingly nPCR also detected parasitaemias in this species, showing an infection prevalence of 9.4% (Palmer et al., 2013).

For little penguins (*Eudyptula minor*) data regarding avian malaria are scarce. No positive nPCR results for *Plasmodium* spp. have been reported in the literature. However, antibodies were detected in 92% (11/12) of captive little penguins from the Napier aquarium and 63% (7/11) of little penguins (*Eudyptula minor*) from the South Island of New Zealand (Graczyk et al., 1995b). The little penguin is the world's smallest penguin. It is locally common on the New Zealand mainland and its surrounding islands, around Tasmania and on the South coast of Australia (Heather and Robertson, 2005). Although numbers are gradually declining (Flemming, 2013; Miskelly et al., 2008), with predation by dogs and mustelids and traumatic injuries responsible for a significant proportion of deaths (Hocken, 2000), the species is currently classified as "least concern" according to the IUCN redlist (Birdlife International, 2012). This clinical communication describes the clinical and pathological findings of avian malaria in a captive little penguin from Wellington Zoo. In addition, a retrospective analysis of post mortem records and molecular analysis of archived tissues from New Zealand little penguins was carried out.

4.3 Case history

A 2.5 year old adult male little penguin of wild origin had been in care of Wellington Zoo for over 2 years. A traumatic fish hook injury and ocular trauma, resulting in permanent blindness, lead to admittance to the Wellington Zoo Wildlife Hospital (henceforth called "The Nest Te Kōhanga"), followed by captive placement within Wellington Zoo. The bird was housed in an outdoor enclosure with one female and two other male little penguins. The enclosure was open air, and consisted of a pond, rocks, sandy beaches, foliage, artificial burrows and a driftwood shelter.

4.4 Clinical findings

During the last week of March 2013 the blue penguin became inappetant and increasingly lethargic over a period of three days. No clinical signs were seen in the other three penguins which shared the same outdoor enclosure. Supportive care and diagnostic tests were performed at The Nest Te Kōhanga. On admission to the hospital, the penguin's weight had reduced to 837 grams from 870 grams two days prior and its body condition, as judged by pectoral muscle condition scoring, was moderate (3/9). The bird had pale mucous membranes, appeared weak and was mildly dehydrated as assessed by decreased skin turgor on the legs. The penguin spent most time in sternal recumbency, but could stand when assisted. An increased respiratory effort was evident. During auscultation, mild wheezing without rales was noticed over both lung fields on both inspiration and expiration. Biliverdinuria was present.

Haematology (Table 4.1) showed no abnormalities in total plasma solids and packed cell volume (PCV). An estimated white cell count and differential, performed by microscopic examination of a modified Wright's stained blood smear, were within the ISIS-reference ranges for blue penguins (Teare, 2013). Despite the absence of anaemia, a regenerative response in the erythrocytes was observed with ~35% reticulocytes present and occasional earlier erythroblastic forms. Evaluation of Giemsa stained blood smears revealed round and ovoid pale-blue bodies with occasional basophilic stippling in the cytoplasm of approximately 0.5% of mature and immature erythrocytes. These bodies

were 0.5 to 1.5 times the size of the non-displaced cell nuclei, and based on morphology, were indicative of an infection with *Plasmodium* spp. (Figure 4.1a).

Table 4.1. Haematological parameters of a little penguin (*Eudyptula minor*) with avian malaria at Wellington Zoo.

	Patient value	Reference range[a]	Units
Total plasma solids	50	39-82	g/L
PCV	42	29-58	%
Reticulocytes	35	1-5	%
Total white cell count	17.3	2.9-34.6	x 10^9 cells/L
Heterophils	6.23	0.6-19.8	x 10^9 cells/L
Lymphocytes	10.5	1.0-16.2	x 10^9 cells/L
Monocytes	0.63	0.0-2.1	x 10^9 cells/L
Eosinophils	0	0.0-0.5	x 10^9 cells/L
Basophils	0	0.0-1.0	x 10^9 cells/L

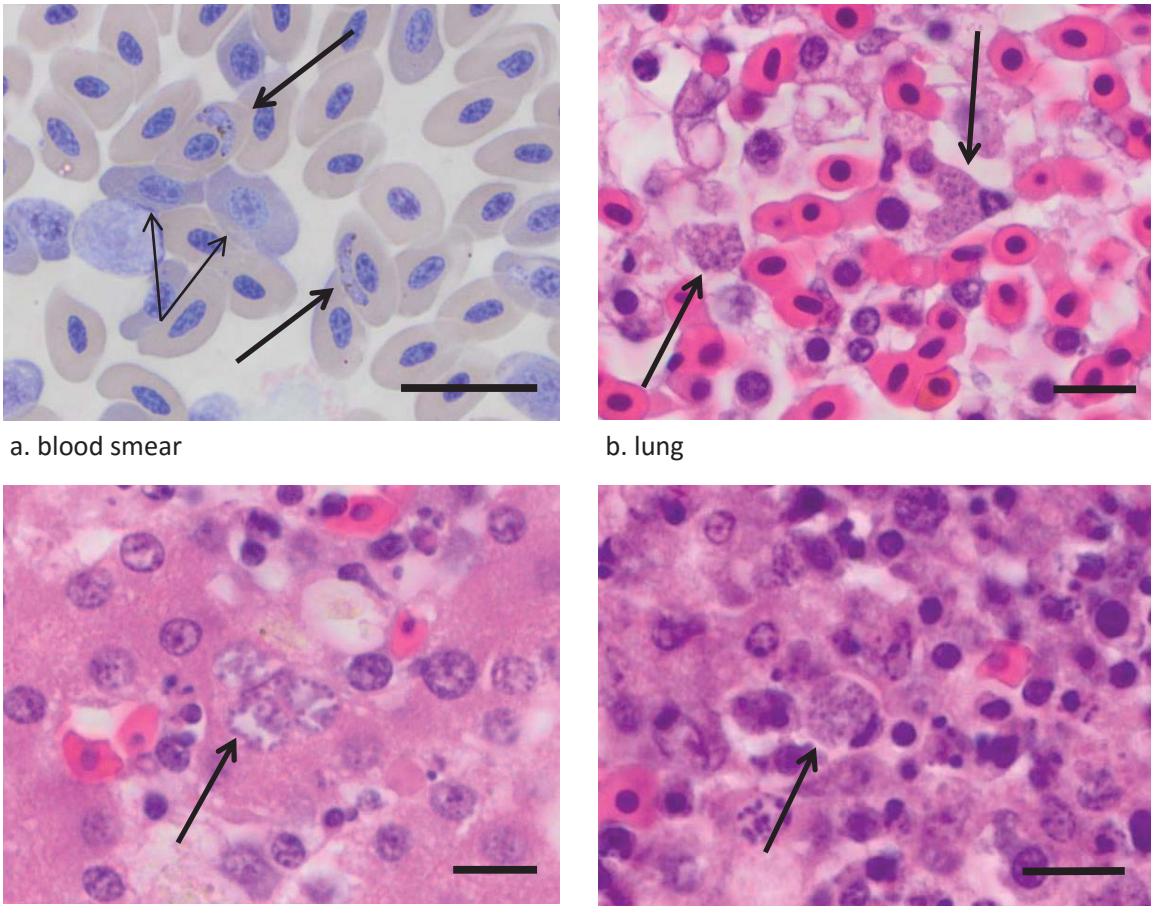
[a] Reference ranges are derived from the International Species Information System (ISIS) (Teare, 2013), except for the reference range for reticulocytes (Ritchie et al., 1994).

The penguin was hospitalised at a room temperature of 22^0C and fluid therapy was provided by crop tubing, using an electrolyte solution (Vytrate, Jurox, Rutherford, Australia) at 40 ml/kg PO BID. Trimethoprim sulfamethoxazole (AFT Pharmaceuticals Ltd, Takapuna, Auckland, New Zealand) was given at 25 mg/kg PO BID. As nutritional support, 20 ml of blended whole salmon slurry with electrolytes was given by crop tubing in the evening. Despite supportive care and initial treatment, the animal died within 24 hours. Blood smears and heparinised blood samples were collected from the three other penguins and sent to Massey University in Palmerston North for nested PCR for *Plasmodium* spp. Until nested PCR results were available, these three in-contact birds were given a preventative treatment for malaria with a combination of 15 mg atovaquone and 6 mg proguanil (Malarone®, GlaxoSmithKlineNZ, Auckland, New Zealand), corresponding to a minimum dose of 17.5 mg/kg atovaquone and 7 mg/kg proguanil for each penguin, given once daily per os (PO).

4.5 Pathological findings

On post mortem examination, the bird was in moderate body condition (3/9) as assessed by the pectoral muscle condition and showed mild to moderate dehydration based on skin turgor. The lungs were heavy, deep red and moist; on cut surface the parenchyma oozed a large amount of clear frothy fluid while the trachea was clear. Both the liver and spleen were moderately enlarged and slightly firmer than usual. The pericardial sac contained approximately 2mls of clear straw coloured fluid. Moderate subcutaneous oedema was present in the inguinal areas and along the lateral body walls.

