The impact of conservation translocations on vector-borne parasites

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Ecology at Massey University, Palmerston North, New Zealand

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Abstract

Wildlife conservation in New Zealand relies on translocations of endangered species to safe sites. While knowledge of the biology and behaviour of translocated hosts has steadily increased, the role of parasites in wildlife translocations has been largely overlooked. Parasites can affect their host's survivorship during translocations by causing disease. However, failure to translocate or reintroduce a host specific parasite with its endangered host can contribute to the extinction of the parasite with unforeseen consequences for the future of the host or even the whole ecosystem. The main aims of this study were to establish baseline data on the impact of North Island saddleback translocations on their avian malaria (*Plasmodium* spp.) parasites as well as gaining further insight into potential vectors in New Zealand. The study was also intended to contribute to the development of recommendations for future parasite screening programmes for native passerine translocations. Saddlebacks and *Plasmodium* were chosen because of the detailed saddleback translocation history and its known relationship with the parasite.

As a result of this study, several *Plasmodium* lineages previously unrecorded in saddlebacks and New Zealand were identified, for example, the native Kokako01 and one lineage closest related to two lineages from the Americas. Nonetheless, the most frequent lineages found were the cosmopolitan *P. elongatum* GRW6 and LINN1, and *P. vaughani* SYAT05, common in birds introduced to New Zealand. This finding suggests that endemic parasites may have already become rare or extinct. In addition, *Plasmodium* DNA was detected in both native and introduced mosquitoes that may act as vectors. A qPCR assay was developed that was found to be a cost effective and rapid screening tool for the detection of *Plasmodium* in native birds suffering from acute infection, presenting with clinical symptoms, and in birds that were found dead. .

I conclude that future translocations should consider the movement of endemic parasites with their hosts. How this should happen is open for future studies. However, I urge managers to start considering this issue now as New Zealand has already recorded the extinction of one endemic parasite and many more may have already been lost without knowledge.

Acknowledgements

Having fun hunting mosquitoes in the New Zealand bush (picture by courtesy of Gillian Gordon)

Foremost, I have to thank my parents and family who enabled me to come to New Zealand and provided me with ongoing support. I am also very grateful to my wonderful supervisors, Isabel Castro, Laryssa Howe, Daniel Tompkins and Kevin Parker, for their patience and understanding. Without them I might have had many more difficulties and I owe them very much for their help in crossing the hard patches. They were always there when I needed them, to share new discoveries as well as tears, which has earned them my utmost respect and appreciation.

I would like to thank the following iwi for access to their ancestral land to collect samples (Te Arawa, Ngati Wai and Ngati Whatua). It was a wonderful experience to visit these magical places.

I also would like to thank Tamsin Ward-Smith and the team of Cape Sanctuary (Cape Kidnappers, Hawkes Bay) for access to the sanctuary as well as taking me along for the saddleback translocation from Cuvier Island to the Cape. That was a very unique trip!

I am also grateful to Allan Anderson and the Bushy Park Trust for access, logistical support and accommodation at Bushy Park. Bushy Park is such a great pristine treat in the agricultural monotony of the lower North Island.

I would like to thank the Whangarei City Council for permission to sample introduced birds in Mair Park in Whangarei.

This study would not have been possible without the help of many enthusiastic volunteers and colleagues, they are too many and I am in danger of forgetting one of you. So to all of you, thank you very much!!! I especially want to thank Bethany Jackson for assisting me with mosquito sampling on Tiritiri Matangi Island and Josie Galbraith for letting me join her for birds sampling in Auckland.

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Thanks also goes to Chris Good from the Massey library team for his help with Endnote and Massey University International Student Support and Victoria Sibley for help and advice on English spelling, grammar and punctuation.

Preface

This thesis is formatted in a series of distinct research manuscripts ready for publication. As a consequence, the individual chapters contain unavoidable repetition. This thesis is the original work of the author, unless otherwise stated in the references, methods and acknowledgements.

The field methods used in this study were the same as those used by Dr Isabel Castro and collaborators when studying the epidemiology of avian malaria in New Zealand passerines in 2007/2008.

The animal ethics protocol for this study was approved by the Animal Ethics Committee at Massey University, MUAEC Protocol 11/59. Birds from different offshore islands were sampled under the following Department of Conservation permits: NO-33680-FAU, TW-32756-FAU and ECBP-32634-RES. Birds were banded under institutional banding permit No. 2012/009.

This study would not have been possible without the generous funding by the Morris Animal Foundation, study grant ID number D13ZO-811: Do Translocations for Species Restoration Cause Pathogen Pollution?

I would like to advise the reader that this thesis started out as a study on wildlife translocations and their potential to cause pathogen pollution. During the work, and after receiving the first results, the emphasis shifted away from the potential impact of parasites on wildlife translocations towards the impact of wildlife translocations on native parasites, which can potentially cause extinction of rare parasites. This shift can be noted through the earlier chapters of this thesis, in particular as I carried out a review of the pathogen pollution literature and developed hypotheses and predictions that were directed towards explaining pathogen pollution. Please consider this when reading.

The raw data for this thesis can be found at the back of this document, in Appendix 6.

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With few exceptions, all the pictures in this thesis have been taken by the author, Ellen Schoener. Photographs from other sources have been acknowledged in the legends.

List of tables

List of abbreviations

°C	Temperature in degrees centigrade
µl	Microlitres
µm	Micrometres
µM	Micromoles
BLAST	Basic local alignment search tool
bp	Base pairs
CDC	Center for Disease Control
cm	Centimetres
CNS	Central nervous system
CO_2	Carbon dioxide
Cq value	Quantification cycle value
Ct values	PCR crossing points
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
DoC	Department of Conservation
EDTA	Ethylenediaminetetraacetic acid
et al.	Et alia/and others
g	Grams
H&E	Haematoxylin and eosin stain
ha	Hectare
hr	Hour
HRM	High resolution melting
IUCN	International Union for the Conservation of Nature and Natural resources
IVABS	Institute of Veterinary, Animal and Biomedical Sciences (Massey University)
kDa	Kilodalton
mg	Milligrams
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
min	Minute
MIR	Minimum infection rate
ml	Millilitres
mm	Millimetres
mM	Millimoles
MUAEC	Massey University Animal Ethics Committee

MYA	Million years
n	Number
ng	Nanograms
NI	North Island (of New Zealand)
NZ	New Zealand
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR/real-time PCR
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
spp.	Plural of species
SSC	Species survival commission
T_m	Melting temperature

Chapter 1: Introduction

1.1 Importance and aims of this study

"Considering these and many other major and still growing impacts of human activities on earth and atmosphere, and at all, including global, scales, it seems to us more than appropriate to emphasize the central role of mankind in geology and ecology by proposing to use the term “Anthropocene” for the current geological epoch. The impacts of current human activities will continue over long periods.”
(Crutzen, 2002)

Today we are living in a world more and more removed from an original ”natural” state, with humanity as an unstoppable changing force of geological proportions (Crutzen, 2002). The biosphere is rapidly changing and biodiversity worldwide is in decline due to human impacts, including pollution, habitat destruction and the overexploitation of resources. Recently, scientists started asking whether we are now experiencing the sixth mass extinction (Wake and Vredenburg, 2008, Barnosky *et al.*, 2011, Pievani, 2014). The earth has undergone mass extinctions previously in its history; the best known one happened at the end of the Cretaceous period and wiped out the non-avian dinosaurs. These extinction events have shaped the biosphere as we know it today and once nearly wiped out life altogether when 96% of all species died at the end of the Permian period (Barnosky *et al.*, 2011). The proposition of a new mass extinction event, this time caused by humanity, appears plausible since the current extinction rate is dramatic, and is far above the estimated background extinction levels (Barnosky *et al.*, 2011). For example, a whole class of animals (the amphibians) is now threatened by extinction (Wake and Vredenburg, 2008).

This problem appears overwhelming, with more bad news published continuously. In addition to direct human impact on biodiversity, climate change may amplify the effect and accelerate extinction risks (Urban, 2015). Together with the big charismatic vertebrate species, there is an even wider range of species disappearing: invertebrates of poorly known and researched taxa (Regnier *et al.*,

2015) and species depending on other species to survive, like mutualists and parasites (Dunn *et al.*, 2009).

But all is not lost. Worldwide, conservation efforts are becoming more elaborate and are widely supported by the public. Knowledge of current conservation issues is becoming more and more widespread and books on these topics are on the bestseller lists and are winning prestigious prizes like the Pulitzer prize, as happened in 2015 with ”The Sixth Extinction: An Unnatural History" by Elizabeth Kolbert.

This thesis is a very small part in the global effort to preserve biodiversity, and it is trying to create baseline data which may be helpful guiding future conservation decisions. The topic I examine herein is wildlife translocations, the intentional movement and release of a living organism for conservation purposes (more details in Chapter 3). Wildlife translocations are a vital part of wildlife conservation today and have become more important on a global scale as more restoration areas become available and captive breeding programmes for wildlife reintroductions become more successful. Translocations are a vital tool to mitigate man-made threats for at least some endangered species. In New Zealand, species reintroductions and translocations for conservation purposes have become the preferred methods in species recovery programmes (Craig *et al.*, 2000, Cunningham, 1996, Mathews *et al.*, 2006). This is because of progress in pest control and the creation of large mainland islands that are either predator free or intensively controlled, which allows the its recolonizing of these areas with native species (Craig *et al*., 2000). In the past, parasites infecting translocated animals have often been overlooked, for example when introducing chytrid fungus (*Batrachochytrium dendrobatidis*) to a wild population with captive bred Mallorcan mid wife toads (*Alytes muletensis*) (Walker *et al.*, 2008). Therefore, reintroductions and translocations can pose a threat to the pre-existing avifauna through the introduction of pathogens (“pathogen pollution”), and to the translocated birds through exposure to pathogens already present in the release area and its inhabitants (Cunningham, 1996, IUCN guidelines 1998). On the other hand, parasites specific to the translocated hosts can get lost during a translocation. It can be assumed that parasites make up the unseen majority of species extinctions (Dunn *et al.*, 2009, Lafferty, 2012); and biodiversity loss may reduce parasite diversity more than previously thought (Lafferty, 2012). A loss of parasites may be

positive for the individuals in the short term, but it may lead to a loss of genetic variation in immunity within the species and therefore make the species more susceptible to disease outbreaks in the long term (Sainsbury, 2015, Altizer *et al.*, 2003, Smith *et al.*, 2009). In addition, parasites play a key role in ecosystem stability and their demise would be generally detrimental (Lafferty *et al.*, 2008, Hudson *et al.*, 2006)

The main objective of this study is to examine the impact of wildlife translocations on the parasite communities of the translocated hosts. For this, I am using the North Island saddleback (*Philesturnus rufusater*) and its infections with different species of *Plasmodium* spp., as well as their potential mosquito vectors, as a model system.

1.2 Structure of this thesis

This thesis will be divided into seven chapters, including two review and three research chapters.

Chapter 2- Avian malaria (global and New Zealand perspectives): Avian malaria parasites (*Plasmodium* spp.) have been chosen for this study as They are common vector borne pathogens in passerine birds worldwide. They have the ability to cause disease and mortality in their hosts and infections can therefore become a serious issue for bird conservation. In this chapter I examine the history of Avian Malaria infections as well as taxonomy, lifecycle, epidemiology, pathology, diagnosis and impact of these parasites in birds worldwide and in New Zealand. Part of this chapter was published as (Schoener *et al.*, 2014).

Chapter 3- Wildlife translocation and parasites- Pathogen pollution and loss of parasites: Wildlife translocations for conservation are becoming more and more important and frequent, but they are also the potential source of a diverse suite of problems. Here I review the history of wildlife translocations internationally and in New Zealand and examine issues that have appeared in previous years. The translocation history of my study species, the North Island saddleback, is presented in detail. The main focus of this chapter is on the impact translocations may have on pathogens, and therefore wildlife health, a question I will also examine in detail in my own research in Chapters 5 and 6.

Chapter 4- A new real time PCR for the rapid diagnosis of the four most important lineages of avian malaria in New Zealand: Molecular methods have become the most widespread way of diagnosing *Plasmodium* infections. They require less experience and time than previously used microscopic examinations, but they are also more expensive and less specific. Molecular methods today are underestimating concurrent infections by several parasite species/lineages (mixed) and require sequencing to determine parasite species identity. To make rapid screening of native birds in New Zealand cheaper and faster, I developed a new method for diagnosing the four most common and ubiquitous lineages of avian malaria in New Zealand. This method uses real time PCR and high resolution melt (HRM) technology and is able to detect mixed infections.

Chapter 5- A bottleneck not just for the hosts: the impact of translocations of North Island saddleback on the diversity of their *Plasmodium* parasites: Their well documented translocation history makes the North Island saddleback an excellent bird species to examine the impact of wildlife translocation on associated parasites. The main emphasis of this study was to find out the possibility and extent of saddleback translocations moving *Plasmodium* spp. that could cause disease at the release site (pathogen pollution) versus the loss of parasite species and lineages after translocations.

Chapter 6- New insight into avian malaria vectors in New Zealand: Insect vectors are compulsory for malaria parasites to complete their life cycle and therefore should not be overlooked when studying *Plasmodium* spp. So far, mosquitoes as vectors in New Zealand have not been studied in any detail and baseline data is needed. My study is the first in New Zealand to identify potential *Plasmodium* vectors around the North Island.

Chapter 7- General discussion: Does the translocation of parasites with their host pose a threat to the host population? Or is the loss of parasite species due to conservation translocations a greater threat to biodiversity? These questions and possible answers are discussed and the results of my research are concluded. I also suggest steps for future conservation management options as well as ideas for future research.

1.3 Bird species used in this study

1.3.1 Natural history of the North Island saddleback *Philesturnus rufusater*

The saddleback is a range restricted endemic bird that belongs to the family of wattlebirds (Callaeidae) which is endemic to New Zealand. Until recently, the North Island saddleback (*Philesturnus rufusater*) and the South Island saddleback (*P. carunculatus*) were seen as two subspecies, but there appears to be sufficient morphological, behavioural and genetic evidence to justify the classification as two separate species (Parker *et al.*, 2014). They are now listed as two separate species in the checklist of the birds of New Zealand (Gill *et al.*, 2010).

Characteristic of this family are the colourful fleshy wattles at the gape of the beak, and short, rounded wings that limit their flight (Colour Plate 1; Hanzab, 2006). Other members of this family are the kokako (*Callaeas cinerea*) and the extinct huia (*Heterolocha acutirostris*). This family has no close relatives anywhere in the world; the closest group taxonomically is the family Notiomystidae with a single species *Notiomystis cincta* that separated from saddlebacks 34 MYA during the Oligocene (Ewen *et al.*, 2006, Driskell *et al.*, 2007).

The North Island saddleback has a body length of 25cm, with males weighing on average 80.0 (70—88) g and females 66.7 (61-75) g. The South Island saddleback is slightly larger, with the males weighing on average 83.8 (80-88) g and the females average around 73.8 (71-78) g (Higgins, 2006). Saddlebacks are forest birds that prefer the middle and lower layers of vegetation. They feed on invertebrates, but also take fruit and nectar when available, and possess a brush tip tongue (Higgins, 2006).

Both species of saddleback are vulnerable to the destruction of their natural habitat and introduced mammalian predators (Hooson and Jamieson, 2003). The North Island saddleback became extinct on the mainland of the North Island in 1910. A single population of less than 500 birds (Merton, 1973) survived on Hen Island (Hauraki Gulf, off shore North Island) from where individuals were sourced for several successful translocations to other islands. Currently, more than 6000 birds (in 2008; (Parker, 2008)) survive on offshore and mainland islands (Table 1) (Hooson and Jamieson, 2003). For the well documented translocation history of the North Island saddleback, see Chapter 3.

The South Island saddleback became initially confined to Big South Cape Island and two small islets near Stewart Island, but these were invaded by rats in 1962. A transfer of the surviving 36 saddleback to the rodent free islands Kaimohu and Big Island saved the birds from extinction. Today, around 1200 South Island saddleback (data from 2003) survive on 15 offshore islands around Stewart Island, Fiordland and the Marlborough Sounds (Table 2; Higgins, 2006; Hooson and Jamieson, 2003).

1.3.2 New Zealand saddleback and avian malaria

The New Zealand saddleback is known to be a host to *Plasmodium* spp., (Castro *et al.*, 2011) with a recent disease outbreak in 2007 in the South Island saddleback on Long Island (Hale, 2008). Recent studies by Castro et al. (2011) and Howe et al. (2012) report three different *Plasmodium* spp. isolates which belong to two different taxonomic clades (*P. elongatum* and *P. relictum*). These parasites cause subclinical and potentially chronic infections (Section 2.1.7.4) in North Island saddleback and some are possibly endemic to New Zealand (Castro, Howe, pers. comm.). In their 2011 paper, Howe et al. (2012) found that one *Plasmodium* isolate from North Island saddleback had 100% sequence homology with isolates found in a dead blackbird (*Turdus merula*) and great spotted kiwi (*Apteryx haastii*). Therefore, the isolate has been found in the two major bird superorders (paleognathous and neognathous), and while this parasite has an apparent pathogenicity to both great spotted kiwi and blackbird, North Island saddleback show only low level parasitaemia, possibly indicative of chronic infections and seem to either be resistant or have adapted to this parasite (Howe *et al*., 2012). However, there may also be mortality in the acute phase of the disease which may be undetected (Chapter 5). Following these results, one question to be asked is if the translocations of saddleback from their source populations to islands around New Zealand have introduced lineages of *Plasmodium* spp. into naïve environments and hosts.

1.3.3 Blackbirds (*Turdus merula*) in New Zealand

The blackbird is an introduction from Europe in the late 1800s and a member of the Muscicapidae family. It is a medium sized thrush with a length of about 25cm with a weight of around 90g, and is widespread in New Zealand. Blackbirds inhabit mainly urban and other settled areas, preferring modified habitat with dense cover of shrubs, usually parks, gardens and farmland. The adult male is entirely black, with an orange-yellow bill and orbital ring. The adult female is of dark brown colour.

Blackbirds are omnivorous and mainly take invertebrates from the ground, but also fruit and seeds. In this regard they are similar to saddlebacks.

Blackbirds in New Zealand have a high prevalence for *Plasmodium* spp., which supports an existing hypothesis that this species is a maintenance host which thus allows spill over infection to native birds (Tompkins, 2008).

Colour Plate 1: Study species, the North Island saddleback, Philesturnus rufusater. A) Saddleback on Tiritiri Matangi Island; picture by courtesy of Melanie Leech. B) Saddleback male close-up. Note the large fleshy wattles.

Colour Plate 2: Bird sampling (1). A) Mist nets are erected in the forest. Here the mist net is set up in a waterhole frequented by birds on Mokoia Island. B) Birds that are caught are placed in cloth bags. C) Every bird is checked for health status, here the author (E.S.) is examining the body condition of a Tui (*Prosthemadera novaseelandiae*). D) Birds are marked with metal bands to enable identification of the individual.

Colour Plate 3: Bird sampling (2). A) Sampling station in a historic building on Cuvier Island. B) Every bird is weighed. Picture by courtesy of Allan Anderson. C) After blood sampling, saddlebacks are given the opportunity to drink some nectar solution

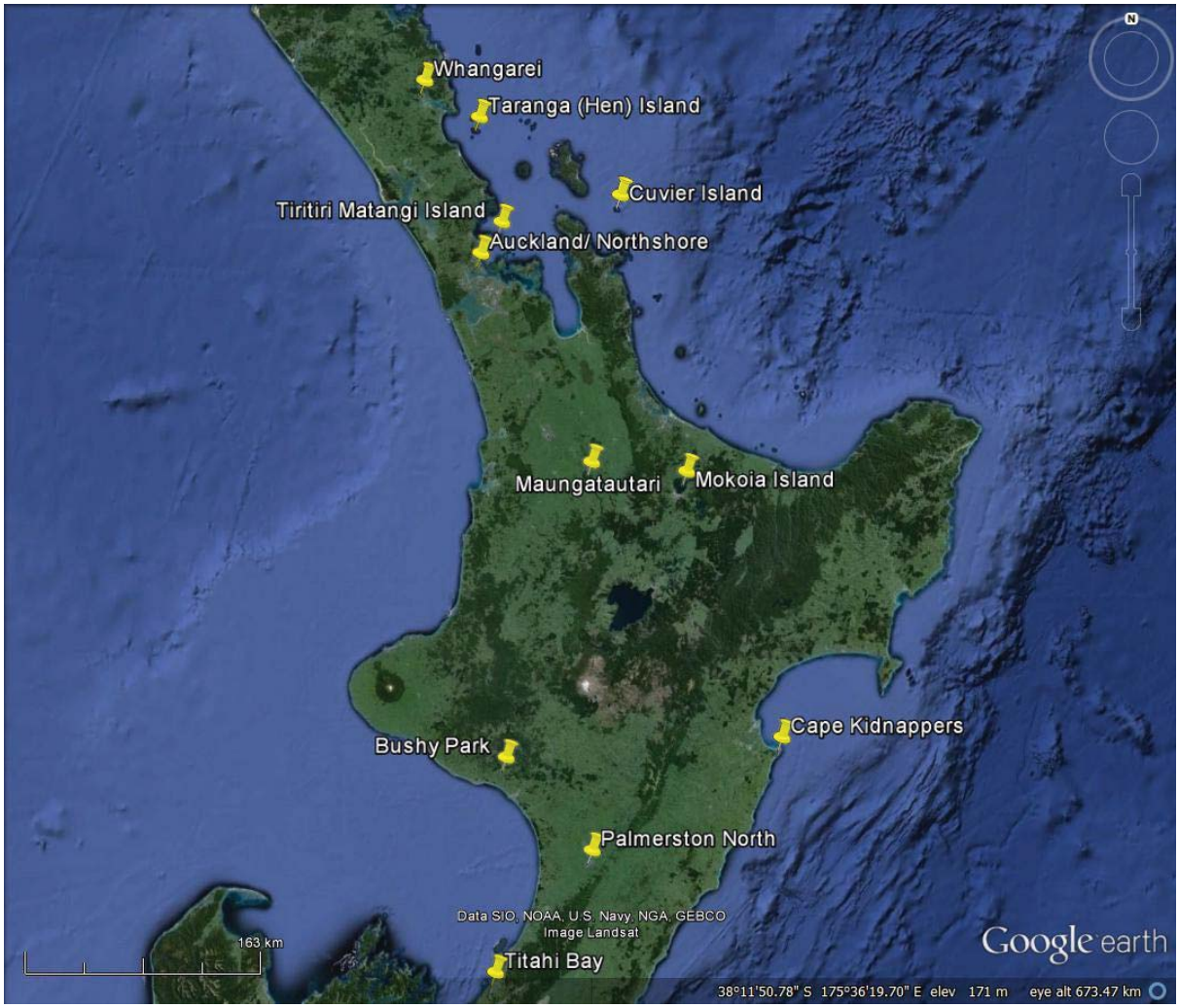
1.4 Study sites

All current populations of the North Island saddleback originate from a single source population on Hen Island, an island in Northland off the shore of Whangarei city. All other populations have been translocated to offshore and mainland islands in a well-documented serial translocation history (Chapter 3).

The sites visited to collect bird blood and mosquito vectors during this study are as follows (Colour Plates 2, 3, 4 and Figure 1):

1. Hen Island in the Hauraki Gulf (source population of all later North Island saddleback translocations).
2. Cuvier Island, Hauraki Gulf.
3. Tiritiri Matangi Island, Hauraki Gulf.
4. Mokoia Island in Lake Rotorua
5. Bushy Park near Whanganui.
6. Maungatautari/Waikato (samples taken before saddleback translocation occurred in 2013)
7. Cape Sanctuary, Cape Kidnappers (Hawkes Bay) (samples taken before saddleback translocation occurred in 2013).
8. The cities of Auckland, Northshore and Whangarei for comparison of protected island sites with urban environments dominated by introduced passerine species, for the collection of samples of introduced passerines and mosquitoes.
9. Palmerston North for the collection of mosquitoes at an urban site.
10. Titahi Bay/Porirua for the collection of the native *Opifex fuscus* mosquitoes.

Figure 1.1: Map of the North Island of New Zealand showing the sampling sites used during this study



1.4.1 Taranga (Hen Island)

(data: DoC website: http://www.doc.govt.nz/)

Hen/Taranga Island (latitude 35°91'S longitude 174°75'E) is part of the Hen and Chicken Islands East of the North Auckland Peninsula. The island is the remnant of an old volcano, 4.7km^2 in size and has cultural significance for the Ngatiwai people. In 1883, it was sold to the New Zealand government and was made a scenic reserve in 1908. Today it is home to saddleback, little spotted kiwi (*Apteryx owenii*), red crowned parakeet (*Cyanoramphus novaezelandiae*), kaka (*Nestor meridionalis*) and tuatara (*Sphenodon punctatus*). The Island is covered in coastal broadleaf forest. From first of May 2011 to 31st October 2011, a successful attempt to eradicate pacific rats/kiore (*Rattus exulans*) from Hen Island was made, after which the island became free of introduced mammal pests.

1.4.2 Cuvier (Repanga) Island

(data: DoC website: http://www.doc.govt.nz/)

Cuvier Island (latitude 36°26'S, longitude 175°46'E) is a 170ha island off the East coast of the North Island and 23km Southeast of Great Barrier (Hauturu) Island. It is a wildlife sanctuary managed by the Department of Conservation. Pacific rats or kiore were the only introduced mammals during Maori inhabitation, but after the arrival of European settlers, who used Cuvier Island as a lighthouse reserve and farmland, other mammals were introduced including predators, such as cats and livestock e.g. sheep, cattle and goats. By 1957, the forest had been transformed to open woodland without an understorey, coastal scrub was reduced to eroding grassland and some native plans had become extinct. In addition, mammalian predators eliminated North Island saddleback, tomtit (*Petroica macrocephala*), tui (*Prosthemadera novaezealandiae*) and red-crowned parakeet. In 1961, restoration of the island began, with the eradication of goats, followed by the removal of feral cats by 1964 and a ban on domestic cats from 1970. Pacific rats were eradicated by 1993. In 1968, North Island saddlebacks were reintroduced.

1.4.3 Tiritiri Matangi Island

(DoC website: http://www.doc.govt.nz/, http://www.tiritirimatangi.org.nz/)

Tiritiri Matangi Island (36°60'S, 174°89'E) in the Hauraki Gulf lies four kilometres off the coast of Auckland's Whangaparaoa Peninsula and 20km North-East of central Auckland. It is a 220ha wildlife sanctuary, which was originally cleared for farming after the arrival of the Europeans, when 94% of its native bush was destroyed. During World War II, it was part of the Auckland Harbour defences. In 1970, Tiritiri Matangi Island became a Recreation Reserve within the Hauraki Gulf Marine Park. Between 1984 and 1994, the livestock on the island were removed, and around 280,000 native trees were planted by volunteers. Today, 60% of the island is forest, with the remainder left open for species which prefer an open habitat. Many species were reintroduced and the island is now home to little spotted kiwi, kokako, hihi (*Nothiomystis cincta*), brown teal (*Anas chlorotis*), takahe (*Porphyrio hochstetteri*), North Island saddleback and tuatara.

1.4.4 Mokoia Island

Mokoia Island (latitude 38°05′S longitude 176°16′E) is located in Lake Rotorua in the North Island of New Zealand. It is New Zealand's largest inland island and is 2.1km from the mainland at the nearest point. Mokoia Island possesses a fertile volcanic soil that was used intensively for cultivation by the Te Arawa Iwi for several hundred years (Andrews, 1992). The island was cleared by axe and fire and terraced for this purpose. In addition Maori introduced pacific rats or kiore and dogs (*Canis familiaris*) (King, 2005).

In the early 1800's, European missionaries began to introduce many exotic species of plants and ungulates to the island and, unintentionally, pests such as Norway rats (*Rattus norvegicus*) and mice (*Mus musculus*) (Andrews, 1992). Around 1950, the cultivation of the island stopped and it became a wildlife refuge which enabled the vegetation to regenerate (Andrews, 1992, Christensen, 2007). The regeneration was improved by an eradication programme for rats, goats and sheep from 1989-90. Mice were also eradicated from Mokoia Island by 2001. Mokoia Island is covered with regenerating broadleaf-podocarp forest (Andrews, 1992, Perrot, 2000, Armstrong *et al*., 2002, Christensen, 2007). Nectar sources for honey eating birds are available in the spring and summer (September-February), and many fruits are available in autumn or winter (March-August) (Andrews, 1992, Perrot, 2000, Christensen, 2007).

1.4.5 Bushy Park

(data: info@bushyparksanctuary.org.nz; www.bushyparksanctuary.org.nz)

Bushy park is a 98ha mainland conservation reserve 24km Northwest of Wanganui (latitude 39°57`S longitude 175°1`E). In 1962, programs for weed and mammalian predator control were started, which had developed into a systematic trapping of mammalian predators by 1995. Since 2005, a 4.8km long predator proof fence, as well as pest control measures, protects 90ha of mature lowland rainforest with 150 native trees, shrubs, ferns, orchids and grasses. Mammalian predators, except mice, were eradicated in 2005. Today, Bushy Park is home to more than 17 species of native birds, reintroduced there including North Island saddlebacks which were introduced there in 2006.

1.4.6 Maungatautari

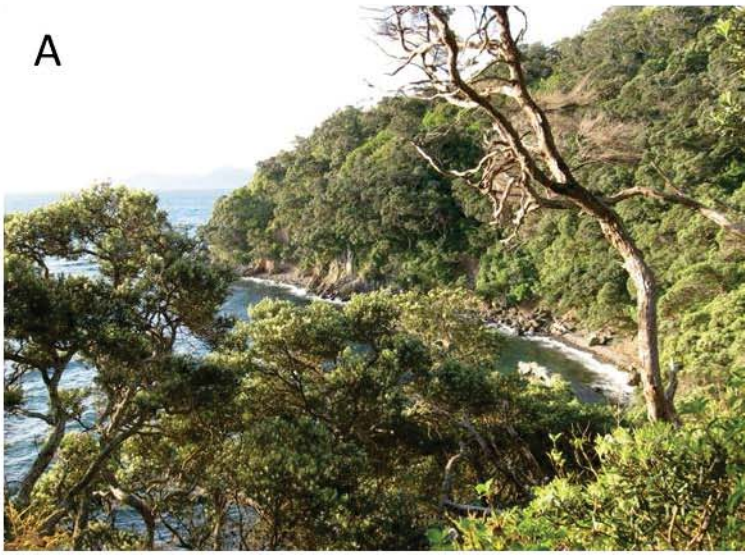
(www.maungatrust.org, history by John Scott)

Maungatautari (38°02'S, 175°57'E) is the largest mainland island in New Zealand with 3400ha enclosed by a 47km long predator proof fence. By 2011, the eradication of mammalian predators was largely complete and re-introduction programmes for native birds had begun. The first inhabitants of the area were the Ngati Kahupungapunga people, which were displaced by the tribes of the Tainui, with Ngati Raukawa being kaitiaki (carers) of this area for centuries. After the land wars in 1873, the European settlement began, and bush was cleared from the lower slopes of Maungatautari to make place for pasture. Livestock as well as cats and dogs and pests like rats and rabbits (*Oryctolagus cuniculus*) were introduced, but the introduction of the possum (*Trichosurus vulpecula*) in the 1950s proved to be devastating with the natural cover of the mountain showing severe damage because of concentrated browsing for 30 years.

1.4.7 Cape Sanctuary, Cape Kidnappers

(Data: http://www.haumoana.com/pages/capesanctuary.html; http://www.poutiri.co.nz/partners/te-matau-a-maui-cape-kidnappers-sanctuary/; http://www.lowecorp.co.nz/conservation/index.htm)

Cape Sanctuary on the Cape Kidnappers peninsula (39.6447° S, 177.0933° E) is the largest privately owned and funded wildlife restoration project in New Zealand, situated 20km from Napier/Hawkes Bay. In 2007, a predator proof fence was constructed to keep 2500ha pest free, supported by an intensive pest control programme with 1,400 traps for mustelids (stoats (*Mustela erminea*), ferrets (*Mustela putorius*) and weasels (*Mustela nivalis*)) and 2,200 bait stations for rodents (rats and mice). These 2500 ha are a mosaic of grazed farmland (650 ha), pine forest (350 ha), regenerating native forest (180 ha), vegetated gullies and coastal cliffs (900 ha), sand dunes (300 ha) and include a DoC (Department of Conservation) reserve (13 ha) as well as a golf course. The first reintroductions of native birds began in 2007, followed by the reintroduction of North Island brown kiwi (*Apteryx mantelli*) and the re-establishment of sea-birds in 2008.



Colour Plate 4: Field sampling sites. A) Dragon Mouth Cove on Hen Island. B) Lighthouse on Cuvier Island. C) Regenerating Forest with tree ferns on Mokoia Island.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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

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

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Please indicate either:

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

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Chapter 2: Avian malaria

(A part of this chapter, Avian Malaria in New Zealand, has been published in the New Zealand Veterinary Journal (Schoener *et al.*, 2014); the paper is included in this thesis as Appendix 1. The following version has many changes to accommodate more recent findings)

2.1 Haemosporidian parasites and avian malaria

2.1.1 The order Haemosporidia: traits and taxonomy

The members of the order Haemosporidia are single-celled, intracellular parasites (Rommel, 2000) that have an apical complex in at least some of their life stages. Their phylum is therefore known as Apicomplexa (Levine, 1982, Ellis *et al.*, 1998), although some authors prefer the older name of Sporozoa (Valkiūnas, 2005). The apical complex is composed of specialized organelles that are believed to be important in host cell penetration (Atkinson, 1991). Haemosporidian parasites originate in hosts with nucleated erythrocytes and adapted to mammals with non-nucleated erythrocytes via a series of host-switching events (Ricklefs and Outlaw, 2010). So far, it has only been possible in the case of one species (*P. lophurae*) to directly transmit avian parasites to mammalian hosts (mice) (Valkiūnas, 2005). In 2008, Martinsen et al. (2008) proposed the hypothesis, that all major taxonomic clades of haemosporidian parasites are associated with shifts to vectors belonging to different dipteran families. At the time, this was well supported by molecular findings. Therefore, the genus *Haemoproteus* was split into two subgenera, one utilising louse flies as vectors, the other using biting midges. The genus *Plasmodium* turned out to be paraphyletic, with one clade including parasites of mammals plus the previously named genus *Hepatocystis,* and another clade including only bird and reptile *Plasmodium* species. All clades also had their own distinct vector groups. Recently, it became apparent that the relationships between parasites and vector groups are not as easily distinguished. It may very well be possible that members of different vector groups can transmit members of different parasite groups, or that the parasites may be able to adapt and perform a vector shift, something that has happened frequently in the past (Molina-Cruz *et al.*, 2013).

The current taxonomy of avian haemosporidian parasites, which most researchers in the field of avian malaria research agree on, is as follows (Valkiūnas, 2005):

- Kingdom Protista
- Phylum Apicomplexa (Sporozoa being the older name for this phylum)
- Class Coccidea
- Subclass Coccidia
- Order Haemosporidia
    - Family Haemoproteidae
        -Genus *Haemoproteus*
            - Subgenera *Parahaemoproteus* and *Haemoproteus*
    - Family Garniidae
        - Genus *Fallisia*
            - Subgenus *Plasmodioides*
    - Family Leucocytozoidae
        - Genus *Leucocytozoon*
            - Subgenera *Leucocytozoon* and *Akiba*
    - Family Plasmodiidae
        - Genus: *Plasmodium*
            - Sub-Genera (only those infecting birds): *Bennettinia Giovannolaia*, *Haemamoeba*, *Huffia* and *Novyella*.

With the advent of molecular techniques, it soon became apparent that the species and lineage richness of haemosporidian parasites was much higher than first thought (Bensch *et al.*, 2009). The taxonomy of haemosporidian parasites and the delineating of new species have become more complex and in some cases doubtful, after new parasitic lineages are only identified by molecular information, in particular using cytochrome b sequence data. This is partly due to ongoing disagreement about the relevance of sequence differences for defining new species (Outlaw and Ricklefs, 2014). As Outlaw and Ricklefs (2014) write, "*Ideally, phylogenetic species should be defined based on multiple (genetic) loci, i.e. relating gene trees to species trees. This can be done with most mammalian Plasmodium parasites owing to the availability of multiple, independent genetic markers, but it is more difficult for haemosporidian parasites of wild birds and reptiles*".

With all the new work done on avian haemosporidian parasites, standards need to be adopted to avoid re-naming identical lineages and therefore creating synonymous

entities. This creates difficulty in literature searches as well as in the comparison of findings of different groups internationally. There are already examples in published papers that contribute to this taxonomic confusion (Perkins, 2014).

To streamline international research and to create an overview of the rapidly growing number of parasite lineages based on cytochrome b sequences, an international initiative was started and a common online database for avian haemosporidian parasites was created. This "MalAvi" database can be found on the World Wide Web at: http://mbio-serv2.mbioekol.lu.se/Malavi/. The MalAvi database is mainly based on the nested Polymerase Chain Reaction (PCR) protocol created by Hellgren et al. (2004) and Waldenstroem et al. (2004), using the resulting 480 basepair amplicon to create the sequences. To avoid confusion with naming recently found haemosporidian parasites, the MalAvi database is an effort to standardise the nomenclature, concentrate all available information on previously found parasites and facilitate access for all researchers in the field (Bensch *et al.*, 2009).

The increased complexity of haemosporidian parasite taxonomy since the introduction of molecular methods has also had an impact on what is seen as a species. Different authors have examined several species concepts to understand apparent discrepancies between what is seen as a morphospecies (the appearance of a parasite as seen under a microscope) and genetic species units (Martinsen *et al*., 2006, Perkins, 2000). The more research is carried out, the more it becomes clear that there are many more genetic lineages (phylogenetic units that form a "clade") and species units than there are distinguishable morphospecies, a concept known as cryptic speciation (Perkins, 2000). One of the most striking examples is the cryptic speciation in the most widespread avian *Plasmodium* species, *P. relictum*, where the lineage GRW4 is genetically distinct from the two other lineages, GRW11 and SGS1 (Hellgren *et al*. 2015). So far, no general agreement exists on the amount of genetic variation that constitutes a species unit when looking at sequences from the most frequently used mtDNA (Bensch *et al*., 2004, Ricklefs *et al*., 2005). While Fallon et al. (2003a) accepted 0.1% sequence difference to distinguish between genetic species units, Perkins (2000) suggested a 3% sequence difference. This wide discrepancy suggests that more research is needed including the examination of sequences from a greater vaiety of different genes.

2.1.2 What is an avian malaria parasite?

There are differing opinions regarding which parasites to include in the term "avian malaria parasite". Traditionally, only the genus *Plasmodium* was included (Perez-Tris *et al.*, 2005). However, over time some authors have also included other genera such as *Leucocytozoon* and *Haemoproteus* within the order Haemosporidia (Perkins and Schall, 2002). This controversy developed because of the incomplete knowledge of phylogenetic relationships and the pathogenicity of nonhuman malaria parasites (Perez-Tris *et al.*, 2005). Phylogenetic work has shown that previously created genera like *Plasmodium* and *Haemoproteus* are indeed paraphyletic and hence grouping them together creates artificial units that do not accurately show real life relationships. Therefore, typological definitions of malaria parasites are useful as working concepts to ease understanding, but they may be misleading when looking at their evolutionary history (Perez-Tris *et al.*, 2005). To simplify matters for this thesis, only parasites of the avian clade of the genus *Plasmodium* will be called avian malaria parasites. For the purpose of the following review however, I will discuss the genus *Plasmodium* as well as other Haemosporidians as close relatives when details of life history traits deem it necessary.

2.1.3 The genus *Plasmodium*

Plasmodium spp. rely on arthropod vectors to complete their life cycle (Section 2.1.6). Part of the parasite life cycle is accomplished in the vectors and thus they are also referred to as definitive hosts. For most species, the definite hosts are mosquitoes of the genera *Culex*, *Aedes, Culiseta* and *Anopheles* (Ritchie *et al*., 1994, Valkiūnas, 2005). *Plasmodium* possess a sexual and an asexual life cycle; the fertilisation and formation of zygotes and the asexual sporogony occur in the definitive (invertebrate) host while the sexual gametogony and asexual merogony happen in the intermediate (vertebrate) host. For a more detailed description of the life cycle see Section 2.1.5. Although more experimental studies are needed, it is generally assumed that if a bird survives initial infection or disease, individuals remain infected for life with low level parasitaemia (Atkinson, 1991, Valkiūnas, 2005). The term "chronic infection" has to be used with caution. Large parts of the literature indifferently call the very commonly recorded low level parasitaemias in wild birds "chronic infections", although it is only possible to label an infection as "chronic" if it has been going on for a while (Section 2.1.7.4). The determination of the duration of

an infection requires continuous sampling of the same birds over a longer period of time something that is very hard to do with wild birds (Section 2.1.7.3). For this thesis, I will only use the term “chronic infection” when referring to longer lasting infections, in the case of single blood sampling events, the term “low level parasitaemia” will be used.