On histopathological examination, the most severe lesions were present in the lungs in which there was a marked, diffuse, sub-acute interstitial histiocytic pneumonia as well as marked congestion and pulmonary oedema. The interstitium was expanded by large numbers of macrophages and fewer heterophils as well as multiple small deposits of fibrin. Small numbers of protozoal organisms were visible within the cytoplasm of macrophages and endothelial cells (Figure 4.1b). Haemosiderin was present in many of the macrophages. Many parabronchi and air-capillaries contained proteinaceous fluid. Within the liver numerous Kupffer cells contained haemosiderin with a small number containing protozoal organisms (Figure 4.1c). The splenic parenchyma was expanded with large numbers of histiocytic-type cells, most of which were situated around sheathed arterioles. Small numbers of these cells contained protozoal organisms (Figure 4.1d), while a larger proportion contained haemosiderin. No abnormalities were noted in sections of heart, kidney, adrenal gland, gastrointestinal tract and testes.



a. blood smear

b. lung

c. liver

d. spleen

Figure 4.1. Photomicrographs of tissues from a little penguin (*Eudyptula minor*)
a. blood smear with multiple sexual stages (gametocytes) of a *Plasmodium* sp. within erythrocytes (solid arrows); the black pigment associated with the parasites is likely haemazoin, a by-product of haemoglobin digestion by the parasite; polychromasia due to many immature erythrocytes (thin arrows) is evident; Giemsa stain. b. asexual stages (arrows) of a *Plasmodium* sp. within macrophages or endothelial cells of the lung; Haematoxylin & Eosin; c. asexual stages (arrows) of a *Plasmodium* sp. within a Kupffer cell of the liver; haematoxylin & Eosin stain; d. asexual stages of a *Plasmodium* sp. within a macrophage in the spleen; Haematoxylin & Eosin stain. All scale bars represent 20 µm.

4.6 Molecular biology

Frozen liver tissue of the deceased little penguin and pre-treatment heparinised blood samples of the three in-contact penguins were tested for avian malaria at Massey University in Palmerston North. A nested polymerase chain reaction (nPCR) based on the protocol by Hellgren et al. (2004), confirmed the liver tissue of the diseased penguin to be positive for *Plasmodium* spp. DNA. The positive amplicon was purified using the Purelink PCR purification kit (Invitrogen, Carlsbad CA, USA) and subjected to automatic dye-terminator cycle sequencing with the BigDye Terminator Version 3.1 Ready Analyser (Applied Biosystems Inc., Foster City CA, USA) to confirm the genomic sequence. The resulting sequence was compared with published sequences available from GenBank (Benson et al., 2014). NCBI BLAST sequence analysis confirmed 100% similarity to *Plasmodium* (Huffia) *elongatum* lineage GRW06 (GenBank DQ368381). The three living penguins tested negative for *Plasmodium* spp. by nested PCR.

4.7 Retrospective review of the Massey wildlife post mortem database

A review of 294 little penguin records from the Massey University post mortem database from 1992 to 2015 revealed one other avian malaria case confirmed by nested PCR, and two suspicious cases based on histology. The confirmed case involved a little penguin from Auckland Zoo which died in 2005, while the two suspicious cases involved wild blue penguins, found on Foxton Beach and Waikanae Beach on the South west coast of the North Island of New Zealand in 2012 and 2013. Post mortem findings in these birds included poor body condition, lung oedema and congestion, hydro-pericardium, splenomegaly, subcutaneous oedema, hydro-coelom, arrested moult, fluid filled blisters on the feet and dilatation of the heart. Histological findings included the presence of schizonts of *Plasmodium* spp. in endothelial cells and macrophages of the spleen and lung, and in hepatocytes and macrophages of the liver. In addition, haemosiderin deposition was present within Kupffer cells, and lympho-plasmacytic cholangiohepatitis and cholestasis were detected in the liver. There was generalised hyperplasia of periarteriolar lymphocytes and plasma cells in the spleen. In the previously confirmed case from Auckland Zoo, a nested PCR was positive for *Plasmodium* spp. and DNA sequencing

(EcoGene®, Auckland) confirmed the presence of *P.* (Huffia) *elongatum* lineage GRW06. For the two wild penguin cases that had been previously diagnosed as suspicious for avian malaria at histology, stored paraffin embedded material was retrieved from the archived collection at Massey University and subjected to nested PCR and DNA sequencing as described above. Both of these samples were positive for *Plasmodium* spp. and when compared to sequences from GenBank by NCBI BLAST analysis, the DNA sequences showed 100% similarity to *P*. (Haemamoeba) *relictum* lineage SGS1 (GenBank AF495571) and *P.* sp. lineage LINN1 (GenBank GQ471953). Based on this additional testing, the overall frequency of avian malaria as a confirmed cause of death in little penguins from the Massey University post mortem database was 1.36% (4/295). Avian malaria related mortality appears to be more frequent in captive (2/20) compared to wild little penguins (2/275) (Yates' $\chi^2 = 6.06$, d.f. = 1, $P = 0.014$).

4.8 Discussion

Morbidity and mortality of birds due to avian malaria are mostly seen during the acute phase of infection, when intra-erythrocytic replication of the parasite causes destruction of erythrocytes, and widespread exo-erythrocytic replication occurs in the endothelial cells of capillaries of many organs including the brain (Valkiunas, 2005). Splenomegaly, hepatomegaly and congested, oedematous lungs, as were described for most of our post mortem cases, have been previously described in penguins that have died due to avian malaria infection (Graczyk et al., 1994a; Grim et al., 2003; Silveira et al., 2013). It is noteworthy that the three penguins from the same enclosure as the one that died did not show any signs of disease and that the PCR for *Plasmodium* spp. for these individuals was negative. However, latent infections could have been present in these birds, with the number of parasites in the peripheral blood reduced to zero or amounts too low to detect by nPCR. Thus, more sensitive molecular methods such as real-time PCR may be required to detect these infections with extremely low parasitaemias (Friedl and Groscurth, 2011; Knowles et al., 2009; Zehtindjiev et al., 2008). Serological tests could reveal the presence of antibodies against *Plasmodium* spp., and thus indicate previous exposure to these

parasites (Graczyk et al., 1995a; Sturrock and Tompkins, 2007), but are not commercially available.

The current preferred treatment for avian malaria in captive penguins is a combination of chloroquine phosphate and primaquine phosphate (Carpenter, 2013). Multiple dosing regimens for penguins have been described, with 5 to 10 mg/kg chloroquine given every 6 hours for 1 day to once daily for up to 7 days, combined with primaquine 0.03 mg/kg once daily for 3 to 10 days. Chloroquine phosphate has a toxic effect on intra-erythrocytic *Plasmodium* stages (Slater, 1993), resulting in a rapid decrease in parasitaemia, while primaquine, besides an intra-erythrocytic gametocidal effect, acts against tissue schizonts (Singh and Vingkar, 2008), leading to a gradual reduction in parasitaemia and reducing the chance of relapses (Sohsuebngarm et al., 2014). The three in-contact penguins were given a preventative treatment with atovaquone and proguanil, a treatment which has shown efficiency against blood stages, but not against tissue stages of *Plasmodium* spp., as was demonstrated in chaffinches (*Fringilla coelebs*) (Palinauskas et al., 2009). Combining this latter treatment with primaquine is potentially a more effective approach to target intra- as well as extra-erythrocytic *Plasmodium* stages. The treatments mentioned above were not available at The Nest Te Kōhanga for the penguin from our clinical case on the day of admission, and treatment with Trimethoprim sulfamethoxazole was initiated. Although trimethoprim sulfamethoxazole is not a preferred treatment for *Plasmodium* spp. infections, this drug has some proven anti-protozoal action. In people, it has shown efficiency as a preventative drug for infection with *Plasmodium falciparum* infection (Thera et al., 2005) and has antimicrobial action against another protozoal parasite, *Toxoplasma gondii* (van der Ven et al., 1996). In birds, it has been used as part of a combination therapy with a protozoal *Sarcocystis* infection (Page et al., 1992). Therefore it seemed a sensible broad spectrum treatment option until more efficient anti-malaria drugs were acquired. Other drugs that suppress the level of parasitaemia and the number of tissue stages of *Plasmodium* spp. in chicken are doxycycline and artesunate, although both drugs do not fully clear infection (Sohsuebngarm et al., 2014). While alternative human drugs might be useful in birds, published reports regarding clinical trials are lacking. Due to the advanced stage of clinical signs at the time of admission of the little

penguin from Wellington Zoo, mortality was likely, even if first choice anti-malaria treatments would have been available on the day of admission (Vanstreels et al., 2014).

The retrospective review revealed another zoo related mortality case in a little penguin due to avian malaria. Zoos are mostly located in urban areas with a high density of introduced passerines as well as mosquitoes, increasing the possibility for spillover of malaria infection from wild passerines to birds within the zoo (Derraik, 2004). In New Zealand introduced passerines like blackbirds and song thrushes generally show a high prevalence of *Plasmodium* infection compared to endemic and native species (999 et al., 2012b; Howe et al., 2012), and are suspected to function as a reservoir for infection of native birds (Sijbranda et al., 2016). Mosquito vectors are likely to infect zoo birds if there is a large pool of reservoir hosts in the same area (Dinhopl et al., 2011). The penguin case at Wellington Zoo occurred during a long dry spell, and water turn-over in the penguin pool had been reduced to 50% for 3 days, potentially creating conditions for a short term increase in hatching and survival rate of mosquito larvae.