A generally accepted characteristic of *Plasmodium* spp. are the small golden brown or black pigment deposits called hemozoin or malaria pigment in infected erythrocytes. These are produced by chemically changing the hosts’ haemoglobin (Atkinson, 1991, Egan, 2002). Hemozoin is highly refractive when examined by dark-field or polarized light microscopy in both stained and unstained blood smears (Atkinson, 1991, Egan, 2002). It is crucial in identifying the parasites microscopically during diagnosis. As it is understood, hemozoin is an important heme detoxification product and anti-malarial drugs like chloroquine inhibit its formation under synthetic conditions (Egan, 2002).

Intraerythrocytic gametocytes of the genus *Plasmodium* also present with refractile pigment granules and some species alter the position of the host cell nucleus. Beside the presence of gametocytes, meroogony is also performed in the peripheral blood, so meronts can also be found in blood cells. Meronts are round to oval intracytoplasmic inclusions that contain dark staining merozoites. Both *Haemoproteus* and *Plasmodium* may reveal small, ring like forms (trophozoites) in the cytoplasm of infected erythrocytes (Ritchie *et al*., 1994).

2.1.4 Worldwide diversity and prevalence of haemosporidian parasites in passerine birds

Avian haemosporidian parasites are a very diverse group of parasites in passerine birds worldwide (Valkiūnas, 2005). There are differences between the diversity of individual genera within the group. For example, the current number of recognised species and estimated diversity of the genus *Haemoproteus* is much higher than for the genus *Plasmodium*, possibly because *Haemoproteus* tend to be more host specific than *Plasmodium* parasites (Clark *et al.*, 2014). Globally, avian haemosporidian parasites are currently represented by approximately 200 morphospecies, of which 38 are recognised species of *Plasmodium* parasitizing birds worldwide (Valkiūnas, 2005). The richest fauna of *Plasmodium* parasites is

found in galliformes (17 species) followed by passeriformes with 16 different species. The genus *Plasmodium* contains several subgenera, namely *Haemamoeba*, *Bennettinia*, *Giovannolaia*, *Huffia* and *Novyella* (Valkiūnas, 2005). Recently, Landau et al. (2010) have proposed a sixth subgenus "*Papernaia*" containing species previously listed as *Novyella,* but without a morphological feature; the refringent globule. Among the species in the subgenus *Haemamoeba* is *Plasmodium relictum*, a pathogenic protozoan which can cause severe infections. The morphospecies of *P. relictum* is to date the most commonly reported avian malaria parasite worldwide with an extensive host range covering many bird orders, and its lineage SGS1 is the most common lineage in birds in Europe (Hellgren et al., 2015).

The subgenus *Novyella* contains the species *Plasmodium* (*Novyella*) *vaughani*, which is the second most common *Plasmodium* species. So far, the pathogenicity of the members of the subgenus *Novyella* has been insufficiently investigated, but they generally seem to produce mild infections with low mortalities. These parasites can infect a range of bird species, but are most commonly found in passerines.

Plasmodium elongatum belongs to the subgenus *Huffia* and it is another common pathogenic parasite, often causing mortalities (Valkiūnas, 2005) in birds from several orders. *P. elongatum* has a very wide host range, and passerine birds are thought to act as reservoir hosts.

Of the current 38 valid species of *Plasmodium*, there are currently 488 recognised cytochrome b lineages (Clark *et al.*, 2014). Many more likely remain to be discovered, as only about 45% of the known bird species worldwide have been examined for blood parasites and parasite diversity positively correlates with host diversity (Valkinuas, 2005). In 50% of the examined bird species, parasites of the genus *Haemoproteus* were found, while *Plasmodium* and *Leucozytozoon* (another genus of avian haemosporidian parasite) infections accounted for 30%. The richest diversity of *Plasmodium* parasites was found in South America and in general the diversity is much higher in tropical "hotspot" areas (e.g. India, Australia, Southeast Asia) than in temperate regions (Clark *et al.*, 2014).

There are also a number of cryptic species of *Plasmodium* in existence, meaning species that cannot be discerned from others morphologically but possess a

distinctive genetic makeup. One such a species has been described recently, *Plasmodium homocircumflexum*, which is morphologically identical to *Plasmodium circumflexum*, but cannot be detected by PCR. It also shows a much higher virulence than it's morphological twin (Palinauskas *et al.*, 2015).

Passerine birds have been used as avian host models for human and avian malaria research for more than a century (Wolfson, 1941). The canary especially has been used widely, because it proved to be susceptible to most known species of *Plasmodium* and is also easily maintained under laboratory conditions (Wolfson, 1941). Most of our knowledge of avian *Plasmodium* today comes from studies of passerine birds, perhaps because this is the largest bird group with over 5000 species worldwide.

Different *Plasmodium* lineages are able to infect various families of passeriform birds. This enables an overall higher encounter rate of individual parasites by the hosts, and compensates for possibly reduced performance in any one host species. The ability to infect a wide variety of hosts may also lead to the ability to be the most prevalent blood parasite in a single host (Hellgren *et al.*, 2009). Therefore, generalist malaria parasites are often more successful or widespread (Valkiūnas *et al.*, 2008b).

Table 2.1: Prevalence of haemosporidian parasites in birds worldwide

Region	no. of bird species examined	no. of birds individuals	Diagnosis method	Prevalence (%) *Haemoproteus*	Prevalence (%) *Plasmodium*	Mixed infections	Reference
American Samoa	8	188	PCR	N/A	59	N/A	Jarvi *et al*., 2003
Australia	1	260	PCR	0-19.2	19.2-46.2	N/A	Clark et al., 2015
Australia	N/A	2038	PCR	13.1	4.0	N/A	Clark et al., 2015
Australia	12	219	PCR	28	14	N/A	Baedell et al., 2004
Australo-Papuan	80	428	PCR	31	13	6.8	Baedell et al., 2004
Cook Islands	10	79	Microscopy	N/A	N/A	N/A	Steadman *et al*., 1990
Papua New Guinea	77	209	PCR	31	10	N/A	Baedell *et al*., 2004
Southeastern Europe	43	460	PCR	48	43	9	Dimitrov et al., 2010
Western Amazon	104	2488	PCR	0-50	0-86.7	N/A	Svensson-Coelho *et al*., 2013
India	1	116	PCR	6	56.9	N/A	Ishtiaq *et al*., 2006, 2007

The prevalence of *Plasmodium* species in passerines varies with the bird species and region examined. Many different studies have been done worldwide, examining the prevalence of malaria parasites and Table 2.1 presents some selected examples with a special emphasis on the Australian/Pacific region. It is notable that the prevalence of both genera *Plasmodium* and *Haemoproteus* is quite variable in the examined regions and depending on the examined bird species. Haemosporidian parasites have been found all around the Pacific region, with the notable exception of the Cook Islands (Table 2.1).

In a study examining four passerine families on the Curonian Spit in the Baltic Sea Krizanaskiene et al. (2006) presented evidence that the genetic diversity of

haemosporidian parasites may be positively correlated to the migratory strategies of the host. The authors found that blackcaps (*Sylvia atricapilla*), a species with three different migration strategies (sedentary population, short distance and long distance migration) harboured the largest proportion of exclusive lineages of haemosporidian parasites observed in any passerine bird thus far (Krizanaskiene *et al*., 2006). Despite these findings, Valkiūnas (2005) adds a word of caution to the interpretation of prevalence and intensity of infection in ecological studies; I support his opinion that the summation and averaging of data on the intensity of infection for ecological research does not have enough validity, because it does not take into account the daily cyclic fluctuations of the parasitaemia. In addition, most birds are caught in mist nets or stationary traps. Birds that are weak due to heavy infections may be under sampled because they remain inactive and will not fly into the nets or traps (Valkiūnas, 2005). See also Behavioural Influence Section 2.1.6.3

2.1.5 Individual factors influencing prevalence and severity of infection in passerine birds

There are different factors that may influence the prevalence and severity of avian malaria in the host. The genetic makeup of the individual host has an impact on infection and disease. When looking at the heterozygosity at putatively neutral loci in the genome of blue tits (*Cyanistes caeruleus*) and their infections with *Plasmodium relictum* (SGS1 lineage), Ferrer et al. (2014) found a significant relationship between probability of infection and host genetic diversity. In Hawaii, where *Plasmodium relictum* (GRW4) was first introduced in the 1800s, it caused mortality and extinction in many bird species. Since then, a change in genetic attributes has occurred in the surviving amakihi (*Chlorodrepanis virens*) (Foster *et al.*, 2007). Looking at recent samples as well as historic museum specimens, a change in allele distributions can be seen. Lowland birds, which are most highly exposed to avian malaria, are now genetically different from highland birds. The high pressure of disease rapidly selected for resistance and created small pockets of resistant birds from which resistance spread to the whole of the lowland. Experimental infections showed that infected low elevation birds had lower mortality, lower reticulocyte counts during recovery from acute infection, lower weight loss, and no declines in food consumption compared to experimentally infected high elevation Hawaii Amakihi in

spite of similar intensities of infection (Atkinson *et al.*, 2013). Foster et al. (2007) also examined two other species of Hawaiian honeycreeper, which did not show this separation, perhaps because these species have different genetic attributes, which are less suitable for malaria resistance and species recovery.

Host species, body condition and host nutritional status have an impact on the dynamics of the infection in addition to the virulence of the parasite. While severely affected birds from Hawaii nearly always suffer a loss of body mass after infection (Atkinson et al., 2000, 2001), wild European passerines experimentally infected with the same parasite species (*P. relictum*), did not show a loss of body mass after infection (Palinauskas et al., 2008). Similarly, surveys of breeding populations of 15 different passerine species have found little correlation between infection with haemosporidian parasites and body mass and fat levels, suggesting that hosts are able to tolerate infection under the physiological stresses of reproduction (Bennett, 1988, Atkinson, 1991). Nutritional status still may also influence infections, as shown by Cornet et al. (2014). They experimentally infected canaries with *P. relictum* (SGS1), while the birds were either provided with either an enriched supplemented diet or a control diet. Birds fed with the poorer quality food had a lower body mass and showed a higher parasitaemia as well as more sexual parasite stages in their blood. In contrast, birds on the supplementary diet appeared to be able to control the infection better, had a higher body mass and a lower parasitaemia, although these birds also showed a significantly decreased haematocrit compared to the controls. It is feasible to assume that a great part of the *Plasmodium*-related cost of infection is due to the immune system, and better nutrition provides the ability to mount a stronger immune response, and therefore clearing of infected red blood cells, which causes immunopathological damage (Cornet *et al*., 2014).

Another factor influencing the prevalence and severity of avian malaria in the host is the sex of the bird. In a study on Hawaiian forest bird communities, Atkinson et al. found a higher prevalence in the native male birds (Atkinson *et al.*, 2005). A similar finding was made in Seychelles warblers (*Acrocephalus sechellensis)*, where the prevalence in males was also higher than in females (van Oers *et al.*, 2010). Van Oers et al. (2010) hypothesized that the influence of the sex on prevalence seems to be related to dispersal with the dispersing sex possibly having more exposure to

vectors and lineages of different blood parasites and therefore they may show a higher prevalence (van Oers *et al*., 2010). This may or not be true when looking at birds where females are generally the dispersing sex (Greenwood, 1980). Further studies are needed to be able to draw definite conclusions.

Age is also a factor. In Hawaiian forest bird communities, the prevalence is higher in adults (Atkinson *et al*., 2005). In contrast, Seychelles Warblers show a reduced blood parasite prevalence with age, which may be caused by either selective mortality or suppression of the infection in older animals, or both. A higher prevalence in juvenile passerine birds may be due to a higher exposure of juveniles to the parasites, a lack of developed immunity and the possibility that they acquired an infection as naked and immobile nestlings (van Oers *et al*., 2010). To define the age of the first infection, Cosgrove et al. tested naked and immunologically naïve blue tit (*Cyanistes caeruleus*) nestlings, a species in which the adults are frequently infected with *Plasmodium* spp.. Although the authors believed that in the nestling stage, the infection should just have reached patency (the threshold of parasite detection), blood parasites could not be found by nested PCR in these nestlings. Possible reasons may be that very young birds are not bitten by the vectors, the nestlings may be infected but may not have reached patency, or they might have reached patency but had only a very low and hard to detect parasitaemia (Cosgrove *et al.*, 2006). In general, adult birds will have a longer period of contact with the vectors than the young ones, and therefore have a greater chance to be infected. After infection, the birds very likely carry the parasites for life (Atkinson, 1991, Valkiūnas, 2005), thus maintaining a chronic infection that is hard to detect (Valkiūnas, 2005).

Phylogeny and life history traits may also play a role. Palinauskas et al. (2008) experimentally infected three species of passerines with *Plasmodium* lineage p-s651 and examined if these birds developed parasitaemia. Starlings (*Sturnus vulgaris*) were resistant to this lineage and did not show any parasites in the blood, while 50% of the house sparrows were susceptible and all the crossbills (*Loxia curvirostra*) became infected and presented with high parasitaemia. The authors discussed whether the relative resistance of sparrows and starlings to some avian malaria lineages may have helped them in their recent worldwide increase in range

(Palinauskas *et al.*, 2008). Susceptible birds on Hawaii like the amakihi (*Chlorodrepanis virens)* showed a mortality rate of up to 65% with a parasitaemia as high as 50% of the total erythrocytes (Atkinson *et al.*, 2000). Valkiūnas also noted that bird species with a nestling period longer than 14-16 days had an increased probability of infection (Valkiūnas, 2005).

Another factor is the time of year. In 1970, Applegate found in house sparrows, that avian malaria parasites are more prevalent in the blood in spring than in other seasons. For a long time, this “spring emergence” was seen as being connected with bird reproduction and hormonal status, but a recent study found no evidence for this and instead suggested that this phenomenon is due to season-specific changes in either host or parasite physiology (Cornelius *et al.*, 2014). Nonetheless, in temperate northern hemisphere climates, most of the parasite transmission happens during the breeding season of the hosts. Breeding season in most birds coincides with an increase of the vector population, the relapse of disease in adult birds with chronic infections and the immunologically naïve juvenile birds hatching and fledging creating ideal conditions for parasite transmission (Atkinson, 1991). In a study in great reed warblers (*Acrocephalus arundinaceus*), a migratory bird that travels between its European breeding sites and wintering sites in Africa, raised the question of why this bird species did not show any ill effects from malaria parasites at the European breeding sites. The authors concluded that the birds become first infected at their African wintering sites and would only present with subclinical chronic infections in Europe (Bensch *et al.*, 2007).

2.1.6 Life cycle

Haemosporidian parasites rely on arthropod vectors to complete their life cycle. The arthropod vector (mosquitoes of the genera *Culex*, *Aedes*, *Culiseta*, and *Anopheles* for *Plasmoidum*) is the definitive host in which sexual reproduction is performed. The vertebrate (e.g. bird) is the intermediate host, into which the vectors inoculate the sporozoites (infective stages). Most studies on the life cycle of *Plasmodium* have been done on the subgenus *Haemamoeba*, especially on *P. relictum*, with only fragmentary knowledge on the subgenus *Novyella*. In addition, most studies and experimental infections were performed on chickens and passerines (mainly canaries) and the life cycle in the vast majority of wild birds is unknown.

In the intermediate host, when infected with *P. relictum* (Valkiūnas, 2005), several asexual generations of exoerythrocytic meront development occur in cells of fixed tissues. These cells are of mesodermal origin, like the endothelial cells of capillaries, haemopoetic cells and cells of the lymphoid-macrophage system, and are often found in organs like lung, liver and spleen or skeletal muscle. This is followed by the erythrocytic merogony, which is performed in cells of the erythrocytic series. There are two described stages of exoerythrocytic merogony in birds, the primary (pre-erythrocytic) and secondary (post-erythrocytic) stage. The primary stage consists of two generations of meronts (cryptozoides/first generation and metacryptozoides/second generation), which is performed in reticular cells of many organs and tissues including the skin and frequently the spleen. The secondary stage has several generations of meronts (phanerozoites), which happens in macrophages in many different organs. The duration of the erythrocytic merogony differs between the *Plasmodium* species and all agamic (not producing reproductive cells) stages can be usually found in the same blood film. Some of the merozoites formed by erythrocytic meronts induce the next cycle of the erythrocytic merogony, as well as the formation of the gametocytes. Others penetrate the capillaries of many organs including the brain and initiate the secondary exoerythrocytic merogony and therefore form meronts containing phanerozoites (see above). Phanerozoites together with erythrocytic meronts produce meroites maintaining the parasitemia during the chronic stage of the infection (Section 2.1.7 and 2.1.7.4). In addition, phanerozoites are responsible for relapses.

After this period, the parasites may be cleared from the host or enter a latent stage whereby they remain in the host tissues (as phanerozoites), if not the blood, for an extended period of time and possibly for life (Atkinson, 1991, Valkiūnas, 2005). These are chronic, low level infections which are usually not seen on blood smears (Atkinson *et al.*, 2000, Jarvi *et al.*, 2003, Castro, 2006). Since low level parasitaemias, including chronic infections, are hard to detect in the blood of wild birds both microscopically and with PCR and the examined wild birds are rarely killed to examine tissue samples, it is difficult to assess how often birds are able to clear the infection and are truly uninfected or carry the infection undetected in the tissues. More work needs to be done experimentally infecting a wider range of bird species as well as develop new alternative and more sensitive detection methods. In general, if a bird is chronically infected, stressful events in the bird's lives can always cause a relapse of disease symptoms (Section 2.1.7.4). If there is a high incidence of apparently chronic infections, relative stability of native land bird communities and the presence of vectors that are considered endemic and capable of transmitting the blood parasites, this may suggest that these parasites or certain lineages are indigenous and have a long coevolutionary history with their hosts (Jarvi *et al*., 2003).

Following the primary pre-erythrocytic merogony (see above) in the vertebrate host, sexual stages, namely gametocytes or gamonts, start to develop in mature erythrocytes, which develop into macro (female) and microgametes (male). These sexual stages are acquired by the vector by feeding on vertebrate blood (Valkiūnas, 2005). Once there, micro and macro gametocytes round up and leave their host cells in the insect gut in a process called gametogenesis (Atkinson, 1991). For this, the change of oxygen and carbon dioxide concentration between vertebrate and vector is the main stimulus (Valkiūnas, 2005). The threadlike flagellated microgametes fertilize the larger spherical macrogametes. This process of sexual reproduction produces the zygote which quickly differentiates into a highly specialised, elongated, motile form called the ookinete. These possess a haploid genome and apical organelles like the conoid. Ookinetes penetrate the epithelial cells of the vectors' midgut, round up and develop into oocysts. Oocysts undergo asexual reproduction (sporogony) which results in the formation of numerous elongate sporozoites which travel through the haemocele of the vector and penetrate the salivary glands. From

there, they are transmitted by the insects to the avian host during feeding on its blood (Atkinson, 1991; Ritchie *et al*., 1994; Gabrisch and Zwart, 2001; Valkiūnas *et al.*, 2002).

2.1.7 Avian malaria as a disease

It is often assumed that all *Plasmodium* parasites show similar, if not the same, traits as *P. relictum* and *P. gallinaceum.* However, this can only be verified by more experimental infections with other *Plasmodium* species in the future. For *P. relictum*, there are four phases in the course of avian malaria as a disease. The first phase is the prepatent phase in which the parasites undergo the initial cycles of merogony and are not circulating in erythrocytes (Atkinson, 1991, Valkiūnas, 2005). In experimentally infected birds, the prepatent phase in *Plasmodium* infections lasted from two days to several months (Valkiūnas, 2005). The second phase is the acute phase in which the parasites begin to appear in the circulation of the bird host and rapidly increase in numbers. This is followed by the crisis phase when the parasitaemia and the physiological stresses reach a peak. Acute infections are generally more severe to hosts which had no previous contact with the parasite lineage. If the bird survives the acute phase, the infection enters the latent or chronic phase and may persist for years in the hosts' body (Atkinson, 1991, Valkiūnas, 2005).

It has been noted that malaria causes little direct mortality to avian hosts in their natural environment and is relatively harmless to wild birds (Bennett *et al*., 1993; Valkiūnas, 2005). In general however, the impact of avian haemosporoidia on host fitness, ecology and life history may be very much underestimated. Avian haematozoa may play a significant role in concomitant infections with other diseases under certain conditions (Bennett *et al.*, 1993) as well as cause serious disease in immunologically naïve and susceptible birds. In Hawaii, amakihi susceptible to *Plasmodium* had a parasitaemia as high as 50% of erythrocytes resulting in 65% mortality (Atkinson *et al*., 2000). Haemosporidian parasites can cause severe disease in domestic birds, with lethal cases of *Haemoproteus* infections in domestic pigeons (*Columba livia*) as well as lethal *Plasmodium* infections in canaries (*Serinus canaria*) (Valkiūnas, 2005). *Haemoproteus* and *Plasmodium* infections are also the main causes of haemosporidiosis in zoos and aviaries, places which have certain

features that make birds prone to infections. In zoos and aviaries, exotic birds imported from areas with or without different lineages of haemosporidian parasites are kept together. These birds have not adapted to local lineages or local vectors and carriers of the parasites are available in the surroundings facilitating transmission and infection (Valkiūnas, 2005).

Several studies have found that *Plasmodium* infections can increase the probability of predation (Navarro *et al.*, 2004, Møller and Nielsen, 2007). These parasites can have an impact on life-history traits in natural populations by affecting survival (Dawson and Bortolotti, 2000, Valkiūnas, 2005), body condition (Valkiūnas *et al.*, 2006b) and reproductive success (Merino, 2000, Marzal *et al.*, 2005, Tomás *et al.*, 2007).

A recent paper by Dinhopl et al. (2015) paints a more alarming picture of the possible effect avian malaria may have on bird populations. In the first large scale study looking at causes of mortality in wild birds, there was an association between severe avian malaria infection and deaths in 14.6% of 233 birds found dead in Austria. This is in contrast with the current assumption that these parasites almost exclusively cause subclinical infections in areas with endemic infection.

2.1.7.1 Clinical signs

The clinical signs shown by birds infected with *Plasmodium* spp. depend on the species of bird as well as the lineage of parasite involved. Infected birds can remain asymptomatic (Ritchie *et al*., 1994). Hawaiian amakihi infected with *P. relictum*, showed a decline in food consumption and body weight and also diminished activity that made them more susceptible to predators (Atkinson *et al*., 2000). In experimentally infected passerines in Europe, no effect on body mass and temperature was seen, but the birds had a significant decrease in their haematocrit value. Hypertrophy of the liver and spleen was seen in siskins (*Carduelis spinus*) and crossbills which were most susceptible to the experimental lineage *P. relictum* SGS1. Starlings proved to be resistant to the lineage, and chaffinches (*Fringilla coelebs*) and house sparrows only showed light parasitaemia (Palinauskas *et al*., 2008). Canaries infected with *Plasmodium* spp. have a decreased ability to thermoregulate as well as a lowered oxygen binding capacity in the crisis phase of the disease (Atkinson, 1991). In general, passerine birds with an outbreak of the

disease show the following signs: Vomiting, anorexia, depression, dyspnoea, anaemia of the regenerative haemolytic type, splenomegaly, hepatomegaly and pulmonary oedema. Hepatomegaly is also known as “black spot disease” in passerines kept as pets, showing an enlarged, dark liver through the skin (Ritchie *et al*., 1994).

Changes in the chemical parameters of the blood include a decreased pH value, elevated plasma protein concentration and decreased oxygen binding capacity of the haemoglobin. In terms of blood cell differential, the haematocrit (total volume of red blood cells) is decreased and the lymphoid-macrophage system is hyperplastic (Ritchie *et al*., 1994).

2.1.7.2 Pathology

To date, hardly any work has been done on the pathological effects of particular lineages of *Plasmodium* spp. on different bird species, and further research in this field is needed (Valkiūnas, 2005).

In general, affected birds present grossly with an enlarged as well as discolored or pale liver and spleen. The primary cause of death is believed to be anaemia (Colour Plate 5; Atkinson *et al.*, 2000; Alley *et al*., 2008; Palinauskas *et al.*, 2008; Atkinson and LaPointe, 2009; Alley *et al*., 2010).

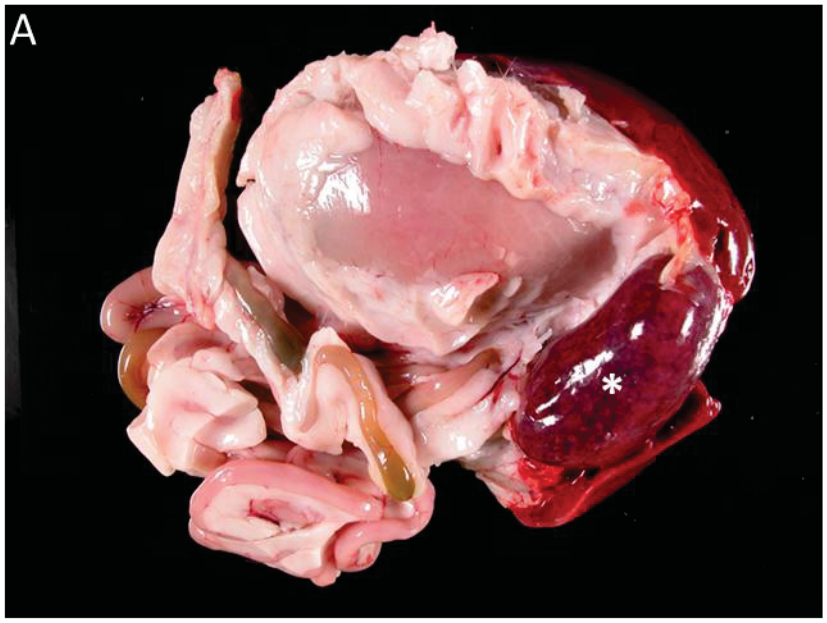
On histopathology (Colour Plate 6), affected birds show mild to moderate interstitial pneumonia (Atkinson *et al*., 2000; Howe *et al*., 2012) with increased numbers of granulocytes in the pulmonary interstitium (Atkinson *et al*., 2000). Large numbers of immature erythrocytes were present in the circulation in affected Hawaiian amakihi, while the bone marrow showed increased cellularity with depletion of erythrocytes (Atkinson *et al*., 2000). Moderate multifocal hepatitis was present in the liver of a range of affected New Zealand birds (Howe *et al*., 2012). In addition, there may be diffuse pigment deposition in the Kupffer cells in the liver and macrophages in the spleen (Atkinson *et al*., 2000). Extramedullary erythropoiesis may be present in the liver and spleen, as well as multifocal extramedullary granulopoesis in the hepatic parenchyma as well as in the red pulp and subcapsular areas of the spleen (Atkinson *et al*., 2000). In affected New Zealand birds, the endothelial cells of tissue capillaries often show an enlarged cytoplasm filled with numerous small basophilic granular structures which resemble protozoal merozoites (Howe *et al*., 2012).

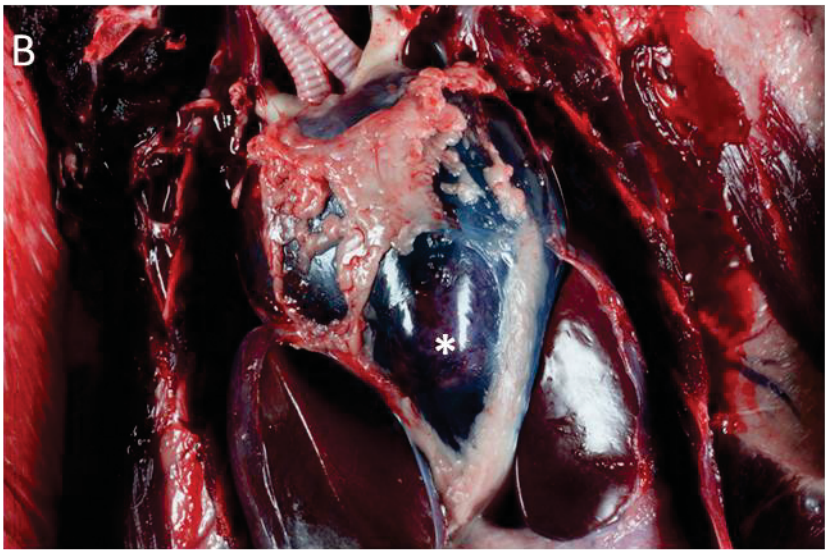
In a severe case of a blackbird (*Turdus merula*) found dead on Mokoia Island (Schoener, 2009), the bird was found to be in very poor body condition, with severe loss of body fat reserves and atrophy of the pectoral muscle. On postmortem, examination the liver was very friable and fell apart on touch. The gallbladder was enlarged and very prominent. The pericardium was filled with a clear yellowish fluid. The spleen was swollen and enlarged. The kidney was enlarged, pale and granulated with yellow pinpoint lesions over the whole organ. Most of the organs examined on histopathology (lung, liver, spleen, heart, brain, skeletal muscle and kidney) showed extensive change in the capillary endothelial cells. These cells were often enlarged and their cytoplasm was filled with numerous small basophilic granular structures resembling protozoal merozoites. Each organism measured about 2-3 microns in diameter and was slightly elongated and separate from one another. The endothelial cell nucleus was often crescent-shaped because of compression to one end of the cell by the intracytoplasmic meronts. The organisms were particularly numerous in the lung, heart and brain (Colour Plate 6). The changes in the endothelial cells of the kidney were best visible in the glomeruli and both liver and kidney showed active haematopoesis. There was mild haemosiderin deposition in the hepatocytes and within the histiocytes of the spleen which was congested and showed moderate lympholysis. The thymus and bursa however, were active with many lymphocytes present and only mild lympholysis. The ventricular myocardium showed eosinophilic degeneration in some fibres with swelling, eosinophilic fragmentation and vacuolation of sarcoplasm and in some muscle bundles. A PCR performed on tissues from this bird identified the infective agent as a unique *Plasmodium elongatum* lineage (the report of *P. relictum* in Schoener, 2009 was based on an earlier, inconclusive PCR; however, the PCR was repeated at the time of publication and found to be *P. elongatum* LINN1).

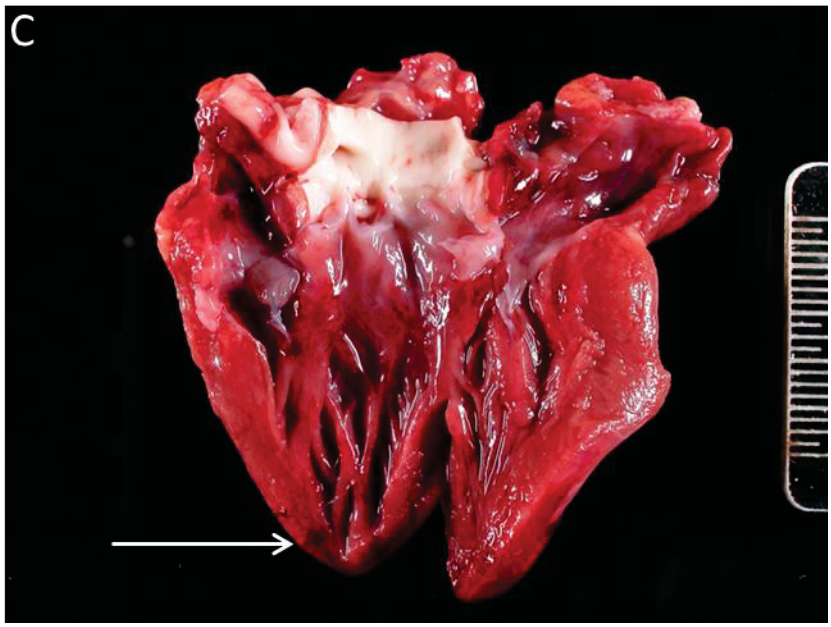
In the case described above, no clinical signs were observed prior to death, because the bird was not marked or monitored. The histological finding of a recent cardiomyopathy in this bird suggests that this may have been contributed to death. Large numbers of *Plasmodium* meronts were present in myocardial endothelial cells which were both hyperplastic and hypertrophic raising the possibility that a sub-acute vasculitis was responsible for ischaemic changes in the ventricular myocardium. However, the numbers of organisms present in cerebral endothelial cells were

sufficiently high as to speculate that intravascular disturbances and perhaps ischaemia may have occurred in the brain, which very likely was the main cause of death.

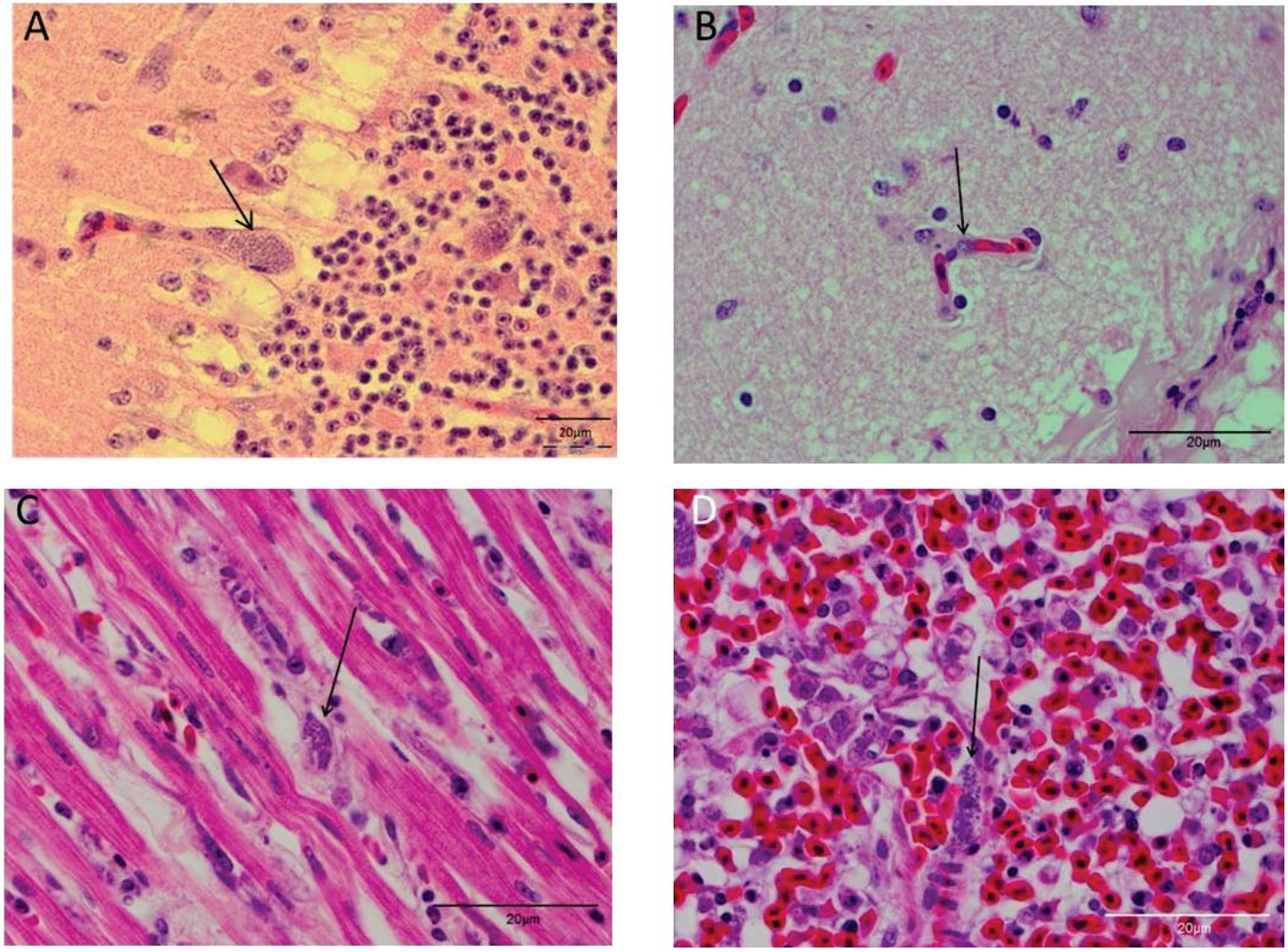
Cerebral malaria as seen above is known to occur in experimentally infected canaries (Valkiūnas, 2005), but nothing is known about its incidence in wild birds. Cerebral malaria is a serious cause of death and disability in humans worldwide (Mishra and Wiese, 2009), and is one of the most severe complications of malaria due to *Plasmodium falciparum*. The principal cause seems to be the blockage of cerebral microcirculation by infected erythrocytes (Aikawa, 1990). It is most common in young children living in malaria-endemic sub-Sahara Africa. The main clinical features consist of seizures often preceding deep coma resulting from cerebral oedema, microhaemorrhages and ischaemia (Bruzzone *et al.*, 2009). There is a limited involvement of parasites or of leucocytes within the CNS parenchyma itself as the inflammation and immune mediated events remain essentially intravascular (Bruzzone *et al*., 2009). In the chicken, extensive infection of the endothelia of brain capillaries is considered a sign that the host has not adapted to the malaria parasite (Frevert *et al.*, 2008). *P. gallinaceum* is the primary malaria parasite of the Indian jungle fowl, where it produces only subclinical infections with low mortality. In contrast, this parasite can produce large exoerythrocytic stages in brain capillary endothelia in untreated domestic white leghorn chickens causing fatal paralysis by occlusion of the infected brain capillaries (Frevert *et al.*, 2008).







Colour Plate 5: Gross Pathology. A) Kiwi with marked splenomegaly (asterisk). B) Yellow-eyed penguin with haemopericardium and severe myocardial haemorrhage (asterisk) C) Kiwi with myocardial haemorrhage (arrow). Pictures by courtesy of Stuart Hunter, Pathology, Institute of Veterinary Animal and Biomedical Sciences, Massey University.



Colour Plate 6: ***Plasmodium spp.*** **tissue merogony (arrow) in the brain of a blackbird (A) and the brain of a yellow-eyed penguin (*Megadyptes antipodes*) (B), heart muscle of a kiwi (C) and lung of a yellow -eyed penguin (D); H&E stain.**

2.1.7.3 Behavioural influences

It has been observed that haemosporidian parasites may influence the competitiveness of birds in the wild. The males of some bird species, when infected, may show a decrease in reproductive activity copulating with less success and less often, choosing young inexperienced females and breeding late in the breeding season which, is less favourable in terms of food availability and climate (Valkiūnas, 2005). Valkiūnas (2005) noted that due to the traditional methods of bird sampling, like mist nets and fixed traps, mainly birds with subclinical and chronic infections with low parasitaemia are sampled. Therefore, birds in the acute stage of the infection remain under sampled, most likely because of changes in their behaviour. Young affected by avian malaria revert back into a behaviour normally associated with fledglings. They will hide, prefer to hop away from danger instead of flying and will not feed. In general, acute parasitaemia in affected birds lowers the locomotion activity, so they are less likely to be caught. Therefore, much of the information acquired in avian malaria in the past decade represents subclinical and chronic stages of disease, not acute stages, and conclusions drawn in relation to host behaviour have to be regarded with this in mind.

Overt behavioural changes may only be evident during prepatent, acute and crisis phases of infection when physiological stress reaches its peak. In very intense infections, these typically manifest as signs of discomfort and include changes in posture and decreases in physical activity. Behavioural changes might not be noticeable in infections of low intensity (Atkinson, 1991). One study found some evidence that haemosporidian infections sometimes have a negative effect on dominance in captive red-winged blackbirds (*Agelaius phoeniceus*) (Weatherhead, 1995). Uninfected individuals tended to be dominant over infected individuals, but the pattern was variable and there may be other unmeasured influences. While infections with *Leucocytozoon* and *Plasmodium* influenced the singing behaviour of White-crowned sparrows (*Zonotrichia leucophrys oriantha*), infections with *Haemoproteus* had no detectable effects. Birds that were infected with *Plasmodium* spp. sang fewer songs (Gilman *et al.*, 2007), but the authors did not quantify parasitaemia in their birds and were most likely low level parasitaemias. Malaria infection also had an impact on song development in infected juvenile canaries, thus

affecting adult male song (Spencer *et al.*, 2005). Males that were infected as juveniles developed simpler songs as adults and had reduced development in the high vocal centre nucleus of the brain. It is thought that the infection interrupted the early development of the underlying brain nuclei.

In a study on infection with haemosporidian parasites and the risk of predation in Europe, findings suggest that predation is an important factor affecting the parasite-host dynamics. Prey species with a high prevalence for blood parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) were found to have a higher risk of predation (Moller and Nielsen, 2007). In Hawaii, experimentally infected juvenile Apapane (*Himatione sanguinea*) (Yorinks and Atkinson, 2000) spent less time on movements and were more stationary. This loss of activity made acutely infected birds vulnerable to predation.

Plasmodium may have an impact on host reproduction. A study by Podmokla et al. (2014) found that infected blue tit breed later in the season but had no difference in clutch size compared to uninfected birds. Of note was the finding that infected birds produced heavier and larger offspring with possibly stronger immune reactions.