Studies suggest that penguins in captivity may be more susceptible to *Plasmodium* infections compared to wild penguins (Beier and Stoskopf, 1980; Brossy, 1992). Stress in penguins has been described as an important immuno-suppressive factor, increasing the susceptibility to avian malaria (Brossy, 1992; Cranfield et al., 1994). In captive African black footed penguins in the USA, latent infections could be converted to active parasitaemias by experimentally injecting the birds with dexamethasone, imitating elevated corticosterone levels due to stress (Cranfield et al., 1994). Likewise, injured or oiled captive African black footed penguins exposed to stressful situations showed a significantly higher prevalence of parasitaemia compared to the wild population on the Southern coast of South Africa (Brossy, 1992). Stress and resulting immuno-suppression can be related to housing in enclosures with physical properties that differ from the species' natural surroundings, excessive noise, crowding, or concurrent infections with other diseases like avian pox or intestinal parasites. Due to the increased likelihood of recrudescence of latent *Plasmodium* spp. infections in penguins, avian malaria screening is advised in rehabilitation scenarios, such as oil spill responses. In our clinical case from

Wellington Zoo, possible stress factors included a recent moult, the relatively recent adaptation of the previously wild bird to the captive environment, social stresses from con-specifics, the close proximity of zoo visitors and a recent long spell of high environmental temperatures.

All three *Plasmodium* lineages causing mortality in the four New Zealand little penguins are globally widespread generalist parasites. Their pathogenicity in New Zealand bird species is currently not well understood. *Plasmodium* (Huffia) *elongatum* lineage GRW06 is the most common lineage in New Zealand's wild birds, and has been detected in a wide range of bird species throughout New Zealand and Australia (Alley et al., 2010; Baillie and Brunton, 2011; Beadell et al., 2006; Castro et al., 2011; Ewen et al., 2012b; Howe et al., 2012; Marzal et al., 2011). The GRW06 lineage has been associated with sporadic deaths in endemic birds in New Zealand (Alley et al., 2010; Banda et al., 2013; Schoener et al., 2013). This indicates that although the pathogenicity of this lineage in exotic passerines appears to be moderate to low, its impact on endemic species could be severe, especially when other factors suppress the birds' immune response. *Plasmodium* sp. lineage LINN1 has been isolated from a wide range of avian species and mosquitoes throughout Europe, Asia and America (Bentz et al., 2006; Cosgrove et al., 2008; Ferraguti et al., 2013; Hellgren et al., 2011; Kimura et al., 2010; Szoellosi et al., 2011; Wood et al., 2007), but has so far not been reported in Australia (Ewen et al., 2012b). This lineage was also found in passerines and penguins from an Austrian zoo in Europe (Dinhopl et al., 2015). Within New Zealand, the lineage LINN1 has been reported to cause mortality in blackbirds, great spotted kiwi (*Apteryx haastii)*, and to infect other passerines such as song thrushes and bellbirds (*Anthornis melanura*) (Ewen et al., 2012b; Howe et al., 2012), New Zealand robins and silvereyes (Sijbranda et al., 2016). *Plasmodium* (Haemamoeba) *relictum* lineage SGS1 is found in various bird species around the world and it has been suggested that European house sparrows (*Passer domesticus*) may play a role as carriers and have introduced the parasite to New Zealand (Marzal et al., 2011). In New Zealand, the SGS1 lineage has been previously found in the saddleback (*Philesturnus carunculatus*), sparrow, common myna (*Acridotheres tristis*), and yellowhammer (*Emberiza citronella*) (Beadell et

al., 2006; Ewen et al., 2012b; Howe et al., 2012), but there have been no previous reports of mortality. SGS1 has not been reported in penguins previously.

Prevention of avian malaria in captive institutions is based on removal of stagnant water reservoirs, bio-control (importing natural predators for mosquito larvae), trapping of insects or use of insecticides, exclusion of vectors through use of mosquito netting or window screening, high water turnover in aquatic animal enclosures, reduction of stress factors, and potentially an increased air flow. An example of a successful implementation of a prevention strategy was the use of pesticide strips in the indoor portion of the Baltimore Zoo (Maryland, USA) exhibit, which proved effective in eliminating mosquitoes from the enclosure (Stoskopf and Beier, 1979).

4.9 Clinical relevance

This clinical case, combined with the retrospective analysis of blue penguin mortality cases from the Massey University post-mortem database, suggests that infections with *Plasmodium* spp. cause sporadic mortality in New Zealand's little penguins, both in the wild and in captivity. The overall frequency of avian malaria as a confirmed cause of death in little penguins in the Massey University post mortem database is 1.46% (4/295), but we currently have no estimates of the prevalence of avian malaria infection or exposure in wild little penguin populations. To improve our understanding of the geographical spread and parasite dynamics of *Plasmodium* spp. in blue penguins in New Zealand, targeted surveys of wild populations should be performed.

CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5: GENERAL DISCUSSION

5.1 Overview of major findings

Knowledge regarding the pathological impacts and host-parasite dynamics of *Plasmodium* spp. in New Zealand birds is still scarce. The results of this research revealed interesting new data regarding the prevalence of infection for various *Plasmodium* lineages and the level of parasitaemia in endemic, native and introduced bird species of New Zealand. These results have increased our understanding of the susceptibility, resistance and tolerance of various bird species towards avian malaria; however, there are many interesting questions that remain unresolved.

5.1.1 Avian malaria in introduced, native and endemic New Zealand bird species in a mixed ecosystem

The results of the study described in Chapter 2 show an overall prevalence of infection with *Plasmodium* spp. of 20.3% in selected bird species from the Waimarino Forest detected by PCR. Infection prevalence differed significantly between endemic, native and introduced bird species (3.5%, 19% and 80.5%, respectively). The significantly higher infection prevalence in introduced bird species was consistent with findings from previous New Zealand studies (Ewen et al., 2012b; Howe et al., 2012; Ishtiaq et al., 2006; Tompkins and Gleeson, 2006). These results suggest that introduced bird species may be an important reservoir for avian malaria in this area of New Zealand. *Plasmodium sp*. lineage LINN1 was the most commonly detected lineage, followed by *P.* (Huffia) *elongatum* lineage GRW06, reflecting their generalist nature; *P*. sp. lineage SYAT05 was the most bird species specific one. These three detected lineages have been reported throughout New Zealand previously, but this is the first report of *P*. sp. lineage LINN1 infection in the brown kiwi, silvereye and North Island robin. Infection with the potentially pathogenic lineage *P.* (Haemamoeba) *relictum* GRW4 was not detected in any of the birds during this research. Based on these results, the likelihood of spreading *Plasmodium* lineages from the

Waimarino Forest to new areas by wildlife translocations was statistically very small, and the area was judged to be a suitable current source site for New Zealand robin translocations.

5.1.2 Use of real-time PCR to explore disease dynamics of avian malaria in a mixed New Zealand ecosystem

The newly developed qPCR protocol described in Chapter 3, resulted in a molecular test with a higher sensitivity and specificity (96.7% and 98.0%) than the nPCR (80.9% and 85.4%) described in Chapter 2, as determined by latent class analysis. The qPCR had a higher sensitivity than the nPCR for detecting low parasitaemias due to a lower detection limit of approximately one *Plasmodium* DNA copy per 100,000 cells for the qPCR, compared to 3 DNA copies per 1,000 avian blood cells for the nPCR. In endemic bird species, the qPCR detected twenty-two low parasitaemias with negative nPCR results. Thus, based on qPCR results the overall prevalence of *Plasmodium* spp. infections for birds from the Waimarino Forest was 28.7%. The prevalence of *Plasmodium* spp. in introduced species (80.5%) was significantly higher than in native (11.0%) and endemic species (21.0%). In addition, the mean parasite load was significantly higher in introduced bird species than in endemic and native species (2063 as opposed to 29.5 and 2.8 *Plasmodium* copies per 10,000 avian cells, respectively). The combination of a high prevalence of infection and a high level of parasitaemia in introduced species implies a high susceptibility and a high load tolerance towards *Plasmodium* spp. infections. These data further support that introduced bird species are an important reservoir for avian malaria in New Zealand.

5.1.3 Mortality of little penguins (Eudyptula minor) in New Zealand due to avian malaria

The clinical report presented in Chapter 4 is the first to confirm parasitaemia and tissue stages for *Plasmodium* spp. in little penguins by histology and nPCR. In addition, a retrospective examination of the Massey University post mortem database revealed an overall frequency of avian malaria as a confirmed cause of death in little penguins of

1.36% (4/295). The data set included avian malaria related mortalities in two out of 275 wild penguins and two out of twenty captive penguins. Both captive penguins were infected with *P*. (Huffia) *elongatum* lineage GRW06, while a *Plasmodium* (Haemamoeba) *relictum* SGS1 and a *P*. sp. lineage LINN1 infection were detected in the two wild penguins. These results demonstrate that little penguins are susceptible to avian malaria, that sporadic deaths in little penguins occur in wild as well as captive birds, and that mortality rates due to avian malaria may be higher in little penguins in captive situations.