2.1.7.4 Chronic infections and immunity

A bird that has been infected once with a malaria parasite and has survived the acute stage of the infection, may maintain the infection for years if not for life. Indeed, antibodies against *Plasmodium relictum* persisted as long as 1248 days after the initial infection in experimentally infected Hawaii amakihi (Atkinson *et al.*, 2001). It has also been shown for *P. relictum* (lineage GRW4) in Hawaii that chronically infected birds had no increases in peripheral parasitemia or declines in food consumption or weight when rechallenged by mosquito bites with a homologous isolate of the parasite. This phenomenon is known as “premunition” (Atkinson *et al*. 2001). In these cases, chronically infected birds are functionally immune to superinfection in areas where transmission of a certain lineage of parasite has become endemic, like in Hawaii (Atkinson *et al*., 2001). It has also been proposed that individual resistance against avian malaria parasites is correlated to the diversity of MHC genes and that these play a role in humoral and cell mediated immune responses. Evidence for this so far has been circumstantial (Atkinson et al., 2000, Jarvi et al., 2004) and more research needs to be done in this field.

The majority of infected birds sampled in the wild will carry chronic infections (Valkiūnas, 2005, Zehtindjiev et al., 2008). Avian malaria parasites remain dormant in the hosts' organs, and may be very hard to detect on blood smears (Atkinson, 1991, Scheuerlein, 2004, Valkiūnas, 2005). The hosts' immune system is able to develop some degree of resistance to the lineage it is infected with and therefore parasitaemia is very light as a result of an efficient immune system. In these cases, parasitaemia may be as low as one parasite in one million examined erythrocytes (Zehtindjiev *et al.*, 2009). These chronic asymptomatic infections can resume the haemoparasitic/acute stage again if the host becomes stressed and this can cause mortalities (Atkinson et al., 2001) Stressors may include the onset of the breeding season (spring relapse), a change in environmental conditions, and other causes of physiological stress (Atkinson, 1991, Scheuerlein, 2004). Therefore, chronically infected birds provide a source for the annual initiation of infection if it is consistent with seasonal peaks in vector abundance (Garvin *et al.*, 2003).

Chronic infections that remain subclinical for years (Atkinson, 1991) may result in some continuous, small costs to the host, affecting immune defences, activity, behaviour and life expectancy. These costs may diminish the bird's ability to adapt to stressful events in its environment (e.g. lack of food, bad weather conditions, predation, and onset of the breeding season), causing a higher mortality and reduced reproductive success (Atkinson, 1991, Garvin, 2003, Bensch *et al*., 2007). For example, adult Hawaiian amakihi chronically infected with *Plasmodium relictum* had a 17% lower survival rate compared to uninfected birds (Kilpatrick *et al.*, 2006). Chronically infected blue tits (*Cyanistes caeruleus*) treated with the anti-malarial drug malarone had an almost complete clearance of parasites from the blood, and medicated females had higher hatching success, higher provisioning rates and higher fledging success when compared to un-medicated infected females (Knowles *et al.*, 2010). In another study Asghar et al. (2011) found that chronically infected females of the migratory great reed warblers (*Acrocephalus arundinaceus*), which did not show clinical signs of disease, but nevertheless presented with higher parasitaemia, arrived later in the breeding season. They also found that females had a higher parasitaemia than males and that parasitaemia was higher in infected juvenile birds compared to infected adults.

A recent study found that chronic infections may have an even bigger hidden cost. Asghgar *et al.* (2015) found that while low level chronic avian malaria infections did not have a direct short term cost for the birds, it reduced life span as well as the lifetime number and quality of offspring in great reed warblers. This process is mediated by faster degradation of telomeres in infected birds (Asghar *et al.*, 2015). This may pose a serious future threat for the conservation of rare endemic birds.

2.1.7.5 Mixed infections

Mixed infections with two or more different malaria parasites are common in the wild (Jarvi *et al.*, 2008) and it is possible to find several haemosporidian parasite lineages in both a population and in individuals (Hellgren, 2004). Beadell et al. (2004) found mixed infections in 29 out of 428 individuals (6.78%) in their study on the prevalence of two avian blood parasite genera (*Plasmodium* and *Haemoproteus*) in the Australo-Papuan region. In this study, one individual harboured two *Plasmodium* lineages, 11 harboured two *Haemoproteus* lineages and four birds had a mixed infection with *Haemoproteus* and *Plasmodium*. A study of the genetic diversity of blood parasites of passerines in South East Europe presented a similar result: 460 birds from 43 different species were examined, and 9% showed mixed infections with both *Haemoproteus* and *Plasmodium* lineages. In a recent study in New Zealand, double infections with *P. relictum* and *P. rouxi* were found in introduced blackbirds (Gudex-Cross, 2011).

There is some discrepancy in the literature about the effect of co-infections with different lineages of *Plasmodium,* although these are generally considered more virulent than infections with just a single lineage (Arriero and Moller, 2008, Marzal *et al.*, 2008, Palinauskas *et al.*, 2011). Palinauskas *et al.* (2011) found heavy parasitaemia (over 35% and up to 90% during peaks) in three species of experimentally infected passerines, but did not note any significant effects in body mass. In contrast, Marzal *et al.* (2008) found a negative additive cost in body condition in individuals from a natural population of house martins (*Delichon urbicum*), experimentally infected by two different *Plasmodium* lineages.

Mixed infections may have a potential impact on mortality because different parasites simultaneously share limited available physiological resources (Atkinson, 1991). In the study of house martins individuals infected with two different parasite

species were in poorer condition and had a lower body mass than uninfected or single-infected individuals. These individuals also suffered from a higher intensity of chewing lice in the feathers (Marzal *et al.*, 2008). Double infections also had an impact on bird reproduction: against expectations, individuals harbouring a double infection invested more in current reproduction, despite being in poor physical condition. Double infected birds as well as uninfected birds initiated clutches earlier than single-infected ones, and double-infected birds also laid larger clutches than both uninfected and single-infected birds. This may be the result of the "terminal investment strategy" that predicts that individuals should continue to invest more resources in reproduction throughout their life, because of an ever-decreasing residual reproductive value (Cluttonbrock, 1984).

In humans, Bruce and Day (2002) found that the clinical outcome of infection with multiple species and lineages of *Plasmodium* is determined by the hosts' ability to regulate the density of malaria parasites in the blood. Interestingly, most infections do not cause symptoms of malarial disease after a degree of immunity is acquired. They showed that the total parasite density of *Plasmodium* species oscillated around a threshold and that peaks of infection with each species did not coincide. There seemed to be a cross-species mechanism of parasite regulation; a density-dependent regulation on the side of the parasites seemed to act together with specific immune responses to produce stability in the dynamics and a sequential pattern of infection with different species.

Co-infections of *Plasmodium* parasites with other vector-borne diseases, such as avian pox virus, are also possible. For example, South-Island saddlebacks (*Philesturnus carunculatus carunculatus*) translocated to two offshore islands in the Marlborough Sounds of New Zealand presented concurrent infections with *Plasmodium elongatum* and avian pox virus in the summers of 2002 and 2007 with a concurrent severe decrease in population numbers (Alley *et al.*, 2010). In addition, a New Zealand dotterel (*Charadrius obscurus*) has also been reported as co-infected with both avian pox virus and *Plasmodium* (Reed, 1997).

2.1.8 Diagnosis of avian malaria

Detection of *Plasmodium* parasites can be undertaken by one of three methods: 1) microscopic examination of blood smears for unique life stages (i.e. merozoites,

microgametocytes and macrogametocytes) developing in peripheral blood, 2) histological evidence of meronts in the tissues of dead birds, and 3) molecular diagnostic tools. Although some authors still uphold microscopy as the gold standard of malaria diagnosis (Ochola *et al*., 2006), most researchers in the field recommend that, where possible, both microscopic methods and molecular tools should be used (Valkiūnas *et al*., 2006a). While molecular tools are of advantage when not all parasite stages are visible under the microscope and a low parasitaemia is present, microscopy is still superior for identifying mixed infections (Perkins *et al.*, 2011). Furthermore, some *Plasmodium* species cannot be detected with the currently available molecular tools (Zehtindjiev *et al.,* 2012) and can only be detected in smears.

2.1.8.1 Microscopic diagnosis

Traditionally, avian malaria is diagnosed with the help of Leishman-, Giemsa- (most commonly used) or Wright-stained blood smears (from live birds) or impression smears of liver and spleen (of dead birds) using light microscopy (Colour Plate 7) (Ritchie *et al*., 1994, Fallon *et al*., 2003b, Waldenstrom *et al*., 2004, Cosgrove *et al*., 2006). Usually, a smear is examined using a light microscope under high magnification (x1000) and immersion oil. Blood smears are examined for any parasites within the blood cells. If parasites are found, they can be identified by their shape in the blood cells, the presence or absence of asexual merogony in the circulating blood and the presence or absence of hemozoin pigment granules (highly refractile small golden-brown or black deposits) (Atkinson, 1991). Identification is done by sketching or photographing what is observed under the microscope and comparing the images to already universally documented and accepted morphological characteristics (blood stages and morphometric analyses) unique to each parasite (Valkiūnas, 2005).

There are several possibilities for preparing a satisfactory blood smear which are described in detail by Ritchie et al. (1994) and Hume (1995). It has long been recommended to fix blood smears in absolute methanol if staining is delayed more than 48 hours. However, according to Hume (1995), unfixed smears can be stored for up to 3 months without staining deficiencies if they are kept clean and dry. Hume (1995) also notes that a delay between fixing and staining reduces the quality of

staining of the leucocyte granules markedly and that it is advised not to fix smears until the actual time of staining. Furthermore, a blood sample should be dried very quickly to prevent the loss of the main diagnostic characters of the parasites from exposure to air (Valkiūnas *et al*., 2008).

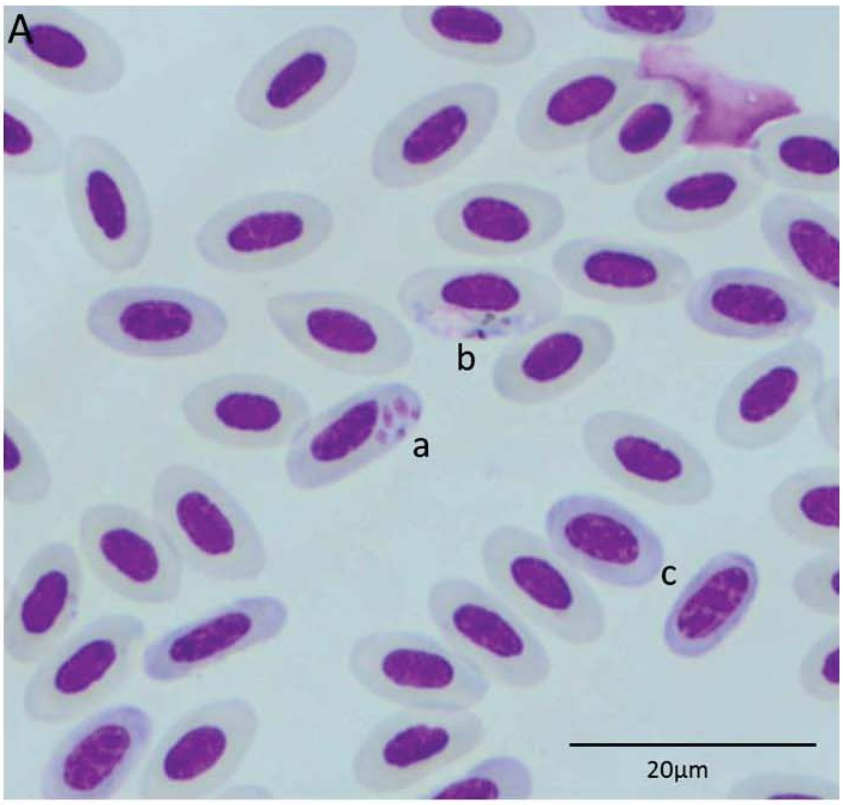
The normal mature avian erythrocyte is oval with a centrally positioned oval nucleus. The cytoplasm is abundant and stains a uniform orange-pink, resembling the cytoplasm of mammalian erythrocytes. The nucleus of the mature erythrocyte is condensed and stains dark purple. The nuclear chromatin is uniformly clumped. The red cell nuclei vary with age, becoming more condensed and darker staining as the cells age (Colour Plate 7) (Ritchie *et al*., 1994).

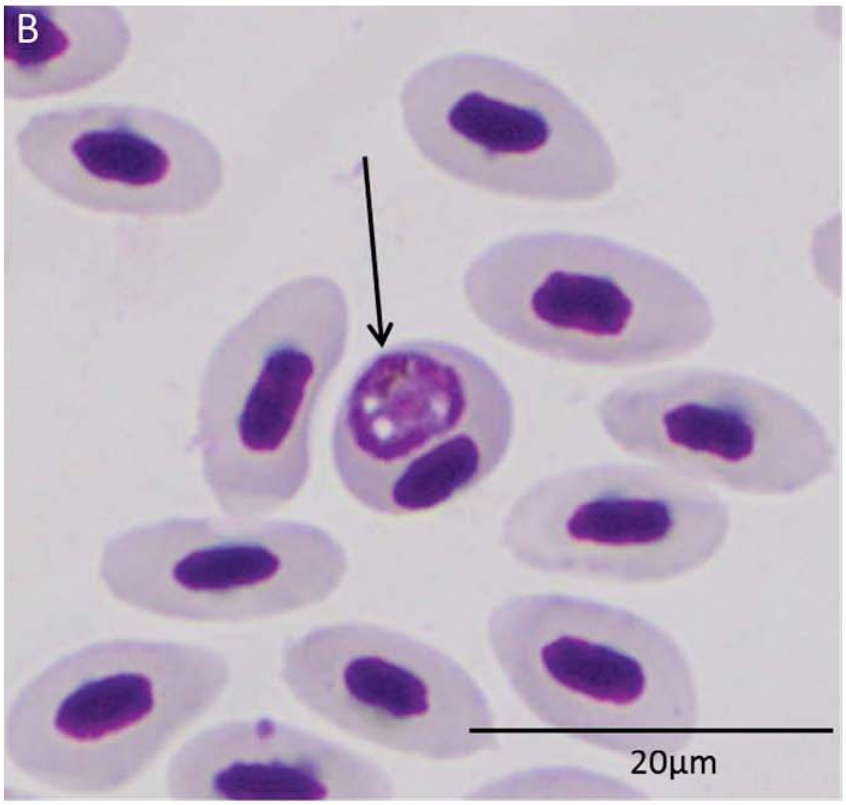
In blood smears, *Plasmodium* spp. can be identified by the hemozoin pigment that they produce during the intraerythrocytic state (Gabrisch and Zwart, 2001). *Plasmodium* gametocytes usually occupy less than 50% of the host cell cytoplasm and those of some species alter the position of the red cell nucleus. It is rare for more than one mature gametocyte to occur in one cell. Macrogametocytes have pigment granules dispersed throughout the cytoplasm of the parasite. In the smaller microgametocytes, granules appear in spherical aggregates. Another key feature aiding the detection of *Plasmodium* is the presence of merogony in red blood cells (Ritchie *et al*., 1994).

Using microscopy, 175 species of haemosporidian parasites of the genera *Haemoproteus* and *Plasmodium* had been defined up to 2007 (Palinauskas *et al*., 2007). For blood smears to be useful in the identification of genus and species, it is crucial that the blood smear is of good quality and that the correct staining technique is used (Bruce and Day, 2002). The commonly recommended stain is Giemsa, because rapid stains such as Field's stain, are less stable, bleach out more easily and cannot be used for taxonomic examinations (Valkiūnas, 2005).

Although, microscopy remains essential in the diagnosis of mixed infections, there are some significant disadvantages when compared to molecular techniques. These include the labour-intensive examination of slides and the potential for missing low level parasitaemia (Jarvi *et al*., 2002, Fallon *et al*., 2003b). In addition, the identification of parasite morphology is often difficult, and avian malaria parasites are known for their cryptic diversity (where unrelated species may look similar). This is

sometimes made worse by distortion of the cells on the smears due to air drying and fixation, environmental conditions at the sampling site or slight differences in the stains used (Perkins *et al*., 2011). Furthermore, microscopy often does not take into account potential differences between the skill and experience of individual technicians (Valkiūnas *et al*., 2008).





Colour Plate 7: Blood stages of *Plasmodium spp*.; Giemsa stain. A) *Plasmodium vaughani* SYAT05 in the blood of a blackbird, with a meront (a) and a gametocyte (b). Note immature erythrocytes (c) with darker cytoplasm and less dence nuclear chromatin. B) *P. relictum* gametocyte in the blood of a blackbird

2.1.8.2 Serology

Serological examination of blood samples is also possible. Blood plasma samples can be analysed with standard immunoblotting techniques to detect antibodies in the host's body against a crude red blood cell extract of the parasites. According to Jarvi et al. (2002), these tests are more sensitive (97%) than either nested PCR (61-84%) or microscopy (27%) and give the most accurate estimate of an infection, being able to detect low parasitaemia that cannot be detected by PCR or microscopy (Baedell, 2004). Despite these advantages, serological methods are difficult to interpret because they are only genus specific and so it is impossible to distinguish species or different parasite lineages. In addition, these methods may not be comparable and useable across different host species (Baedell, 2004).

2.1.8.3 Cytology and histopathology

Histopathological examinations of birds that have died from avian malaria typically display lesions in the lungs, liver, spleen, and occasionally other organs (Schmidt *et al.* 2003). Thus, rapid diagnosis at necropsy can often be accomplished using routine impression smears of liver, lung or spleen stained with Giemsa or difquik (Harrison, 2006, Alley *et al.* 2008).

2.1.8.4 Molecular diagnosis

New molecular methods to detect and identify avian blood parasites are all based on PCR that targets and amplifies specific parts of the parasites´ genome. PCR based methods have improved the detectability of haemosporidian parasite infections in birds (Krizanaskiene *et al.*, 2006). Owing to their large number of molecular targets, DNA-based diagnostics have the ability to detect parasites at densities too low for detection by conventional microscopy (Freed and Cann, 2003). PCR is considered a specific and sensitive method because it detects as few as 1 malaria parasite/µl of blood (Ribeiro *et al.*, 2005), and it is even possible to detect parasite DNA using DNA from stained blood smears (although fixing in methanol and staining with Giemsa affects DNA stability) (Ribeiro *et al*., 2005). Recently, PCR has also been used successfully to detect pre-erythrocytic stages of malaria parasites in faeces of the host (Abkallo *et al.*, 2014). Generally, the PCR products are separated with electrophoresis and scored as absent or present on an agarose gel. In addition to improving the detection rate, another advantage of the PCR-based methods is the possibility of obtaining a reliable genetic identification of haemosporidian species and

lineages. There are different techniques of PCR that can be performed, and the detection rate varies considerably among them (Waldenstrom *et al*., 2004).

Some assays are designed to detect the nuclear-encoded 18S small subunit (SSU) of ribosomal DNA and others detect the mitochondrially encoded cytochrome b gene. The assays which use the cytochrome b gene have an advantage. The RNA region of the mitochondrial genome of avian malarial parasites is highly conserved, making it an excellent location to design PCR primers for detecting infections (Fallon *et al*., 2003b). For example, they are able to detect both *Haemoproteus* spp. and *Plasmodium* spp. that differ by up to 12% sequence divergence in the cytochrome b gene.

In addition to the classic PCR, there are also so called “nested” assays that add a second amplification to the already amplified DNA using another, different primer. The performance of nested PCR-methods is considerably improved by the addition of an extra PCR round to the protocol. With a nested PCR, it is also possible to provide sequence-based data that enables the identification of parasites to their genus and species/lineages (Waldenstrom *et al*., 2004).

Besides the conventional PCR methods, real time PCR methods can also be used. Real time PCR includes a fluorescent dye in the reaction mix, which intercalates with amplified DNA. Fluorescence in the reaction increases with increased amount of DNA produced during the length of the PCR and can be observed in “real time”. Because the intensity of fluorescence differs with the amount of DNA in the reaction, this method is often used to determine the amount of starter DNA in the original sample and can therefore be used to determine the number of malaria parasites in the patient’s blood. Therefore his method is also known as “Quantitative PCR” (qPCR) (Madigan, 2015).