5.2 Scope and limitations

One of the main aims of this study was to evaluate the presence and prevalence of lineages of *Plasmodium* spp. in NZ robins and other bird species in the Waimarino Forest. For this purpose, the study area was treated as one epidemiological unit. Identification of micro-niches within the forest with different densities of *Plasmodium* spp. infections was beyond the scope of this research, due to restraints on the number of birds that was to be screened, as outlined in the agreements with the New Zealand Department of Conservation (DOC) and the Massey University Animal Ethics Committee. A second aim was to quantify the parasite load in birds from different species through qPCR. The developed qPCR protocol was successful in acquiring these data; however, each parasite load reflects only one moment in time, which is most likely during the chronic phase of infection. Therefore, these data highlight the high parasite load in introduced compared to native and endemic bird species, but do not provide detailed information regarding within-host parasite dynamics over the course of infection. In addition, negative nPCR and qPCR results cannot be used to rule out infection with *Plasmodium* spp.; during the latent phase of infection, parasites are still present in internal organs, while molecular techniques may not detect *Plasmodium* DNA in blood samples. Third, the little penguin study with retrospective analysis of the Massey University post mortem database offered a good report of avian malaria related post mortem signs in this species, and demonstrated the occurrence of incidental deaths due to *Plasmodium* spp. infections. It does not however provide any information regarding the prevalence of avian malaria infections in wild populations of little penguins.

5.3 Implications of the study

Based on data from this study the Waimarino Forest was evaluated to be a suitable current source site for NZ robin translocations. Since 1991, translocations have been used as an important tool to safeguard North Island robin numbers by establishing new founder populations on predator-free islands, and on mainland sites where predators are eradicated or controlled (Armstrong, 2010; Lincoln Park Zoo, 2015). After European settlement, the geographical range of North Island robins was reduced from the entire North Island of New Zealand to a band over the midrange of the North Island and Kapiti and Little Barrier Island. However, translocations resulted in new established populations on islands such as Tiritiri Matangi, Mokoia, Mana, Great Barrier and Matiu (Somes) Island, and mainland islands such as Zealandia (Karori Sanctuary) and Bushy Park Reserve. Besides the original central North Island robin source sites such as Pureora Forest and the Mamaku plateau, islands such as Tiritiri, Kapiti and Mokoia are now also used as source sites for translocations.

Depending on reproductive success and the capacity of a population, commonly 20 to 60 birds are translocated every one to three years without causing declines in population size (Armstrong, 2010; Zoo). Nationally, the average number of NI robin translocations per year has varied from 1 to 3, with 4 to 5 registered during some years (Lincoln Park Zoo, 2015). The current main source site for NZ robin translocations in the southern half of the North Island is Kapiti Island. The inclusion of the Waimarino Forest as an extra mainland source site with a large established NZ robin population will simplify cost and logistics of robin translocations in this area. Additionally, the Waimarino Forest is a privately owned site used for logging, and the infrastructure that is in place makes many areas of this forest more accessible than most other mainland NI robin breeding sites.

The prevalence of infection with *Plasmodium* spp. and the level of parasitaemia differed between introduced and endemic or native bird species from the Waimarino Forest, prompting interesting theories regarding susceptibility, tolerance and resistance towards *Plasmodium* spp. in these various bird groups. It has long been suspected that introduced bird species, such as blackbirds, were already adapted to *Plasmodium* spp. in their native

European home range before their introduction to New Zealand (Ewen et al., 2012b). The detection of a very high prevalence of infection in healthy blackbirds supports this theory, and at the same time indicates their high susceptibility to *Plasmodium* spp. (Westerdahl, 2012). Their high parasite load compared to endemic and native bird species implies that these introduced bird species have a higher load tolerance to *Plasmodium* spp. However, high parasite loads have previously been reported to coincide with increased mortality rates in birds (Valkiunas, 2005). Post mortem examination of exotic passerines is seldom performed, and consequently avian malaria as a cause of death in introduced bird species may be underdiagnosed in New Zealand. This theory is tentatively supported by a recent study that indicated that *Plasmodium* spp. infections are a frequent cause of mortality in European blackbirds (Dinhopl et al., 2015), and a report of avian malaria related mortality in a New Zealand blackbird (Schöner et al., 2009). In the latter case, only one diseased blackbird was examined; so a caveat is that estimates of the blackbird mortality rate in New Zealand due to *Plasmodium* spp. can only be made when post mortem examination is performed on significant numbers of birds.

In endemic and native bird species, the combination of low levels of parasitaemia and low infection prevalences could reflect two different scenarios. Firstly, it could mean that these bird species are highly susceptible and have very little resistance or load tolerance towards *Plasmodium* spp. (Westerdahl, 2012). In this scenario the acute high parasitaemia phase of infection would coincide with a high mortality rate, which would result in a short timespan during which parasites could be transmitted to other birds; the reduced transmission time and the low numbers of birds surviving to the chronic low parasitaemia phase of infection would result in a low prevalence of infection in this species. This scenario is plausible if, within the habitat of these endemic and native species, the density of infected reservoir hosts, such as blackbirds, is low. A high density of infected reservoir hosts would lead to a high infection pressure and consequently to a high mortality rate in susceptible endemic and native species, as was demonstrated in Hawaiian birds (Atkinson et al., 1995). The second scenario that could explain a low prevalence and level of parasitaemia in endemic and native birds is that in which these species have a low susceptibility and high resistance towards *Plasmodium* spp. (Westerdahl, 2012). This

would mean that parasitaemia wouldn't often develop in birds of these species, even if they were surrounded by many infected birds and appropriate mosquito vectors. However, when a parasitaemia would develop, the birds' immune system would manage to keep this at a very low level. An argument supporting this second theory is that some lineages of *Plasmodium* spp. may be endemic to New Zealand, and could potentially have been here for ~2.9 million years (Baillie and Brunton, 2011). This implies that NZ bird species have been in contact with certain *Plasmodium* spp. for a long time, and had time to develop genetic resistance towards these parasites (Ewen et al., 2012b). However, even though immunity may extend to closely related parasite lineages (Atkinson et al., 2001a), the detection of frequently occurring co-infection of *Plasmodium* spp. indicates that cross-immunity between *Plasmodium* spp. is not common (Valkiunas, 2005), making this theory less likely. In addition, outbreaks of avian malaria have been reported in endemic bird species, confirming their susceptibility (Alley et al., 2008; Alley et al., 2010; Banda et al., 2013; Reed, 1997). However, these events were mainly reported in birds in captive management situations or birds with concomitant infections, potentially leading to suppression the birds' immune system due to stress related elevated steroid levels (Cranfield et al., 1994).

For little penguins, our study confirmed the susceptibility to *Plasmodium* spp. of captive as well as wild birds. The fact that the frequency of avian malaria related mortality appeared less in wild little penguins, supports the theory that recrudescence of latent avian malaria infections or increased susceptibility to *Plasmodium* spp. infection is more likely in captive situations (Parsons and Underhill, 2005). An immune response against *Plasmodium* spp. has been demonstrated in wild and captive little penguins, suggesting that these birds have been previously infected and that consequently latent avian malaria infections may be present (Graczyk et al., 1995b). Resistance due to previous infections with *Plasmodium* spp. has been described before in African black-footed penguins (Beier and Stoskopf, 1980). Based on these previous reports, the low frequency of avian malaria related mortalities in little penguins may be due to an acquired resistance within the New Zealand population. Nevertheless, penguins in captivity should be routinely screened for avian malaria and action should be taken to minimise exposure to mosquitoes. This applies to

zoos as well as rehabilitation settings, such as temporary holding facilities during oil spill responses.

An important consideration is that co-adaptation between *Plasmodium* parasites and introduced bird species has likely resulted in immune modulation, leading to less damaging inflammatory responses in these bird species after infection (Atkinson et al., 2013). Down regulation of the immune system to infection with *Plasmodium* parasites in highly susceptible bird species could result in high levels of parasitaemia, without eliciting highly pathological reactions or causing high mortality rates. However, endemic and native bird species have only been exposed to introduced *Plasmodium* lineages for a relatively short time and immune modulation may not as yet have taken place. Thus, when infection occurs, damaging and excessive inflammatory reactions possibly take place rapidly at low levels of parasitaemia, and high parasite loads will likely lead to mortality. As *Plasmodium* spp. and introduced bird species both occur throughout New Zealand, avian malaria has likely become endemic in most areas and many endemic and native bird populations are likely not immunologically naïve to these parasites anymore. Even though genetic adaptation is expected to only be in its initial phase, a certain level of acquired resistance can reduce disease symptoms in case of superinfection with a homologous lineage (Atkinson et al., 2001a). This theory is supported by the qPCR results presented in this study, which reflected a much higher prevalence of avian malaria in endemic species than the nPCR, implying a higher survival rate in these species than was initially suspected.

In summary, introduced blackbirds continue to be an important reservoir for avian malaria in New Zealand and questions regarding susceptibility, tolerance and resistance to *Plasmodium* spp. in native and endemic bird species can only be answered further through the exploration of alternative future research options.

5.4 Future research

Besides ongoing monitoring of the occurrence of avian malaria in New Zealand birds, future research should encompass the use of different diagnostic techniques and research options, as well as evaluate ways to optimize the current molecular protocols.

5.4.1 Molecular protocols

A disadvantage of the qPCR protocol from Friedl and Groscurth (2012) is that the assay cannot be used to differentiate between *Plasmodium* spp. and lineages. Currently, *Plasmodium* spp. and lineages are routinely identified by DNA sequencing of nPCR products. However, in case of co-infection with multiple *Plasmodium* spp. or lineages, nPCR primers tend to multiply the lineage for which they have the highest affinity. Therefore, the resulting DNA sequencing data cannot be extrapolated to results of the qPCR, which may be quantifying the total amount *Plasmodium* DNA copies of all lineages that a bird is infected with. The use of lineage specific primers for qPCR has been described previously (Zehtindjiev et al., 2008). Exploring options to design qPCR primer sets that amplify more variable DNA target sequences, specific for *Plasmodium* spp. or lineages within New Zealand, may lead to a better understanding of parasite dynamics and virulence of these varies *Plasmodium* spp. and lineages. Sequencing whole genomes or full length cytochrome b genomes of the different *Plasmodium* spp. in New Zealand may be needed to design these species or lineage specific primers (Knowles et al., 2010a). Such primers will be extremely useful during controlled studies, or when targeting specific species lineages. For general screening, finding primers for highly variable target regions that enable the differentiation of *Plasmodium* species based on differences in melt-curve would also be valuable (Knowles et al., 2010b). In addition, whole genome sequencing would provide a deeper knowledge of phylogeny of *Plasmodium* lineages, and could help identify genes associated with infectivity and pathogenicity.

5.4.2 Serological screening of wild birds

Serological screening can reveal the true exposure of bird populations to *Plasmodium* spp. While a PCR could be negative for birds that are in the latent or chronic phase of infection,

with parasitaemias below the PCR detection limits, serological screening would detect anti-malarial antibodies, indicating previous exposure (Palmer et al., 2013; Sturrock and Tompkins, 2007). Low seroprevalences combined with low nPCR detection in a bird colony could mean that either the exposure to *Plasmodium* was low or that the infection is highly pathogenic and most infected birds don't survive the acute phase of infection. On the other hand a high seroprevalence, even in case of low PCR detection of *Plasmodium* spp., indicates that a high percentage of birds survives the acute phase of infection and has developed an acquired immunity (Atkinson et al., 2001b). Examples of tests that could be used in New Zealand birds are western blot (immunoblot), which has been used for serological screening of Hawaiian Iiwi (*Vestiaria coccinea*) (Atkinson et al., 1995), or indirect ELISA which has been used for serological screening of various international and New Zealand penguin species (Graczyk et al., 1995b; Palmer et al., 2013). The ELISA is genus specific, which means that it differentiates between antibodies against *Plasmodium* and *Haemoproteus* spp., but that it is unable to distinguish between antibodies for different *Plasmodium* species (Graczyk et al., 1994c). Thus these assays could help to determine naïve and exposed populations, but to identify compatible source and recipient locations for translocations, identification of de infecting *P*. species and lineages still has to take place.

5.4.3 Controlled challenge studies

Controlled challenge studies where naïve and exposed birds from various species are infected with a known *Plasmodium* lineage and the level of parasitaemia is measured over time by qPCR, are invaluable in getting a clear understanding of parasite dynamics, pathological signs and mortality. Combining prevalence data with data for parasite dynamics and levels of parasitaemia can reveal valuable information regarding tolerance and resistance to avian malaria in various bird species (Atkinson et al., 2013; Dimitrov et al., 2015; Westerdahl, 2012). Combining sequential measures of antibody response with parasite load using qPCR could be used to explore host response to malarial infection in greater detail and to examine the response to different malarial strains.

5.4.4 Long term effects of avian malaria

To study the long term effects of chronic *Plasmodium* infections on reproductive success, body condition and behaviour of New Zealand bird species, known positive birds would have to be monitored over multiple years and data of infected populations would have to be compared to those of uninfected birds groups. In addition, studies during times of high energy demands or limited food intake, such as during breeding, after migration, or during rehabilitation processes should be included in future research projects. It is plausible that a larger impact of *Plasmodium* spp. infection on BCI or reproduction will be seen when New Zealand birds are exposed to higher energy demands or restricted food intake, e.g. during breeding season or migration, as was demonstrated for female incubating American kestrels (Dawson and Bortolotti, 2000), breeding aquatic warblers (*Acrocephalus paludicola*) in Poland (Dyrcz et al., 2005) and migrating passerines in the Gulf of Mexico (Garvin et al., 2006). A negative correlation between clutch size and the level of parasitaemia was described for female Tengmalm's owls (*Aegolius funereus*) (Korpimaki et al., 1993) and yellow legged gulls (*Larus michahellis*) (Bosch et al., 1997).

5.4.5 Genetic studies

Susceptibility and tolerance to infection with *Plasmodium* spp. are for a large part influenced by a bird's genetic make-up. Whether certain immuno-alleles are present or absent can influence a bird's resistance to infection in a qualitative way (preventing establishment of the parasite in a bird) or in a quantitative way (reducing deleterious effects of a parasite on the host or reducing infection intensity) (Jarvi et al., 2013a; Westerdahl, 2012). Host-pathogen interaction is considered an important factor driving the diversity in these genes (Spurgin and Richardson, 2010). On the other hand, the variety in genes responsible for this innate immunity, which in turn plays an important role in adaptive immunity (Fearon and Lockskey, 1996), is likely an important driver for natural selection (Grueber et al., 2012). Traditionally, studies have been focused on genes of the major histocompatibility complex (MHC). For example, great reed warblers (*Acrocephalus arundinaceus)* were protected against lethal malaria infections if they carried either one specific MHC allele or a large number of different ones (Westerdahl et

al., 2005). A reduction in the heterogeneity of MHC genes has been demonstrated in NZ bird species that went through genetic bottlenecks, such as the South Island Saddleback (Alley et al., 2010; Sutton et al., 2013; Sutton et al., 2015), the NZ Black robin (*Petroica traversi*) (Miller and Lambert, 2004), and the little spotted kiwi (Miller et al., 2011), but the potential concurrent effect on susceptibility to *Plasmodium* spp. in these species is still unclear. Recent studies have focused on the diversity of toll-like receptor (TLR) genes to assess the genetic diversity and the potential of New Zealand birds to adapt to new pathogens (Jamieson, 2015). Toll-like receptors (TLRs) play a role in the recognition of different pathogens such as Gram-negative or positive bacteria, or fungi (Grueber et al., 2012). These innate immune responses are expected to play an important role in the evolution of resistance to novel diseases in wild populations, but the role of TLRs in resistance against *Plasmodium* spp. is currently unclear. Low levels of within-population variation at TLR loci may reflect a reduced potential of a population to adapt over time to changing environments or new pathogens (Grueber et al., 2012). Little is currently known regarding the role that TLR gene diversity plays in the disease susceptibility of threatened bird species; however, in a bottlenecked population of Stewart Island robins (*Petroica australis rakiura*) a sufficient level of TLR diversity was detected to justify future studies into this subject (Grueber et al., 2012). New Zealand has a threatened species history of small population sizes and historical inbreeding. The integration of genetic study data in wildlife management and translocation projects will help to predict the number of individuals needed to retain genetic diversity so that founder populations of threatened species can adapt to future environmental changes (Jamieson, 2015). Evaluating the presence and variety of MHC and TLR genes in NZ bird species and comparing these findings with *Plasmodium* spp. infection prevalence data, parasitaemia levels, pathological signs and mortality rates during controlled studies would greatly increase the understanding of avian malaria susceptibility and disease dynamics.

5.4.6 Social network analysis

Social network analysis, showing the actual overlap of habitats of various bird species within the research area and measuring mosquito densities and transmission-competence in different locations and in various seasons, is an interesting tool to achieve a more

accurate assessment of the expected level of avian malaria transmission between various bird species and would add another dimension to infection risk assessment. Bird species which prefer native forest as a habitat may not actually be in close contact with a high density of species that prefer farmland, even though these different geographical landscapes occur in the same region. How does the physical distance between species micro-habitats correlate with the transmission risk of avian malaria? How far will mosquito vectors travel to spread *Plasmodium* spp? The ecology of competent vectors for avian malaria can have a huge impact on New Zealand's avifauna. Patterns of mosquito aggregation among microhabitats could lead to highly localized differences in the transmission of *Plasmodium* lineages, while differences in phenology between mosquito species could lead to a varying infectious pressure during different times of the year (Kimura et al., 2010). *Culex quinquefasciatus*, the vector for *P. relictum* lineage GWR4 in Hawai'i, has spread over most parts of New Zealand, but data regarding the transmission-competence of the New Zealand mosquito populations are lacking. Mosquito vector related data could be highly valuable for disease management and risk analysis.