In general, after DNA amplification, a “melt” step is included in a real time protocol. The double strands of DNA dissociate under high temperatures, and depending on the amount and proportion of the different base pairs in a DNA sequence, the melt temperature differs from others and causes a unique melt “peak”. The High Resolution Melt (HRM) technique takes advantage of this and can discriminate even between the different genotypes of a certain sequence (Madigan, 2015). Real-time PCR and HRM are in general faster than conventional PCR, for example the

observation of “real time” amplification on a computer screen and subsequent unique melt peaks substitutes the use of an electrophoresis gel and sequencing after DNA amplification. These methods are also cheaper because they use smaller amounts of reagents, do not require subsequent sequencing and are usually more sensitive in picking up target DNA. With the use of HRM, it is also possible to detect mixed infections in a sample, something which is very difficult with conventional methods.

Although PCR has improved the detection of haemosporidian parasite infections, it is still far from perfect. Jarvi et al. (2002) noted that PCR diagnostics underestimated the prevalence of avian malaria in experimentally infected passerines. In chronic cases of infection, the parasites can virtually disappear from the circulation and drop to intensities below detectability. Therefore, PCR is less sensitive than serology for chronic infections (Jarvi *et al*., 2002). It may also be difficult to link the morphology of a parasite seen under the microscope with genetic information acquired by PCR. The acquired sequence may be of a light infection that amplifies better with the PCR protocol used, but it is not identical to the parasite seen under the microscope (Valkiūnas *et al*., 2007). Conventional PCR assays also underestimate double infections, which are common in the wild, as they may amplify the DNA of one parasite better than another (Valkiūnas *et al*., 2006a). To determine the true species composition of haemosporidian parasites in each individual host, PCR diagnostics need to be improved and specific primers for *Haemoproteus* spp. and *Plasmodium* spp*.* should be developed.

According to Freed and Cann (2003) the way DNA extraction is performed may also influence PCR output. Commonly used DNA extraction kits like the Qiagen DNeasy system are designed for plasmid minipreps or are adapted from cultured cell assays (or both). They assume narrow ranges of cell types or concentrations of DNA within tissue types. Because avian red blood cells are nucleated, processing viscous sample lysates of whole blood can be difficult using certain DNA purification kits. In addition, these kits do not seem to produce the pure DNA required for sensitive PCR tests for avian malaria parasites and therefore the use of phenol/chloroform extraction methods should be preferred (Freed and Cann, 2003).

2.1.8.5 Online databases

The MalAvi and GenBank databases contain sequence data of avian blood parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* which are identified by a partial region of their cytochrome b sequences (Bensch *et al.*, 2009, Sayers *et al.*, 2012). The MalAvi database (http://mbio-serv4.mbioekol.lu.se/avianmalaria/) currently includes more than 950 parasite lineages. It uses a harmonised classification and all available information about each parasite is present in a public reference location, which enables easy access for all researchers. This database is updated by researchers and new lineage names remain unique which is done via an automated online system (Bensch *et al.,* 2009). Similarly, the GenBank sequence database (http://www.ncbi.nlm.nih.gov/genbank/) is an open access, annotated collection of all publicly available nucleotide sequences and their protein translations (Benson *et al.*, 2011). This database is created and monitored by the National Centre for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database Collaboration (INSDC) (Sayers *et al.,* 2012).

2.2 Avian malaria in New Zealand

Avian malaria parasites have been known to exist in New Zealand for decades, and blood stages in birds were first described by Laird (1950), who remarked that indigenous birds in New Zealand were remarkably free of haemosporidian parasites. He found no evidence of haematozoa in endemic birds, although he suggested that a far wider study would be needed as his sample size was low. Nevertheless, Laird (1950) examined blood smears from 50 different bird species, including six species of passerines, mostly introduced. Another large scale blood-parasite survey of 43 bird species in New Zealand, including native and introduced, performed in the 1970's was unable to detect avian malaria in the examined birds, possibly due to a low sample size of n=1-10 per bird species (Fallis, 1976). The findings of Laird (1950) and Fallis (1976) are in contrast to studies undertaken in the last decade which suggest that avian malaria may be an emerging threat to New Zealand avifauna (Tompkins and Gleeson, 2006, Howe *et al*. 2012, Ballie and Brunton 2011). An alternative explanation is that these earlier studies used microscopy to identify the parasites, a technique which can overlook low levels of parasitaemia (Section 2.1.8.1).

2.2.1 Lineages Identified

To date, avian malaria parasites belonging to 17 lineages have been found in 37 different bird species in New Zealand (Table 2.2; Castro *et al.,* 2011, Howe *et al.,* 2012, Ewen *et al.,* 2012b). The most common lineages infecting endemic/native New Zealand bird species and also introduced passerines are *Plasmodium (Huffia) elongatum* lineages GRW06 and LINN1 and *P. (Novyella) vaughani* lineage SYAT05; with *P. elongatum* (GRW6) having the widest host range (see Section 2.1.4 for general information on these lineages). Other lineages of *Plasmodium* detected in endemic species are the *Plasmodium relictum* lineages GRW4 and SGS1 (Castro *et al*., 2011, Ewen *et al.,* 2012a&b, Howe *et al.,* 2012). In addition, although not common, the lineages *Novyella* (AFTRU08) and *P. relictum* (LINOLI01) have been found in bellbirds (*Athornis melanura*) (Baillie *et al*., 2011). All the lineages mentioned above are not known to be endemic to New Zealand and show a high prevalence in European birds introduced to New Zealand (Ewen *et al.,* 2012b). In contrast, a lineage, labelled "Kokako01" has to date only been found in kokako (*Callaeas cinerea*) (Howe *et al.,* 2012, Ewen *et al.,* 2012b) and lineage called Bell01 has only been found in native passerines (Baillie *et al*., 2011). These may be endemic *Plasmodium* lineages (Ewen *et al*., 2012b).

Interestingly, parasites of the closely related genus *Haemoproteus* have not been recorded in recent studies in New Zealand (Castro *et al*., 2011, Ewen *et al.,* 2012b, Howe *et al.,* 2012, Sijbranda *et al*., 2016). This is surprising, because *Haemoproteus* spp. parasites are more prevalent and common in European passerines, including in species introduced to New Zealand, than parasites of the genus *Plasmodium* (Valkiūnas, 2005). Indeed, Laird (1950) described *Haemoproteus* parasites on blood smears from introduced song thrushes and blackbirds in New Zealand. Although it is possible that Laird (1950) misidentified these parasites, this is unlikely because he was very experienced at morphological parasite identification and he used voucher specimens deposited at the National Museum to compare his findings with. It is unclear if these parasites have since disappeared from the country, maybe due to incompatible vector species, or if these parasites are not detected by molecular means and are overlooked on blood smears by less experienced researchers today.

Table 2.2: *Plasmodium* spp. lineages identified in New Zealand and their respective avian hosts (updated from Banda, 2012 and (Schoener *et al.*, 2014), excluding the present study).

Subgenus	*Plasmodium* lineage (GenBank #)	Avian species	Reference(s)
Huffia	***Elongatum* sp. GRW06** (DQ659588, DQ368381)	NI saddleback	Castro *et al.* 2011
		SI saddleback (Dead)	(Baron *et al.*, 2014)
		Silvereye	Howe *et al.* 2012
		Brown kiwi	
		Exhibition Budgerigar	(Baron *et al.*, 2014)
		Fjordland crested penguin (Dead)	(Hunter, 2015)
		Blackbird	
		Brown kiwi (dead)	Banda *et al.* 2013
		House Sparrow	Marzal *et al.* 2011
		Bellbird	Baillie and Brunton 2011
		Blackbird	
		Song Thrush	
		NI saddleback	Ewen *et al.* 2012b
		House Sparrow	(MalAvi Database)
		Yellowhammer	
		Whitehead	
		NI robin	
AFTRU5/LINN1 Cluster	**LINN1** (GQ471953)	**Blackbird (Dead)**	Howe *et al.* 2012
		Great Spotted Kiwi (Dead)	Alley et al 2012
	AFTRU5 (MalAvi)	NI Saddleback	Castro *et al.* 2011
		Exhibition Budgerigar	(Baron *et al.*, 2014)
	WA39 (EU810610)	Bellbird	Ewen *et al.* 2012b
		Blackbird	(MalAvi Database)
		Song Thrush	
Haemamoeba	**KOKAKO01** (MalAvi)	Kokako	Howe *et al.* 2012
			Ewen *et al.* 2012b
			(MalAvi Database)
	***Relictum* sp. GRW04** (AY099041)	House Sparrow	Ewen *et al.* 2012b
			Marzal *et al.* 2011
			(MalAvi Database)
		Exhibition Budgerigar	(Baron *et al.*, 2014)
	NZ Hihi (HQ453996)	**Hihi (Dead)**	Howe *et al.* 2012
	***Cathemerium* sp.** (AY377128)	Red-billed gull	Cloutier *et al.* 2011
	***Relictum* sp.**	Saddleback	Howe *et al.* 2012

	SGS1 (AF495571)	House Sparrow	Marzal *et al.* 2011 (MalAvi Database)
		Myna	Beadel *et al*. 2006 (MalAvi Database)
		House Sparrow Yellowhammer	Ewen *et al.* 2012 (MalAvi Database)
Novyella	***Vaughani* sp. SYATO5** (GenBank DQ847271)	Blackbird Kereru Blackbird Tomtit Exhibition Budgerigar	Howe *et al.* 2012 Ewen *et al.* 2012b (MalAvi Database) (Baron *et al.*, 2014)
	LIN3 (GenBank JN415758)	Bellbird	Baillie and Brunton 2011
Unresolved (NZ native *Plasmodium* spp.*?)*	**BELL01** (JQ905572)	Tui Saddleback Bellbird	Ewen *et al.* 2012b (MalAvi Database)
	LIN1 (JN415756)	Bellbird	Baillie and Brunton 2011
	NZRobin30 (JN565686)	North Island Robin	Banda 2012
Unresolved	**PADOM02**	Yellowhammer	MalAvi database (Ruth Brown, unpublished)
Unresolved	**HIHI01**	Hihi	MalAvi database (Ruth Brown, unpublished)
Unresolved	**LIN4** (GenBank JN415759	Bellbird	Baillie and Brunton 2011

[1] Species identified in bold indicate mortality due to *Plasmodium* infection.

2.2.2 Prevalence

The prevalence of these parasites among avian hosts can vary significantly between sex, region, season and year (Table 2.3). For example, Tompkins and Gleeson (2006) reported a very high prevalence of *Plasmodium* in introduced passerines in the North Island of New Zealand, with up to 100% of the examined blackbirds infected in some regions, and the authors therefore hypothesised that introduced birds like blackbirds may act as reservoirs of infection for native passerines. However, the prevalence of *Plasmodium* was markedly lower in the south of the South Island with four blackbirds caught in the Blue Mountains (Otago) not being infected with *Plasmodium*. In addition, Ballie *et al*. (2012) sampled bellbirds from Tiritiri Matangi and Little Barrier (Hauturu) Island and found that female bellbirds had a statistically lower *Plasmodium* prevalence than males, with prevalence in males up

to 2.8 times higher than in females. The overall prevalence of infections ranged from <1% to >45% across the seasons, with prevalence being highest in the winter at Hauturu (Little Barrier Island). The prevalence in males tended to peak during autumn and spring at all sites, with the prevalence in females lower all year round, but with a pronounced peak at the end of the breeding season on Tiritiri Matangi Island. A cluster of infection may also occur in one year but infection may not be detected in the same location the following year as observed in North Island brown kiwi in an Operation Nest Egg (ONE)* facility in Rotorua (Banda *et al.*, 2013). This suggests that a seasonal variation of vector abundance may have been responsible in establishing conditions suitable for the cluster event. The prevalence of each *Plasmodium* species can also vary within host species. For example, Ewen et al (2012b) reported a higher prevalence (~12%) of lineage GRW06 than lineage Bell01 (~4%) in the same population of North Island saddlebacks which may reflect evolutionary relationships between native and exotic lineages of avian malaria.

* Operation Nest Egg (ONE)- New Zealand kiwi conservation initiative where kiwi eggs are taken out of the wild, hatched in captivity, the chicks reared in a créche until they weight ~1200g and then released back into the wild. Birds at the facility in Rotorua (hatching and créche) are new individuals every year

Table 2.3: Prevalence of infection in *Plasmodium* positive introduced and endemic species reported during 2006-2013 at different sites and dates in New Zealand.

Reference	Host Species	Prevalence	Number sampled	Method of Detection
Tompkins and Gleeson, 2006	Blackbird	0-100%	85	PCR and blood smear
	Starling	0-35%	56	
	House sparrow	0-33%	147	
	Song thrush	4-19%	59	
Ishtiaq et al, 2006	Myna	10%	92	PCR
Sturrock and Tompkins, 2008	Blackbird	7%	60	PCR
	Song thrush	11%	27	
Cloutier et al., 2011	Red-billed gulls	7.8%	243	PCR
Baillie and Brunton, 2011	Bellbird	13.4%	693	PCR
Castro et al., 2011	NI saddleback	2.8-10.6%	363	PCR and blood smear
Ortiz-Catedral et al., 2011	Red-fronted parakeets	40.9%	22	PCR
Gudex-Cross, 2011[#]	Bellbird	38.6%	132	Blood smear
	Tui	20%	15	
	Tomtit	13%	8	
	Myna	50%	2	
	Blackbird	100%	4	
	Song thrush	25%	4	
	Silvereye	9.2%	224	
	Fantail	5.3%	19	

Howe et al., 2012	Blackbird	100%	7	PCR and blood smear
	Silvereye	100%	1	
	Hihi (Stichbird)	9%	11	
	Kokako	25%	8	
	Kereru	100%	1	
	NI brown kiwi	10%	10	
Ewen et al., 2012b*	Tui	~5%	30	PCR
	Saddleback	~12%	36	
	Tomtit	~7%	22	
	North Is. Robin	~2%	78	
	Whitehead	~10%	42	
	Bellbird	~7%	78	
	Song thrush	~25%	19	
	Blackbird	~40%	44	
	House sparrow	~7%	60	
	Yellowhammer	~17%	23	
Baillie et al., 2012	Bellbird	<1%->45%	457	PCR
Banda et al., 2013	NI brown kiwi	0-78%	32	PCR and blood smear
Sijbranda et al., 2016	Blackbird	82%	34	PCR
	NI brown kiwi	5%	20	
	Dunnock	100%	1	

Nth. Is. robin	4%	100
Silvereye	21%	33
Song thrush	100%	4

#Master's Thesis, Massey University, 2011.

*Prevalence in host estimated based on data presented in bar graph form. Level of co-infection within host is unclear.

2.2.3 Pathogenicity

Recent outbreaks of avian malaria in NZ passerines caused by a variety of *Plasmodium* spp. have been recorded in captive yellowhead/mohua (Alley *et al.* 2008), European blackbird (Schoener, 2009) and South Island saddleback (*Philesturnus carunculatus carunculatus*) (Alley *et al.,* 2010). In addition mortality events have also be reported in captive dotterel chicks (Reed 1997) and in brown (*Apteryx mantelli*) and great spotted kiwi (*Apteryx haastii)* (Howe *et al.,* 2012, Alley *et al.,* 2012, Banda *et al.,* 2013). This suggests that although most lineages only cause subclinical and chronic infections (Gudex-Cross, 2011), they are also capable of causing mortality under the right conditions.

These findings are compatible to those from studies in other parts of the world. Generally *Plasmodium* spp. cause little direct mortality in their natural environments and are believed to be relatively harmless to wild birds (Bennett *et al.,* 1993, Valkiūnas, 2005), although this does depend on the *Plasmodium* species in question (Section 2.1.7).

2.3 Avian malaria and climate change

With the advent of global climate change there is discussion about the possible impact that changed climate patterns may have on parasites, vectors and disease outbreaks. There is a fear that climate change may increase the distribution and prevalence of infectious diseases. Indeed, increased temperatures speed up biochemical reactions, increasing many of the key processes like activity, growth, development and reproduction in ectothermic organisms. On the other hand, a faster

metabolism may also limit survival as more nutrition is needed to maintain a higher metabolic rate (Lafferty, 2009, Gallana *et al.*, 2013). In the case of malaria parasites, a higher metabolism of the vector means that a mosquito has to increase its biting rate which in turn increases the chance of parasite transmission. In addition, the time for development of the parasite in the vector is decreased. In higher temperatures, the mortality rate of the vector increases, which may cause the vector to die before the parasite can be transmitted (Lafferty, 2009). In addition to temperature, other climate related variables need to be taken into account. These variables should encompass the whole ecological context of the host-pathogen-vector relationships (Gallana *et al.*, 2013). Therefore making definite predictions about the impact of climate change on disease is difficult, and complex, non-linear responses of disease systems are to be expected (Gallana *et al.*, 2013).

One example of the possible impact of climate change that has been extensively studied is the situation of avian malaria (caused by *P. relictum*) and the susceptible endemic birds on Hawaii. Today, many of Hawaiis's endemic birds survive only at high altitude in forest refuge areas. The transmission of avian malaria does not occur at elevations above 1500m, because sporogonic development of the parasites in the vector slows down at 15°C and ceases at 13°C. This isotherm closely follows the 1500m contour line on the Hawaiian Islands (Atkinson and LaPointe, 2009). The models of temperature increases predict a steep decline in available malaria-free forest habitat in the future, by 57% on Maui Island and 96% on the Island of Hawaii. On the island of Kaua'i, available habitat is predicted to disappear completely (Atkinson and LaPointe, 2009). According to Atkinson et al. (2014), there is already an increase in avian malaria prevalence in the previously safe high altitude refuge areas on the island of Kaua'i, causing a decline in two of the susceptible bird species (Atkinson *et al.*, 2014).

Disease modelling has become an important tool in the future prediction of the impact of climate change on avian malaria in places outside of Hawaii. A modelling study on the Island of Tenerife found that of the environmental factors, the minimum temperature of the coldest month of the year was the most important predictor of avian malaria infection. In addition, human impact factors such as distance to artificial water reservoirs and distance to poultry farms were important predictors

(Gonzalez-Quevedo *et al.*, 2014). Another modelling study from mainland Spain (as opposed to Tenerife which is a Spanish island) predicts a rearrangement of high prevalence and richness of haemosporidian parasites in Spain. Based on this model, parasites of the genera *Haemoproteus* and *Leucocytozoon* will decrease, while *Plasmodium* parasites will increase their range on the Iberian Peninsula in years to come. The prevalence of multiple infections as well as parasite richness will be reduced and population differences in parasite prevalence and diversity will decrease, creating a more homogeneous and impoverished parasite landscape (Perez-Rodriguez *et al.*, 2014).

In conclusion, climate change may have an impact on avian malaria, but there are other important factors, especially other stressors created by human impact and introduced species which play more important roles. To minimize the impact of avian malaria, independent from the severity of the actual climate change situation, one of the most important counter measures will be the control and reduction of the mosquito larval habitat (Atkinson and LaPointe, 2009).

2.4 Vectors

2.4.1. Transmission and vectors

Insect vectors are compulsory for malaria parasites to complete their life cycle. In Hawaii, where avian malaria is believed to have contributed to the extinction of many native bird species, *Plasmodium* spp. did not reach epizootic proportions before the spread of the exotic mosquito *Culex quinquefasciatus* (van Riper III *et al.*, 1986). For most species of *Plasmodium*, the insect hosts are known to be mosquitoes of the genera *Culex*, *Aedes* and *Anopheles* (Ritchie *et al.,* 1995; Valkiūnas, 2005). It was proposed by Martinsen *et al.* (2008), that the major clades of haemosporidian parasites are associated with vector shifts in the different dipteran families. *Plasmodium* spp. parasitizing birds and reptiles rely on mosquitoes of the family Culicidae, excluding the genus *Anopheles* which is known to transmit parasites that infect mammals. Although this view has been widely accepted, recent findings may lead to its revision. Biting midges of the genus *Culicoides*, formerly thought to be only able to transmit haemosporidian parasites of the genus *Haemoproteus*, have recently been found to be infected with *Plasmodium*, but the viability of these vectors

to transmit the parasite still needs to be confirmed experimentally (Santiago-Alarcon *et al.*, 2012a).

Vector competence for transmitting avian malaria varies between mosquito species, and each *Plasmodium* species may use a number of different mosquito species as vectors (Kimura *et al.*, 2010). However, a specific list of vectors for *Plasmodium* spp. has not yet been determined (Valkinuas, 2005, Glaizot *et al.*, 2012). So far, the genus *Culex* seems to provide the most successful vectors worldwide; in different studies it has been found that mosquitoes of this genus contained the biggest diversity of different *Plasmodium* lineages (Kimura *et al.,* 2010, Glaizot *et al.,* 2012). It has also been discovered that the diversity of *Plasmodium* lineages infecting mosquitoes is greater than that infecting birds, thereby including lineages that have not yet been recovered from birds (Kimura *et al.,* 2010). Kimura *et al.* (2010) suspected that this is due to most malaria studies being biased towards small common passerines that are easily caught in mist nets. It is therefore possible that more malaria lineages wait to be found in rarer, more elusive bird species.