5.4.8 Disease ecology in a changing world

The most important thing to remember is that avian malaria in New Zealand is not a static picture. The geographic range and abundance of Plasmodium spp. and their mosquito vectors are likely to change with ongoing climate change, landscape changes and species evolution. Slight increases in temperature and local rainfall can lead to increased malaria prevalences by extending the mosquito vectors' breeding season and thus increasing the window for avian malaria transmission, and by shortening the incubation period of Plasmodium stages within the mosquito (Garamszegi, 2011). Consequently, transmission of highly pathogenic Plasmodium lineages that so far failed to establish at a high prevalence in New Zealand could become more successful. Although the presence of 17 Plasmodium lineages has currently been demonstrated in New Zealand, our understanding of Plasmodium dynamics and the impact of avian malaria on our endemic and native bird species is still rudimentary. Future research on parasite-host interactions will open a vast array of knowledge and will result in a whole new understanding of avian malaria in New Zealand, knowledge that is important to safeguard numbers of threatened

endemic species and to implement efficient and pro-active wildlife management strategies.

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APPENDICES

Appendix 1. Nested PCR protocol for the detection of *Plasmodium* spp.

Nucleic acid extraction	Kit
DNA	Qiagen DNeasy Blood and Tissue kit

Primers	Name	Sequence (5'-3')	Size	Target
Forward	NF1	5'-CATATATTAAGAGAAITATGGAG-3'	619	Cytochrome b gene (Hellgren et al., 2004)
Reverse	NR3	5'-ATAGAAAGATAAGAAATACCATTC-3'		
Forward	F	5'-ATGGTGCTTTCGATATATGCATG-3'	480	
Reverse	R2	5'-GCATTATCTGGATGTGATAATGGT-3'		

PCR kit	Invitrogen Taq Polymerase

First Round Reagent mix	Volume (µl) 25µL total
Sterile distilled	15.3
10x PCR buffer	2.55
MgCl2 (50mM)	0.75
dNTPs (10mM)	0.8
10 µM NF1 primer	1.5
10 µM NR3 primer	1.5
Taq	0.1
DNA	2.5

Second Round Reagent mix	Volume (µl) 50µL total
Sterile distilled	36.7
10x PCR buffer	5
MgCl2 (50mM)	1.5
dNTPs (10mM)	1.6
10 µM F primer	2
10 µM R2 primer	2
Taq	0.2
DNA	1 (from first round)

PCR controls	Description
Positive	*Plasmodium* σGSG (AFTRU5/LINN1)
Negative	Nuclease free water

PCR Program Name: Heam

Cycling parameters	Temp (°C)	Time	No. cycles
Hold	5	3 min	1
Denature	94	30 sec	
Anneal	50	30 sec	35 (1st round)
Extension	72	45 sec	40 (2nd round)
Hold	72	10 min	1
	4	hold	

Electrophoresis	Description	Size of amplicon (bp)
Agarose gel	1.5%	480
MW marker	100 bp	

Appendix 2. Real-time PCR protocol for the detection of *Plasmodium* spp.

Nucleic acid extraction	Kit
DNA	Qiagen DNeasy Blood and Tissue kit

Preparation of standard samples

Primers	Name	Sequence (5'-3')	Size	Target
Forward	L1	5'-GACCTGCATGAAAGATG-3'	595	large subunit ribosomal-RNA (LSU-rRNA)
Reverse	L2	5'-GTATCGCTTTAATAGGCG-3'		

Vector kit	*pGEM®-T Easy Vector Systems (Promega, Madison, USA)*
Cell line	E.coli JM109 high efficiency competent cells (Promega, Madison, USA)
Plasmid extraction	PureLink® Quick Plasmid Miniprep Kit (Invitrogen, Life technologies, Carlsbad USA)
Plasmid size	3610 bp (3.836 x 10^{-6} pg)

Real-time PCR

Primers	Name	Sequence (5'-3')	Size	Target
Forward	Plasmo474for	5'-AGGCTAATCTTTTCCGAGAGTCC-3'	85	large subunit ribosomal-RNA (LSU-rRNA)
Reverse	Plasmo558rev	5'-ACATACTACTGCTTTAGGATGCGA-3'		

Mastermix	PerfeCTa® *SYBR® Green FastMix* (Quanta Biosciences, Gaithersburg, USA)

Reagent mix	Volume (µl) 20µL total	Final concentration
Mastermix (2*x*)	10	1*x*
10 µM Plasmo474for primer	0.5	0.25µM
10 µM Plasmo558rev primer	0.5	0.25µM
DNA	*X*	100ng total DNA
Sterile distilled water	up to 20 µL	

PCR controls	Description	Volume added (µl)
Positive	7 10x standard dilutions, concentrations 1 to 10^{-6} ng/µl (LINN1)	1
Negative	Sterile distilled water	8

PCR system: Eco™ Real-Time PCR system (Eco™ Software V4.0.07; Illumina Inc., San Diego, CA, USA)

Cycling parameters	Temp (°C)	Time	No. cycles
Hold	50	2 min	1
Denature	95	10 min	1
Denature	95	15 sec	45
Anneal/Extension	60	1 min	
Melt Curve	95	15 sec	pre-melt conditioning
	55-95	15 sec	over 0.3deg increments wait 5 sec after each temp change
	95	15 sec	Final denature

Appendix 3. Bird data regarding weight, tarso-metatarsal length, packed cell volume (PCV), nested (nPCR) and real-time PCR (qPCR) results, including quantification cycle (Cq), dissociation temperature (Tm) and *Plasmodium* (*P.*) copies per 10.000 avian blood cells and DNA sequencing results

Band type	Band number	Species	Weight (g)	Tarsus (mm)	PCV (%)	nPCR	qPCR	Cq value	Tm	*P.* copies per 10.000 cells	Sequencing results	% GenBank Identicality
CP	4301	Bellbird	28.5	34.1	58	Neg	Neg	n/a	n/a	n/a		
DP	3306	Blackbird	94.0	36.2	40	Pos	Pos	21.0	77.	16148.31	LINN1	100
DP	3305	Blackbird	92.0	33.9	43	Pos	Pos	21.4	77.	12150.28	LINN1	100
DP	3329	Blackbird	93.0	35.8	37	Pos	Pos	23.9	77.	4938.41	SYATO5	99
DP	3325	Blackbird	77.0	32.0	46	Pos	Pos	24.3	77.	3714.70	SYATO5	100
DP	3302	Blackbird	97.0	38.9	37	Pos	Pos	24.6	77.	3937.16	LINN1	99
DP	3316	Blackbird	116.0	32.0	43	Pos	Pos	24.6	77.	2543.14	elongatum GRW6	99
DP	3399	Blackbird	86.0	34.4	44	Pos	Pos	25.0	77.	941.15	elongatum GRW6	99
DP	3327	Blackbird	89.0	36.9	45	Pos	Pos	25.1	77.	1005.73	LINN1	99
DP	3309	Blackbird	88.0	34.3	48	Pos	Pos	25.1	77.	2367.80	LINN1	100
DP	3333	Blackbird	87.0	35.7	45	Pos	Pos	26.8	77.	790.32	LINN1	99
DP	3334	Blackbird	90.0	33.5	47	Pos	Pos	27.0	77.	270.50	LINN1	100
DP	3320	Blackbird	102.0	34.4	36	Pos	Pos	27.0	77.	415.44	elongatum GRW6	99
DP	3332	Blackbird	89.0	34.0	45	Pos	Pos	27.5	77.	272.46		
DP	3314	Blackbird	88.0	32.5	46	Pos	Pos	28.1	77.	202.42	LINN1	100
DP	3324	Blackbird	89.0	36.2	38	Pos	Pos	28.3	77.	316.74		
DP	3326	Blackbird		36.0	47	Pos	Pos	28.3	77.	94.60	SYATO5	99
DP	3330	Blackbird	84.0	34.2	47	Pos	Pos	28.3	77.	81.61	LINN1	100
DP	3310	Blackbird	97.0	35.6	40	Pos	Pos	28.7	77.	72.28		
DP	3315	Blackbird	86.0	34.1	44	Pos	Pos	28.7	77.	116.25	LINN1	100
DP	3307	Blackbird	83.0	36.3	45	Pos	Pos	29.0	77.	57.19	elongatum GRW6	99
DP	3331	Blackbird	100.0	35.3	46	Pos	Pos	29.2	77.	68.39	LINN1	99
DP	3311	Blackbird	86.0	33.8	46	Pos	Pos	29.4	77.	55.04		

DP	3304	Blackbird	99.0	34.7	40	Pos	Pos	30.2	77.	28.07	SYATO5	100
DP	3319	Blackbird	99.0	33.9	56	Pos	Pos	30.9	77.	20.61	elongatum GRW6	99
DP	3318	Blackbird	96.0	32.8	48	Pos	Pos	32.4	77.	6.98		
DP	3323	Blackbird	105.0	34.5	36	Pos	Pos	34.6	77.	1.23	LINN1	100
DP	3303	Blackbird	94.0	35.2	55	Neg	Neg	n/a	n/a	n/a		
DP	3308	Blackbird	96.0	36.8	40	Neg	Neg	n/a	n/a	n/a		
DP	3312	Blackbird	104.0	36.4	44	Neg	Neg	n/a	n/a	n/a		
DP	3313	Blackbird	86.0	33.8	44	Neg	Neg	n/a	n/a	n/a		
DP	3317	Blackbird	95.0	33.7	50	Pos	Neg	n/a	n/a	n/a		
DP	3321	Blackbird	96.0	32.7	40	Pos	Neg	n/a	n/a	n/a		
DP	3322	Blackbird	86.0	34.8	43	Pos	Neg	n/a	n/a	n/a		
DP	3328	Blackbird	95.0	35.3	43	Neg	Neg	n/a	n/a	n/a		
BD	2	Blue duck	780.0	49.6	49	Neg	Pos	35.7	77.	1.66		
BD	13	Blue duck	890.0	53.6	51	Neg	Pos	38.7	77.	0.25		
BD	1	Blue duck	740.0	49.6	47	Neg	Neg	n/a	n/a	n/a		
BD	3	Blue duck	790.0	52.2	47	Neg	Neg	n/a	n/a	n/a		
BD	4	Blue duck	690.0	52.6	49	Neg	Neg	n/a	n/a	n/a		
BD	5	Blue duck	720.0	50.3	45	Neg	Neg	n/a	n/a	n/a		
BD	6	Blue duck	700.0	51.8	48	Neg	Neg	n/a	n/a	n/a		
BD	7	Blue duck	820.0	53.7	46	Neg	Neg	n/a	n/a	n/a		
BD	8	Blue duck	720.0	47.5		Neg	Neg	n/a	n/a	n/a		
BD	9	Blue duck	750.0	52.7	49	Neg	Neg	n/a	n/a	n/a		
BD	10	Blue duck	840.0	49.1	42	Neg	Neg	n/a	n/a	n/a		
BD	11	Blue duck	1070.0	52.2	47	Neg	Neg	n/a	n/a	n/a		
BD	12	Blue duck	850.0	48.2	52	Neg	Neg	n/a	n/a	n/a		
BD	14	Blue duck	740.0	49.8	53	Neg	Neg	n/a	n/a	n/a		
Pepe	Pepe	Chaffinch	13.0	19.7	53	Neg	Neg	n/a	n/a	n/a		
A	105035	Dunnock	9.0	20.7	44	Pos	Neg	n/a	n/a	n/a		

Appendix 3. Bird data continued

Band type	Band number	Species	Weight (g)	Tarsus (mm)	PCV (%)	nPCR	qPCR	Cq value	Tm	*P.* copies per 10.000 cells	Sequencing results	% GenBank Identicality
AX	406	Fantail	8.0	18.1	52	Neg	Neg	n/a	n/a	n/a		
AX	407	Fantail	6.0	19.3		Neg	Neg	n/a	n/a	n/a		
AX	409	Fantail	8.0	17.4	44	Neg	Neg	n/a	n/a	n/a		
AX	410	Fantail	8.0	18.7	47	Neg	Neg	n/a	n/a	n/a		
A	105022	Goldfinch	18.0	16.3	54	Neg	Neg	n/a	n/a	n/a		
AX	401	Grey warbler	6.5	25.4	61	Neg	Neg	n/a	n/a	n/a		
AX	403	Grey warbler	7.0	24.1		Neg	Neg	n/a	n/a	n/a		
BP	4572	North Island	31.0	36.9	48	Pos	Pos	26.1	77.	455.29	elongatum GRW6	99
BP	4543	North Island	30.0	36.7	46	Neg	Pos	30.2	77.	112.78		
BP	4552	North Island	32.5	36.8	45	Neg	Pos	32.5	77.	64.53		
BP	4571	North Island	31.5	37.8	45	Neg	Pos	33.0	77.	3.60		
BP	4580	North Island	31.0	37.7	44	Neg	Pos	33.0	78.	11.52		
BP	4561	North Island	32.5	37.4	40	Pos	Pos	33.3	76.	41.02	LINN1	100
BP	4596	North Island	29.5	37.2	47	Pos	Pos	33.4	77.	3.18	elongatum GRW6	99
BP	4528	North Island	34.0	41.3	45	Neg	Pos	33.4	77.	7.36		
BP	4551	North Island	30.0	36.3	42	Neg	Pos	33.6	77.	9.19		
BP	4554	North Island	34.0	38.5	44	Neg	Pos	33.8	77.	10.40		
A	105013	North Island	28.5	36.0	50	Neg	Pos	33.9	77.	2.54		
BP	4439	North Island	33.0	36.8	43	Neg	Pos	34.1	77.	4.93		
BP	4433	North Island	33.0	33.8	30	Neg	Pos	34.2	77.	5.33		
BP	4431	North Island	29.0	36.1	u	Neg	Pos	34.7	77.	3.12		
BP	4548	North Island	26.0	34.0	43	Neg	Pos	35.0	77.	13.87		
BP	4430	North Island	35.5	37.4	42	Neg	Pos	35.3	78.	2.36		
BP	4590	North Island	31.0	37.1	45	Neg	Pos	38.0	77.	0.15		
BP	4587	North Island	30.0	36.1	44	Neg	Pos	38.4	77.	0.08		
BP	4555	North Island	30.5	37.0	42	Neg	Pos	38.5	77.	0.27		

BP	4575	North Island	30.5	37.2	47	Neg	Pos	38.6	77.	1.42		
BP	3988	North Island	30.0	38.3	52	Neg	Neg	n/a	n/a	n/a		
BP	3989	North Island	29.0	36.9	57	Neg	Neg	n/a	n/a	n/a		
BP	4403	North Island	28.0	41.5	55	Neg	Neg	n/a	n/a	n/a		
BP	4425	North Island	31.5	35.7	45	Neg	Neg	n/a	n/a	n/a		
BP	4426	North Island	33.0	37.7	45	Neg	Neg	n/a	n/a	n/a		
BP	4427	North Island	32.0	36.9	40	Neg	Neg	n/a	n/a	n/a		
BP	4428	North Island	31.0	37.9	46	Neg	Neg	n/a	n/a	n/a		
BP	4429	North Island	30.0	36.1	42	Neg	Neg	n/a	n/a	n/a		
BP	4432	North Island	31.5	36.2	40	Neg	Neg	n/a	n/a	n/a		
BP	4434	North Island	33.0	36.4		Neg	Pos	35.4	76.	n/a		
BP	4435	North Island	33.5	35.9	40	Neg	Neg	n/a	n/a	n/a		
BP	4436	North Island	35.5	37.0	45	Neg	Neg	n/a	n/a	n/a		
BP	4437	North Island	33.0	37.6	36	Neg	Neg	n/a	n/a	n/a		
BP	4438	North Island	34.5	35.8	48	Neg	Neg	n/a	n/a	n/a		
BP	4440	North Island	30.0	35.8	40	Pos	Neg	n/a	n/a	n/a	elongatum GRW6	99
BP	4441	North Island	33.0	37.0	43	Neg	Neg	n/a	n/a	n/a		
BP	4442	North Island	31.0	35.3	40	Neg	Neg	n/a	n/a	n/a		
BP	4501	North Island	31.5	37.1	54	Neg	Neg	n/a	n/a	n/a		
BP	4502	North Island	29.0	37.2	58	Neg	Neg	n/a	n/a	n/a		
BP	4516	North Island	33.0	36.8	53	Neg	Neg	n/a	n/a	n/a		
BP	4517	North Island	30.5	37.2	49	Neg	Neg	n/a	n/a	n/a		
BP	4518	North Island	29.5	37.5	57	Neg	Neg	n/a	n/a	n/a		
BP	4519	North Island	30.0	37.7	47	Neg	Neg	n/a	n/a	n/a		
BP	4520	North Island	29.0	36.9	48	Neg	Neg	n/a	n/a	n/a		
BP	4521	North Island	30.0	38.9	41	Neg	Neg	n/a	n/a	n/a		
BP	4522	North Island	31.0	41.2	41	Neg	Neg	n/a	n/a	n/a		
BP	4523	North Island	31.0	40.8	50	Neg	Neg	n/a	n/a	n/a		