Plasmodium parasites in birds have successfully optimized their transmission from the vertebrate host to the vector. Cornet et al. (2014) showed *Plasmodium* parasites in chronically infected birds react to the presence of vectors. Parasitaemia in birds exposed to (uninfected) mosquito bites appears to be significantly higher than in unexposed birds during the chronic stages of the infection. This means that there is significantly higher infection prevalence in the mosquito. Chronically infected birds are also more attractive to mosquitoes, and this is not dependent on the infection state of the mosquito, with both infected and uninfected mosquitoes behaving similarly (Cornet *et al.*, 2013). This phenomenon may be explained by the presence of volatile vector attractants that are produced by parasites in the vertebrate host, as was found in a study on *Plasmodium falciparum* and its vector *Anopheles gambiae* (Kelly *et al.*, 2015).

Most transmission of the parasites between birds in the Northern hemisphere occurs during the breeding season when vector populations are increasing with the onset of warm weather. During this time, adult birds with chronic, relapsing infections are available as sources of infection, and non-immune juvenile birds are hatching and leaving the nest (Atkinson 1991). This was also found in a study by Tomkins et al.

(2008) where a high prevalence of avian malaria in birds on Long Island in New Zealand coincided with an unusually high abundance of mosquitoes. In a study on *Culex pipiens* in Switzerland, female mosquitoes caught in summer were more likely to be infected than females caught in spring (Lalubin *et al.*, 2013). Similar results were found in a study in Spain, where parasite prevalence in vectors was highest in autumn and lowest in spring (Ferraguti *et al.*, 2013). Labulin et al. (2013) showed that the dominance of different *Plasmodium* species within the studied vector population varied during the seasons. While the total prevalence increased, infections with *P. vaughani* (SYAT05) decreased from spring to summer to be replaced by different *P. relictum* lineages (SGS1; GRW11, PADOM02). This may be due to seasonal changes in host feeding preferences, the development of immunity in the vector against different lineages or different lineages developing under different environmental conditions.

Annual differences in the prevalence of blood parasites in birds also exist (Allander and Bennett, 1994). This may be explained by annual variation in vector abundance and activity at the time for transmission in the year before the sample was taken. The feeding activity of dipteran vectors depends on weather conditions and can be affected by temperature and wind. Another explanation for the yearly changes may be the winter mortality of the vertebrate hosts. Blood parasites may be stressful for the host, and during wintertime uninfected birds may survive better than infected birds (Allander and Bennett, 1994).

Studies of vector competence and behaviour reveal important aspects of parasite transmission that determine when and where hosts become infected. Insect vectors show preference to different kinds of vertebrate hosts. *Culex quinquefasciatus* is known to feed on birds as well as mammals including humans (Tsuda *et al.*, 2009). A study examining feeding patterns on vertebrates using serology to roughly identify the vertebrate blood in the insects, found that *Aedes notoscriptus* fed on 27 (15.9%) bird species out of 170 possible vertebrate hosts, while *Culex quinquefasciatus* fed on seven species (28%) out of 25 vertebrate hosts (Kay *et al.*, 2007). Riberio and Francischetti (2001) examined the feeding (probing and sucking) mechanics in three genera of mosquitoes (*Aedes*, *Anopheles* and *Culex*). They found that the blood sucking behaviour of *Culex quinquefasciatus* was significantly different from mosquitoes of the genera *Aedes* and *Anopheles.* The blood meals of *C.*

quinquefasciatus were not as fast and the saliva had the least anti-clotting activity. This made its' feeding behaviour less efficient on mammals. The reason for these differences is believed to be in the distinct characteristics of the thrombocytes of birds and mammals, with birds for example having nucleated thrombocytes. Various vector species also display other differences in their behaviour. In Hawaii, *C. quinquefasciatus* was more active during the night, while *Aedes* spp. were found to be more active during the day. In addition, *C. quinquefasciatus* did not feed during the day and was virtually absent from forests higher than 600m above sea level (van Riper III, 1986).

2.4.2 Infection in the vector

Malaria parasites have a negative impact on their insect vectors and can cause morbidity and mortality. Labulin et al. (2014) showed that mosquitoes infected with *Plasmodium*, had a significantly lower starvation resistance under low nutritional conditions (Lalubin *et al.*, 2014). The parasites actively absorb carbohydrates from the haemocoele, disturb the metabolism of amino acids, induce mechanical distortion of the epithelial cells in the midgut and therefore cause an increased mortality rate in the infected female mosquitoes (Valkiūnas, 2005). Different *Haemoproteus* spp. are known to increase the mortality rate of their infected insect host, the mortality being highest for the days 1-2 and 3-4 post-feeding, indicating possible negative effects of the sexual parasite stages on the vector (Valkiūnas and Iezhova, 2004). Infections with *Haemoproteus* parasites have also been found to significantly increase the mortality rate in bird biting mosquitoes (Valkiūnas *et al.*, 2014a). The impact of malaria parasites on mosquitoes and the mechanisms of different pathologies have been reviewed by Ferguson and Read (2002). Interestingly, infection with malaria parasites also changes the behaviour of the mosquito. Infected mosquitoes have a higher biting rate that is dependent on the sporozoite load of the individual, and which increases the chances of transmitting the disease to a vertebrate host (Koella, 1999, Ferguson and Read, 2002).

The malaria stages in the vector can be found in the midgut and salivary gland (see life cycle 2.1.5) (Valkiūnas, 2005). In the mosquito, the infection has probably little effect on the salivary gland cells, in contrast to major damage like apoptosis and expulsion caused by the ookinete in the midgut (Kim *et al.*, 2009a).

2.4.3 Possible vectors in New Zealand

Mosquitoes as vectors in New Zealand have not been studied in detail. New Zealand possesses 12 species of indigenous mosquitoes as well as four introduced species (Derraik, 2004) (Table 2.3). This number may increase in the future for several reasons. One reason is that recent taxonomic and genetic studies into different mosquito species are ongoing, and there is a possibility that, for example, *Aedes notoscriptus* may not be a single species, but a complex of species (Endersby *et al.*, 2013). In addition, there is also the possibility that more vector species will become inadvertently introduced into New Zealand, like the invasive Asian tiger mosquito (*Aedes albopictus*) which is a problem both in Australia (Williams, 2012, Hill *et al.*, 2014) and Europe (Kampen and Werner, 2014). In the future, New Zealand will remain at risk from invasion by this species (Peacock, 2013) because larval habitats (standing clean water) are largely underutilised, providing open niches for new vector species to become established (Laird, 1995).

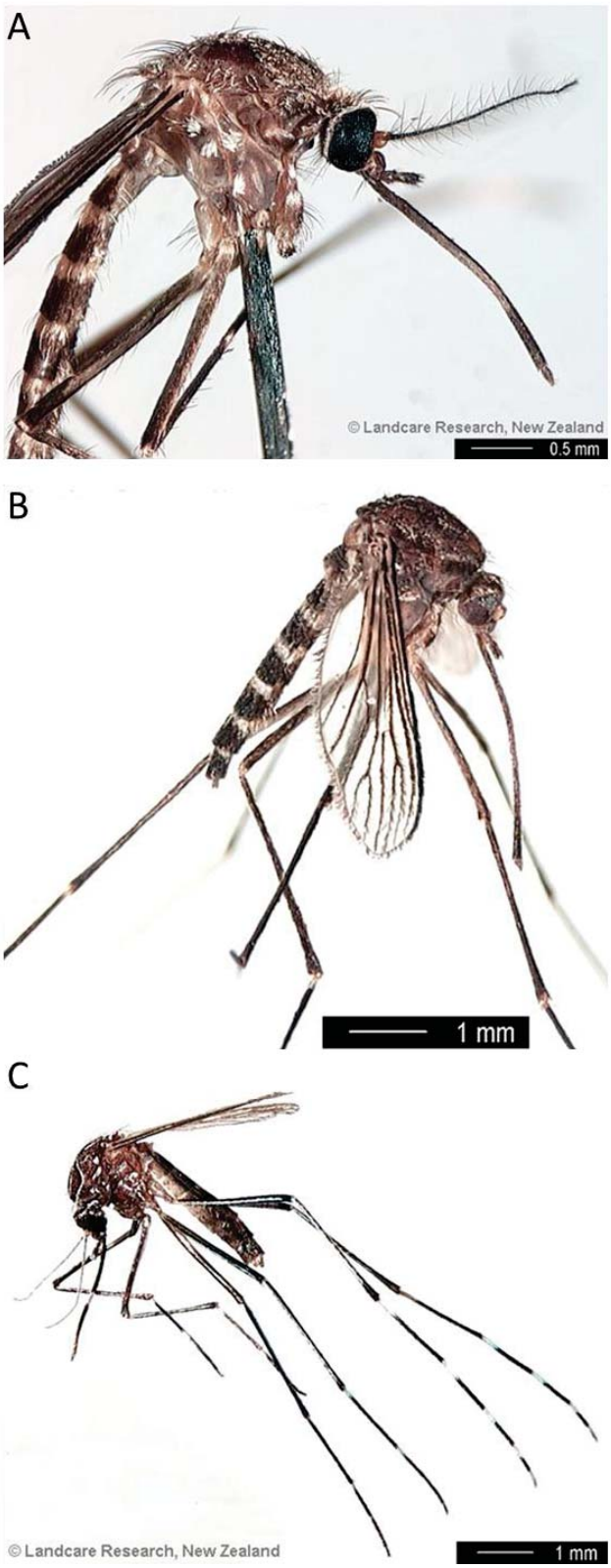
The distribution of different vector species in New Zealand will also change in the future, partly because of global climate change, but also because already introduced exotic mosquitoes have experienced a dramatic increase in their ranges in the past 40 years. For example the native species *Opifex fuscus* has already been replaced by *Aedes australis* in the south-east of the South Island (Cane, 2010).

The only mosquito which so far has been found to carry *Plasmodium* DNA in New Zealand is *Culex pervigilans*, a native mosquito (Massey *et al.*, 2007), although this finding was only in a single engorged female, therefore vector competence is unknown. Other possible vectors are the exotic mosquito *C. quinquefasciatus,* that has spread rapidly in New Zealand (Tompkins and Gleeson, 2006), and *Ochlerotatus (Aedes) australis* (Derraik and Slaney, 2007). In an outbreak of avian malaria in mohua in Orana Park in Christchurch, *C. pervigilans* was identified as the most likely vector which showed a peak population in midsummer (February) (Derraik and Slaney, 2007).

Table 2.4: List of established mosquito species in New Zealand, their origins and associated pathogens (modified after Derraik, 2004)

Origin	Species	Associated pathogens
Endemic	*Coquillettidia (Austromansonia) tenuipalpis* (Edwards)	unknown
	Coquillettidia (Coquillettidia) iracunda (Walker)	unknown
	Culex (Culex) asteliae (Belkin)	unknown
	Culex (Culex) pervigilans (Bergroth)	WH
	Culex (Culex) rotoruae (Belkin)	unknown
	Culiseta (Climacura) novaezealandiae (Pillai)	unknown
	Culiseta (Climacura) tonnoiri (Edwards)	WH
	Maorigoeldia argyropus (Walker)	unknown
	Ochlerotatus (Nothoskusea) chatamicus (Dumbleton)	unknown
	Ochlerotatus (Ochlerotatus) antipodeus (Edwards)	unknown
	Ochlerotatus (Ochlerotatus) subalbirostris (Klein & Marks)	unknown
	Opifex fuscus (Hutton)	unknown
Exotic	*Culex (Culex) quinquefasciatus* (Say)	AM AP BF CH JE KUN LF MVE RR SIN SLE WNV
	Aedes notoscriptus (Skuse)	BF CH DEN JE RR RVF
	Ochlerotatus (Halaedes) australis (Erichson)	CH DEN RR WH
	Ochlerotatus (Ochlerotatus) camptorhynchus (Thomson)	BF KOK RR SIN

Note:
Arboviruses: BF (Barmah Forest), DEN (dengue), JE (Japanese encephalitis), KOK (Kokobera), KUN (Kunjin), MVE (Murray Valley encephalitis), RR (Ross River), RVF (Rift Valley fever), SIN (Sindbis), SLE (St. Louis encephalitis), WH (Whataroa), WNV (West Nile). Other pathogens: AM (*Plasmodium relictum*, avian malaria), AP (avian pox viruses), CH (*Dirofilaria immitis*, canine heartworm), LF (Wuchereria bancrofti, lymphatic filariasis).



Colour Plate 8: Mosquitoes found in New Zealand. A) *Culex pervigilans*, a native mosquito. B and C) *Aedes notoscriptus*, an introduced species. D) *Opifex fuscus*, a native mosquito. Pictures by courtesy of Landcare Research (A, B, C) and Southern Monitoring Serives Lmd. (D)

2.4.4 Diagnosis of infection in the vector

The malaria stages in the vector can be found in the midgut and salivary gland (Valkiūnas, 2005). To find an infection in the vector, the insect has to be carefully dissected. La Pointe (2005) describes a dissection of mosquitoes in 0.9% saline, with the midguts stained in 1% mercurochrome and examined at 400x. In addition, the molecular diagnosis methods as described in Section 1.1.7.3 can also be applied to the vector.

When performing PCR, it is of advantage to examine the thorax and abdomen of mosquitoes separately, as not all mosquitos are competent vectors for all *Plasmodium* lineages and therefore abort the parasite lifecycle before the production of sporozoites. In accordance with the parasite life cycle (2.1.6), parasites found in the abdomen are assumed to be from a recent blood meal while parasites in the thorax are assumed to have completed the invertebrate host part of their life cycle and be capable of infecting the vertebrate host.

Examining individual mosquitoes would be ideal for detecting the real diversity of *Plasmodium* parasites (Zele *et al.*, 2014, Kimura *et al.*, 2010), but this approach is costly and many research groups use pooled samples (Kim *et al.*, 2009, Njabo *et al.*, 2011, Njabo *et al.*, 2009). In the case of blood-fed mosquitoes, individuals have to be processed individually to also determine the identity of the bird host (Kim *et al.*, 2009, Hamer *et al.*, 2009). In a previous study, it was possible to detect host DNA for up to three days after the bloodmeal, using primers specific for different bird orders in PCR (Ngo and Kramer, 2003).

For the identification of the vertebrate host of blood-fed mosquitoes, the use of several different subsequent PCR assays and primer sets is recommended to maximise efficiency and therefore minimize the number of unidentified blood meals, as shown by Hamer *et al.*, 2009, and Medeiros *et al.*, 2015.

Chapter 3: Wildlife translocation and parasites- Pathogen pollution and loss of parasites

This chapter is a literature review and provides a short introduction into conservation translocations, with a special focus on pathogen pollution and the possible impacts of parasites on translocations as well as the importance of parasites in ecosystems.

3.1 What are wildlife translocations?

The International union for the conservation of nature and natural resources, species survival commission (IUCN) defines conservation translocation as the intentional movement and release of a living organism. It must intend to yield a measurable conservation benefit at the levels of a population, species or ecosystem, and not only aim to benefit translocated individuals. Conservation translocations can be done for two major purposes (i) reinforcement and reintroduction when the species is translocated within its indigenous range, and (ii) conservation introductions, comprising assisted colonisation and ecological replacement, when the translocation is outside the indigenous range (IUCN/SSC, 2013).

Translocations have been used as a wildlife management tool for a long time. Initially this was mainly through translocations of game birds and mammals for hunting purposes (Massei *et al.*, 2010). The first conservation translocation is thought to have been the reintroduction of American bison (*Bison bison*) into Oklahoma in 1907 (Seddon *et al.*, 2007). Since then, many more translocations have been performed (Soorae, 2008, Soorae, 2010, Soorae, 2011, Soorae, 2013, Weise *et al.*, 2015). Today, the most important goals of translocation include an attempt to solve human-animal conflicts, restock game populations and, as for the context of this thesis, conservation (Fischer and Lindenmayer, 2000).

For decades, conservation translocations have been instrumental in creating metapopulations of endangered species. In that, the survival of the species does not rely on a single population, but instead several other populations are created to

decrease the chance of extinction if a disaster strikes (such as a disease outbreak or severe storm) (Ballou, 1993).

3.2 Translocation as a globally important conservation method

Worldwide, biodiversity is in decline due to human activities, including causing pollution, destroying habitat and overexploiting resources. Another threat is human induced climate change which may accelerate the extinction risk for many species (Urban, 2015). Wildlife translocations are a useful tool mitigating these threats for at least some species.

In the early days, conservation translocations often failed to establish populations of the target species due to a lack of knowledge and experience, resulting in poor planning, the introduction of inappropriate founder animals, low animal numbers and/or lack of resources (Seddon *et al.*, 2007). As a result of the poor success rate of translocations Dodd Jr and Seigel (1991) recommended that it was preferabe instead to deal with the reasons for decline rather than translocating animals. Since the work in the nineties, the success rate of translocations has improved. Still, however, translocations of reptiles and amphibians have a lower success rate than other vertebrates. This is mainly because these translocations are often performed to mitigate human-animal conflicts and then fail due to the released animals homing instinct or migration and the provision of poor habitat (Germano and Bishop, 2009). One of the best examples of a translocation failing due to animals returning to the area they had been translocated from, is that of the Agassiz's desert tortoise (*Gopherus agassizii*) (Edwards *et al.*, 2004, Hinderle *et al.*, 2015, Nussear *et al.*, 2012).

There is a clear species bias for translocation, with birds and mammals being most commonly translocated (Fischer and Lindenmayer, 2000; Seddon *et al.*, 2007). In their 2000 review, Fischer and Lindenmayer assessed the then published results of animal relocations and examined a total of 180 cases. They found that re-introductions (the "attempt to establish a species in an area which was once part of its historical range, but from which it has been extirpated or become extinct") were more successful if the source population was wild, not captive, a large number of animals was released (>100) and the cause of the original decline was removed. For a decision framework around the number of animals used in translocations and the

mitigation of Allee effects, see (Armstrong and Wittmer, 2011). Translocations to mitigate human-animal conflicts mostly failed, a finding also supported by Germano and Bishop (2009).

The success of a translocation is defined by the survival of the translocated species and its ability to establish a self-sustaining population (IUCN guidelines 2013). Therefore, the success depends on the prior evaluation of suitable species, healthy individuals and suitable habitat (Kock *et al.*, 2010). Often, wildlife managers take only first year observations, such as first year survival, establishment and first time breeding, into account when evaluating their success. In contrast, for a project to be viable, long term surveillance has to be implemented (Seddon, 1999, Axel Moehrenschlager and Doug Armstrong, pers. comm.).

In recent literature, translocation has also been suggested for "proactive conservation management" (Morrison *et al.*, 2011). For this, species at risk of extinction are translocated for the purpose of assisted dispersal, migration and/or colonisation (Mawdsley *et al.*, 2009). This can also be seen as a possible strategy to adapt a species to climate change. One example is the case of the island scrub jay (*Aphelocoma insularis*) which today only survives on one island off the coast of California. Morrison et al. (2011) suggested the reintroduction of these birds to Santa Cruz Island where they have existed in historic times, in order to provide a second population and also help adapt the species to climate change. The climate on Santa Cruz Island is predicted to remain cooler than that of their current island habitat. However, despite these suggestions, assisted colonization to mitigate changes due to climate change remains controversial (Hancock and Gallagher, 2014). The prohibitive costs may divert funds from other conservation projects and there is the potential risk of spreading disease by moving species around. In addition, the knowledge about species biology and ecology is often limited and the impact of the assisted movement cannot be predicted, for example a translocated species might become invasive when moved outside of its native range. Therefore it may be more advisable and more important to take action against threats to the species in their current range, restore habitat and increase habitat connectivity to facilitate the migration of organisms on their own (Hancock and Gallagher, 2014).

Another controversial topic has been brought up with the suggestion of "De-Extinction", the resurrection and reintroduction of previously extinct animals. With the advances in molecular techniques, it may soon be possible to "bring back" certain species (Campbell, 2014, Whittle, 2014, Parker, 2014, Seddon *et al.*, 2014). Still, many ethical questions remain, including whether the conservation of current species and their habitat should be more important than chasing a costly dream.

In conclusion, animal translocations may not just serve conservation or research purposes (Seddon *et al.*, 2007), but can also bring benefits for the wider human community (Parker, 2008). With the translocation of iconic species to places where the public can interact with them, the interest of the human community towards conservation issues increases, and as a result public investment in conservation also increases. A good example of this is the huge public support for wildlife sanctuaries like Tiritiri Matangi Island, Karori ("Zealandia") and Bushy Park in New Zealand (Parker, 2008).