Appendix 3. Bird data continued

Band type	Band number	Species	Weight (g)	Tarsus (mm)	PCV (%)	nPCR	qPCR	Cq value	Tm	*P.* copies per 10.000 cells	Sequencing results	% GenBank Identicality
BP	4524	North Island	29.0	41.5	42	Neg	Neg	n/a	n/a	n/a		
BP	4525	North Island	30.0	40.9	40	Neg	Neg	n/a	n/a	n/a		
BP	4526	North Island	32.0	40.7	40	Neg	Neg	n/a	n/a	n/a		
BP	4530	North Island	33.5	34.6	42	Neg	Neg	n/a	n/a	n/a		
BP	4531	North Island	31.5	36.7	40	Neg	Neg	n/a	n/a	n/a		
BP	4541	North Island	27.5	34.5	48	Neg	Neg	n/a	n/a	n/a		
BP	4542	North Island	31.5	37.4	50	Neg	Neg	n/a	n/a	n/a		
BP	4544	North Island	29.0	37.0	43	Neg	Neg	n/a	n/a	n/a		
BP	4545	North Island	30.5	35.2	42	Neg	Neg	n/a	n/a	n/a		
BP	4546	North Island	26.5	36.1	60	Neg	Neg	n/a	n/a	n/a		
BP	4547	North Island	32.0	38.5	45	Neg	Neg	n/a	n/a	n/a		
BP	4549	North Island	29.0	35.0	46	Neg	Neg	n/a	n/a	n/a		
BP	4550	North Island	31.0	33.7	43	Neg	Neg	n/a	n/a	n/a		
BP	4553	North Island	29.5	37.1	42	Neg	Neg	n/a	n/a	n/a		
BP	4556	North Island	32.5	37.2	48	Neg	Neg	n/a	n/a	n/a		
BP	4562	North Island	36.5	39.1	45	Neg	Neg	n/a	n/a	n/a		
BP	4563	North Island	33.0	36.5	50	Neg	Neg	n/a	n/a	n/a		
BP	4564	North Island	33.0	40.3	50	Neg	Neg	n/a	n/a	n/a		
BP	4565	North Island	31.5	36.4		Neg	Neg	n/a	n/a	n/a		
BP	4567	North Island	32.5	37.3	47	Neg	Neg	n/a	n/a	n/a		
BP	4568	North Island	30.0	36.0	45	Neg	Neg	n/a	n/a	n/a		
BP	4569	North Island	34.5	32.2	42	Neg	Neg	n/a	n/a	n/a		
BP	4570	North Island	28.0	35.9	47	Neg	Neg	n/a	n/a	n/a		
BP	4573	North Island	30.5	36.3		Neg	Neg	n/a	n/a	n/a		
BP	4574	North Island	33.0	38.1	46	Neg	Neg	n/a	n/a	n/a		
BP	4576	North Island	31.0	38.7	45	Neg	Neg	n/a	n/a	n/a		

BP	4577	North Island	34.0	36.3	42	Neg	Neg	n/a	n/a	n/a		
BP	4579	North Island	32.5	38.3	45	Neg	Neg	n/a	n/a	n/a		
BP	4582	North Island	31.5	37.3	51	Neg	Neg	n/a	n/a	n/a		
BP	4583	North Island	29.5	37.8	47	Neg	Neg	n/a	n/a	n/a		
BP	4584	North Island	29.5	38.6	52	Neg	Neg	n/a	n/a	n/a		
BP	4585	North Island	30.0	36.8	47	Neg	Neg	n/a	n/a	n/a		
BP	4586	North Island	34.0	36.2	46	Neg	Neg	n/a	n/a	n/a		
BP	4588	North Island	30.0	38.5	48	Neg	Neg	n/a	n/a	n/a		
BP	4589	North Island	29.5	37.0	46	Neg	Neg	n/a	n/a	n/a		
BP	4591	North Island	31.0	36.5	51	Neg	Neg	n/a	n/a	n/a		
BP	4592	North Island	28.0	36.5	38	Neg	Neg	n/a	n/a	n/a		
BP	4593	North Island	27.0	37.2	56	Neg	Neg	n/a	n/a	n/a		
BP	4594	North Island	30.0	37.5	47	Neg	Neg	n/a	n/a	n/a		
BP	4595	North Island	30.0	38.5	46	Neg	Neg	n/a	n/a	n/a		
BP	4597	North Island	30.0	37.5	47	Neg	Neg	n/a	n/a	n/a		
BP	4598	North Island	26.0	37.2	45	Neg	Neg	n/a	n/a	n/a		
BP	4599	North Island	28.0	36.9	48	Neg	Neg	n/a	n/a	n/a		
BP	4600	North Island	29.0	37.3	49	Neg	Neg	n/a	n/a	n/a		
B	88933	North Island	35.0	35.4	45	Neg	Neg	n/a	n/a	n/a		
A	105006	North Island	28.5	37.6	48	Neg	Neg	n/a	n/a	n/a		
A	105007	North Island	26.5	35.6	51	Neg	Neg	n/a	n/a	n/a		
A	105008	North Island	27.5	35.0	52	Neg	Neg	n/a	n/a	n/a		
A	105009	North Island	29.5	37.3	48	Neg	Neg	n/a	n/a	n/a		
A	105010	North Island	28.5	35.5	47	Neg	Neg	n/a	n/a	n/a		
A	105012	North Island	28.0	36.4	51	Neg	Neg	n/a	n/a	n/a		
A	105014	North Island	30.0	36.5	57	Neg	Neg	n/a	n/a	n/a		
A	105015	North Island	29.0	36.7	49	Neg	Neg	n/a	n/a	n/a		
A	105029	Silvereye	12.0	17.9	54	Pos	Pos	32.1	77.	8.47	LINN1	100

Appendix 3. Bird data continued

Band type	Band number	Species	Weight (g)	Tarsus (mm)	PCV (%)	nPCR	qPCR	Cq value	Tm	*P.* copies per 10.000 cells	Sequencing results	% GenBank Identicality
A	105024	Silvereye	13.0	19.8	60	Pos	Pos	35.0	77.	1.28		
A	105041	Silvereye	12.0	18.8	45	Pos	Pos	35.1	77.	1.04		
A	105016	Silvereye	23.0	22.0	45	Pos	Pos	39.1	77.	0.27	LINN1	100
AP	9001	Silvereye	13.0	21.6	44	Neg	Neg	n/a	n/a	n/a		
AP	9098	Silvereye	10.0	18.1	50	Neg	Neg	n/a	n/a	n/a		
A	105017	Silvereye	13.0	18.2	25	Neg	Neg	n/a	n/a	n/a		
A	105018	Silvereye	13.0	18.8	30	Neg	Neg	n/a	n/a	n/a		
A	105019	Silvereye	11.0	18.5	47	Neg	Neg	n/a	n/a	n/a		
A	105020	Silvereye	13.0	18.5	30	Neg	Neg	n/a	n/a	n/a		
A	105021	Silvereye	8.0	18.0	58	Neg	Neg	n/a	n/a	n/a		
A	105023	Silvereye	13.0	18.9	52	Pos	Neg	n/a	n/a	n/a		
A	105025	Silvereye	11.0	18.2	53	Neg	Neg	n/a	n/a	n/a		
A	105026	Silvereye	12.0	19.7	52	Neg	Neg	n/a	n/a	n/a		
A	105027	Silvereye	11.0	18.0	52	Neg	Neg	n/a	n/a	n/a		
A	105028	Silvereye	12.0	18.2	49	Neg	Neg	n/a	n/a	n/a		
A	105030	Silvereye	11.0	18.2	50	Neg	Neg	n/a	n/a	n/a		
A	105031	Silvereye	13.0	19.5	47	Neg	Neg	n/a	n/a	n/a		
A	105032	Silvereye	13.0	17.9	45	Neg	Neg	n/a	n/a	n/a		
A	105033	Silvereye	13.0	17.8	58	Pos	Neg	n/a	n/a	n/a		
A	105034	Silvereye	13.0	18.5	45	Neg	Neg	n/a	n/a	n/a		
A	105036	Silvereye	11.0	19.5	48	Neg	Neg	n/a	n/a	n/a		
A	105037	Silvereye	12.0	19.1	45	Neg	Neg	n/a	n/a	n/a		
A	105038	Silvereye	12.0	19.7	50	Neg	Neg	n/a	n/a	n/a		
A	105039	Silvereye	13.0	18.9	48	Pos	Neg	n/a	n/a	n/a		
A	105040	Silvereye	12.0	18.1	46	Neg	Neg	n/a	n/a	n/a		
A	105042	Silvereye	12.0	18.9	46	Neg	Neg	n/a	n/a	n/a		

A	105043	Silvereye	12.0	19.0	48	Neg	Neg	n/a	n/a	n/a		
A	105044	Silvereye	10.0	19.8	45	Neg	Neg	n/a	n/a	n/a		
A	105045	Silvereye	11.0	19.8	50	Neg	Neg	n/a	n/a	n/a		
A	105046	Silvereye	10.0	19.8	58	Neg	Neg	n/a	n/a	n/a		
A	105047	Silvereye	14.0	18.0		Neg	Neg	n/a	n/a	n/a		
David	David	Silvereye	13.0	18.1		Neg	Neg	n/a	n/a	n/a		
CP	4398b	Song Thrush	69.0	32.0	41	Pos	Pos	21.2	77.	7346.79	LINN1	99
CP	4398a	Song Thrush	69.0	32.0	41	Pos	Pos	22.8	77.	4571.44		
CP	4400	Song Thrush	73.0	33.0	35	Pos	Pos	25.0	77.	2566.57	elongatum GRW6	99
CP	4399	Song Thrush	73.0	31.0	44	Pos	Neg	n/a	n/a	n/a		
AX	402	Tomtit	8.0	24.8		Neg	Pos	36.2	77.	0.54		
AP	9099	Tomtit	11.0	21.6		Neg	Pos	37.3	77.	0.79		
AP	9100	Tomtit	10.0	22.2		Neg	Neg	n/a	n/a	n/a		
DP	3400	Tui	93.0	36.5	52	Neg	Neg	n/a	n/a	n/a		
BP	4401	Whitehead	18.5	35.6	45	Neg	Neg	n/a	n/a	n/a		
BP	4402	Whitehead	13.0	31.7	50	Neg	Neg	n/a	n/a	n/a		
BP	4404	Whitehead	17.0	28.4	50	Neg	Neg	n/a	n/a	n/a		