3.3 Possible problems faced by wildlife translocations

The risks for species going through the translocation process are multiple. They affect in many ways the focal species, their associated communities and ecosystem functions in both the source and destination areas. There are also risks around human concerns. Any proposed translocation should have a comprehensive risk assessment carried out, with the level of effort appropriate to the situation. Where risk is high and/or uncertainty remains about risks and their impacts, a translocation should not proceed (IUCN/SSC 2013).

An overview of problems that are possible in wildlife translocations was presented by Armstong and McLean (1995), using examples from translocations performed in New Zealand until that date (see below). A more recent review by Letty et al. (2007) examined problems encountered in animal translocations worldwide. The movement of animals to a new and unfamiliar place poses a severe challenge for those animals and translocations can introduce high mortality rates, low breeding success or abnormal behaviour, which may lead to failure (Letty *et al.*, 2007). A whole range of problems may occur during the course of an animal translocation.

3.3.1 Introduced predators

Introduced predators are a huge problem to native ecosystems worldwide, often threatening other species with extinction. One of the most striking examples is the introduction of the brown tree snake (*Boiga irregularis*) to the island of Guam which devastated most of the native bird populations. Moving surviving animals out of the reach of those predators is an important way to ensure survival. This has been one of the main reasons for performing translocations in New Zealand. At first, many conservation translocations in New Zealand, failed due to introduced mammalian predators still being present at the dedicated release sites. The success rate increased after better predator control and predator exclusion became available and was enforced (Armstrong and McLean, 1995, Miskelly and Powlesland, 2013).

3.3.2 Habitat suitability and habitat requirements

The challenges of a new habitat can well mean the failure of a translocation. In some cases, food resources may be limited and the animals require supplementary feeding at the new site (Armstrong and Perrott, 2000), this may be especially important at certain times of the year with seasonal variations in food availability (Letty et al., 2007, Makan *et al.*, 2014).

Poor habitat is also the most common reason for failure in amphibian translocations (Germano and Bishop, 2009). Also, in Agassiz's desert tortoises, when animals were translocated to an atypical habitat, they generally moved around until they reached more suitable patches. The tortoises do best in typical Mojave Desert scrub habitats (Nussear *et al.*, 2012), with the best results found in old growth fenced off areas excluding livestock and vehicles (Berry *et al.*, 2014). Hihi (*Notiomystis cincta*) in New Zealand prefer old growth forests, and fledging success is greatest in old and complex habitats (Makan *et al.*, 2014). This is a problem for hihi conservation, because most of the islands and mainland areas which were targeted for a translocation of hihi consist of regenerating second growth forests. It is therefore important that nest boxes and supplementary feeding are provided for these birds after they have been translocated. Certain habitats may also predispose animals for disease, such as in the case of aspergillosis in hihi on Mokoia Island (Alley, 1999). Hihi are very susceptible to infections with the fungus *Aspergillus fumigatus* (a facultative pathogen). It appears that disturbed regenerating forest habitats, as seen in certain areas targeted for translocation, like Mokoia Island and Mt. Bruce National

Wildlife Centre, have a significant higher level of *A. fumigatus spores* which may have caused translocation failure in these areas (Perrott and Armstrong, 2011).

Therefore, the best choice of new habitat is the one that matches best the environment of the source population (Letty *et al*., 2007), if the source population survives in suitable habitat. A popular example is the New Zealand Takahe (*Porphyrio hochstetteri*) which was thought extinct but had survived in alpine habitat unsuitable for long-term survival of the species. Survival for this bird was possible because introduced predators were also rare in this area since they also preferred a different type of habitat (Wilson, 2003).

3.3.3 Behaviour

The behaviour of the translocated animals is also of vital importance. Early learning and socialisation has to be taken into account in reintroduction and translocation projects. Many projects have failed because of behavioural problems of the translocated animals or insufficient post-release monitoring and support (Jones, 2015).

In some cases, such as with the New Zealand saddleback, juveniles are thought to be better suited for translocations than adults, because they are better able to make behavioural adjustments to the new environment (McLean and Armstrong, 1995). However, in other cases it is better to translocate adults (Letty *et al*., 2007). Problems may also occur in cases like the saddleback (Parker *et al.*, 2010) and kokako (*Callaeas cinereus*) (Bradley, 2012, Valderrama *et al.*, 2013), where birds in different populations show song divergence and distinct dialects which might influence the success of translocations.

3.3.4 Stress

Recently, the role of stress in the failure of animal translocations has been recognized in a variety of publications (Letty *et al.*, 2007, Teixeira *et al.*, 2007, Dickens, 2015, Dickens *et al.*, 2009, Dickens *et al.*, 2010, Germano and Bishop, 2009, Guy *et al.*, 2013). Here, the acute stress to animals during the translocation has been taken into account (Letty *et al.*, 2007, Germano and Bishop, 2009), as was also chronic stress, which could occur for months after the actual translocation event (Dickens *et al*., 2010). Translocation is a great challenge because animals are

subjected to stressful events like physical handling, transport and release into an unfamiliar area (Letty *et al*., 2007).

Under natural circumstances, an acute stress response is composed of adaptive physiological and behaviour responses to a stressor and is seen as beneficial to the animal. If the stressor persists or a series of acute stressors cause multiple consecutive stress responses, the animal becomes chronically stressed, suffering all the pathological consequences of this condition (Dickens *et al*., 2010). As in humans, stress has an influence on the cognitive function of animals. It can reduce cognitive abilities and animals with reduced cognitive abilities will have difficulty adapting to a new environment and may experience further problems (Teixeira *et al.*, 2007).

In their review, Dickens et al. (2010) discuss the physiological and pathological consequences of acute and chronic stress for translocated animals. Chronic stress, following capture, handling, transport, captivity and release may lead to a decreased fight-flight response increasing the risk of predation, anxiety, a deregulated metabolism due to increased exposure to glucocorticoids and changes in the hypothalamic-pituitary-adrenal-axis function, altered environmental coping mechanisms and immunosuppression. These factors increase the vulnerability of translocated animals and may contribute to translocation failure due to starvation, reduced reproductive capacity, predation and dispersal as well as disease. Therefore, reducing the impact and time of chronic stress on animals, for example by decreasing handling time, improving transport and captive facilities and supplementary feeding in the new habitat, will increase the likelihood of translocation success (Dickens *et al*., 2010).

The negative impact of stress on translocated animals is emphasised by a translocation experiment by Dickens et al. (2009), using Chukar partridge (*Alectoris chukar*), where a single exposure to a capture and handling event was enough to cause a long term decrease in endocrine responsiveness, ascertained by measuring a significantly decreased acute glucocorticoid (corticosterone) response. Also in reptiles and amphibians, even short holding periods cause significant acute stress, which can persist for up to a month (Germano and Bishop, 2009). Therefore it is important to pay attention to the translocation protocol, its different stages and how long the animals are going to be handled for, held captive or transported for (Letty *et*

al., 2007). “Soft” release methods (the release of animals into a new habitat after a certain acclimatisation period) may also have a negative impact on the survival of the translocated animals. For example Richardson et al. (2015) who looked at the impact of delayed release on the survival of hihi up to seven months after translocation found strong evidence for a negative effect of delayed release on long term survival, which was not evident in the first six weeks after translocation but affected the survival of the birds for up to seven months after the translocation.

3.3.5 Source population

It is important for the success of a translocation to look at factors such as environmental imprinting, captive versus wild animals and conditioning. In addition, the size and composition of the founder group of animals is of importance. In their review, Fischer and Lindenmayer (2000) mentioned that translocations with large numbers of animals (>100) were more successful, and for decades a minimum of 40 individuals has been recommended in New Zealand (Armstrong and McLean, 1995). For a decision framework around the number of animals in translocations and the mitigation of Allee effects, see (Armstrong and Wittmer, 2011).

3.3.6 Genetics

Genetics and possible population bottlenecks in the founder group also have to be taken into account for animal health during and after a translocation (Armstrong and McLean, 1995). Genetic diversity may or may not play a role in the future survival of a species, if the species if confronted with a novel pathogen. For example, increased genetic heterozygosity was not associated with increased fitness and survival among translocated bighorn sheep (*Ovis canadensis*) in respect to different respiratory viruses. For a New Zealand example in respect of genetic heterozygosy in translocated birds, see Section 4.4.1 Translocation history of the North Island saddleback (*Philesturnus rufusater*).

3.3.7 Other

Funding and bureaucracy. Another aspect that must not be ignored is the financial cost and effort connected to every translocation, as well as issues of local and national governance (Guy *et al.*, 2013).

Disease and pathogen pollution. A final important factor that may cause problems and the failure of translocation projects is the presence of disease and introduced pathogens. This will be reviewed more closely below.

In conclusion, many factors have to be taken into account to ensure the success of a wildlife translocation. If there is too much uncertainty around the success of a particular translocation, it will need to be postponed or abandoned. To assist with the decision of whether a translocation is worth pursuing or not, a conceptual decision framework for prioritizing recovery action for species under high risk of extinction has been constructed by Jachowski (2015).

3.4 Wildlife translocations in New Zealand

After the arrival of humans and the introduction of exotic mammalian predators, the islands of New Zealand lost at least 44 endemic species of birds, which was around 50% of its native avian fauna (Holdaway, 1989, Steadman, 1995). To preserve the remaining species, several approaches for the conservation of endemic New Zealand avifauna have been used by New Zealand conservation authorities over the last 50 years. These methods include: the reservation of land area for conservation, the control and eradication of pests to create predator-free offshore islands and mainland areas that are rendered either predator-free or are maintained under intensive pest-control, the reintroduction of endangered species, and the hands-on management of threatened species including captive breeding programmes (Craig *et al.*, 2000). Reintroductions and translocations have become the preferred methods used in many species recovery programmes (Cunningham, 1996, Mathews *et al*., 2006).

Miskelly and Powlesland (2013) in their extensive review collated information about translocations in New Zealand until 2012. The first translocations were performed in 1863, although these were not really for the purpose of conservation and not successful due to mammalian predators being present at the release sites. Since then, 55 species have been translocated in 1100 separate releases. Of those, 41 species have been established and a further seven are in progress. Since the 2000s, the success rate of translocations has increased, with a success rate up to 66.6%, mainly because of tight introduced predator control and the use of predator proof fencing at translocation sites. Translocations of rails had the lowest success rate,

while South Island saddlebacks (*Philesturnus carunculatus*) had the highest. Tiritiri Matangi Island was the restoration site that received the most bird taxa and was also the most successful translocation site, followed by Karori Sanctuary, Kapiti Island and Mana Island.

3.4.1 Translocation history of the North Island saddleback (*Philesturnus rufusater*)

As an example of wildlife translocations in New Zealand, the well documented translocation history of the North Island saddleback/Tieke (*Philesturnus rufusater*), is reviewed here.

All current populations of the North Island saddleback originate from a single source population on Hen Island, an island in Northland off the shore of Whangarei (Chapter 1/Study sites; Figure 3.2). All other populations have been translocated to offshore and mainland islands in a well-documented translocation history. These translocations were performed in a sequence, with the first wave of translocations from Hen Island to Whatupuke Island (1964), Red Mercury Island (1966) and Cuvier Island (1968). The second wave included translocations from Cuvier Island to Stanley Island (1977), Tiritiri Matangi Island (1984), Little Barrier Island (1984, 1987 and 1988) and Moutuhora Island. In the third wave, birds were translocated from Stanley Island to Kapiti Island (1987, 1988 and 1989) and from Tiritiri Matangi Island to Mokoia Island (1992), Karori (2002) and Motuihe Island (2005). The latest wave of translocations was from Mokoia Island to Bushy Park (2006), Tiritiri Matangi Island to Rangitoto/Motutapu Islands (2011), Lady Alice Island, Red Mercury Island, Mokoia Island to Tawharanui (2012), Hauturu (Little Barrier Island) to Rangitoto/Motutapu Islands (2012), Tiritiri Matangi Island to Maungatautari (2013), Cuvier Island to Cape Kidnappers (2013), Hauturu to Rotokare and Rotoroa Island (2014), Bushy Park to Rotokare (2014), Lady Alice Island to Motorua Island and Urupukapuka Island (2015) and Tiritiri Matangi Island to Motorua Island and Urupukapuka Island (2015) (Figure 3.1 and 3.2 and Table1).

Most of these translocations were successful reintroductions, but they generally used only a small number of founders to create new populations (Taylor *et al.*, 2005). The success of these translocations was attributed to the closed nature of the islands (predator-free and individuals were able to find a mate), low mortality rates following

introduction and high growth rates at low density (Taylor *et al.*, 2005). Other threats to small populations are Allee effects (where animals show decreased fitness in smaller population size and density), increased susceptibility to predation as well as inbreeding and loss of genetic diversity (Taylor *et al.*, 2005, Kriger and Hero, 2009). This can lead to high rates of egg failure in the short term and increase susceptibility to new pathogens and environmental changes in the longer term (Kriger and Hero, 2009). Hale (2007) examined twelve saddleback populations and found that those populations that had gone through a severe genetic bottleneck and had a high population density on a small island presented with higher stress levels and higher feather mite loads, a possible indication of a lower immune function. When Hale et al. compared a population of South Island robins (*Petroica australis australis*) that had gone through a severe genetic bottleneck and had a founder size of five, with a population with a higher number of founders, the former had significantly lower leucocyte and lymphocyte counts and showed a lower response in a haemaglutinin test (Hale and Briskie, 2007).

According to Taylor et al. (2005) for the New Zealand saddleback, the number of the released individuals does not seem to significantly affect the extinction probability of a population on offshore islands. During a translocation of South Island saddlebacks, Taylor et al. (2005) found that although body condition in males and ectoparasite load in females had a negative impact on survival, genetic variation did not appear to come into play (Taylor and Jamieson, 2007).

A difference between the genetic variability of two saddleback species does exist. The South Island saddleback went through a much more severe genetic bottleneck with only 36 birds left in the 1960, while the surviving population of North Island birds is thought to have been around 500 individuals (Jamieson, 2011). Still, the genetic variability of the original surviving population of North Island birds on Hen island is low, most likely due to only a small number of original colonizers which settled on the island or a past population bottleneck (Lambert *et al.*, 2005). However, the North Island saddleback still has moderate genetic variability, as seen in examinations of both MHC class IIB (Sutton *et al.*, 2013) and microsatelite alleles (Ruarus *et al.*, 2011). The major histocompatibility complex (MHC) plays an important role in the vertebrate immune system and it is assumed that populations with a low genetic diversity in MHC alleles may have a lower disease resistance. When different island

populations were examined, North Island saddleback had a range of 3-12 functional MHC IIB alleles, while in South Island birds only 1-4 remain, a finding at the very low end of observed genetic diversity in other passerine birds today (Sutton *et al.*, 2013).

In addition to this, it appears that serial population bottlenecks, as experienced by both saddleback species due to the number of translocations, have a more pronounced impact on the genetic makeup than single translocations (Lambert *et al.*, 2005). Lambert et al. (2005) were able to detect a pattern of significant changes in both the number of minisatellite loci per individual as well as the frequency of alleles in Mendelian loci with increasing population bottlenecks. In general, they found a low genetic variability in all North Island saddleback populations studied.

Since saddlebacks are among the most frequently translocated birds in New Zealand, there is a study which has looked at the impact previous translocations have had on the stress response of the translocated birds and their descendants (Adams *et al.*, 2013). In this study in the Summer of 2005/2006, Adams et al. (2013) measured the corticosterone stress response of saddlebacks in a translocation sequence (Hen Island-Cuvier Island-Tiritiri Matangi Island-Mokoia Island (Figure 1), comparing the response in birds from the original populations with birds from populations along the sequence. The observed responses in source population and the sequential translocation site populations were not significantly different from each other, and they detected no evidence of directional selection operating on the corticosterone responses of the saddlebacks.

Beside genetic bottlenecks, saddlebacks also go through cultural bottlenecks following serial translocations (Parker *et al.*, 2012). New Zealand saddlebacks males have a culturally transmitted rhythmical song, presenting different dialects in different areas of an island. It is unlikely that any translocation will carry the entire song repertoire of one island to the next (as with different alleles in genetic variety). Translocations are therefore followed by gradually decreasing song sharing and variation in acoustic characteristics between the source and daughter populations. In addition, birds accumulate learning errors, leading to cultural mutations and any translocation will likely disrupt the social system of the birds and therefore the way in which males learn their song (Parker *et al.*, 2012). This causes new songs to emerge through withdrawal of possible sources from which the original song of the founder

population could be learned. All these factors accumulate in the significant and rapid evolution in structure and diversity of North Island saddleback song, as discovered during a playback experiment on different islands (Parker *et al.*, 2012). These changes affected both repertoire (loss of song diversity) as well as physical acoustic characteristics of the bird songs, with two distinct lineages evolving in less than 50 years, all a consequence of conservation management. In the playback experiment, birds on Motuihe Island showed significant discriminatory behaviour towards songs from their own island as opposed to foreign songs from Hen Island and Cuvier Island. If this continues, it may lead to speciation due to of the rapid divergence of conspecific recognition signals (the birds on different islands might simply not understand each other anymore). The question begs: how may saddleback song have sounded before the mainland population disappeared in the wake of the arrival of Europeans and left the culturally impoverished Island populations behind?

Figure 3.1: History of NI saddleback translocations from Parker, 2008. NI Saddlebacks survived on a single Island, Hen, from where birds were translocated to the following islands, Whautupe, Red Mercury and Cuvier. Birds from these islands have been subsequently translocated to other sites as shown in this figure. Since then, more translocations have been performed, as for example during this study in 2013, when translocations from Cuvier Island to Cape Kidnappers and from Tiritiri Matangi Island to Maungatautari occured.

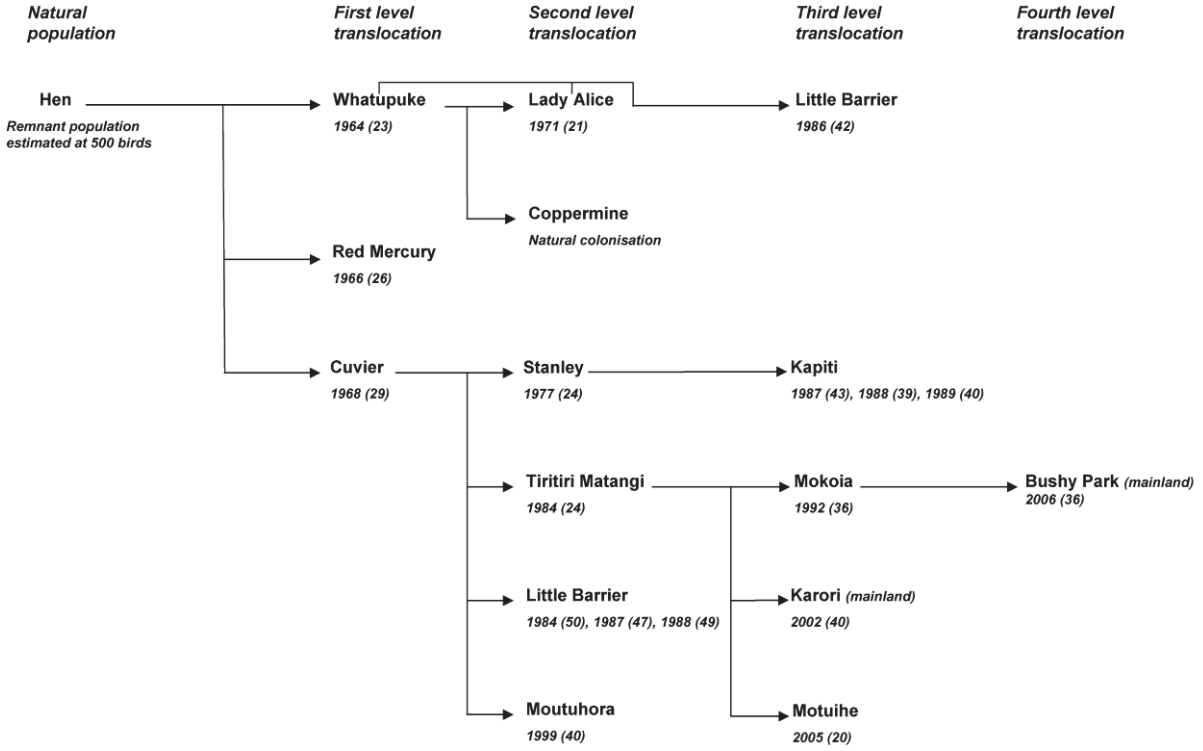


Figure 3.2.: Map of all NI saddleback translocation sites to date. Hen Island (source population) has been marked in red, all others are in yellow).

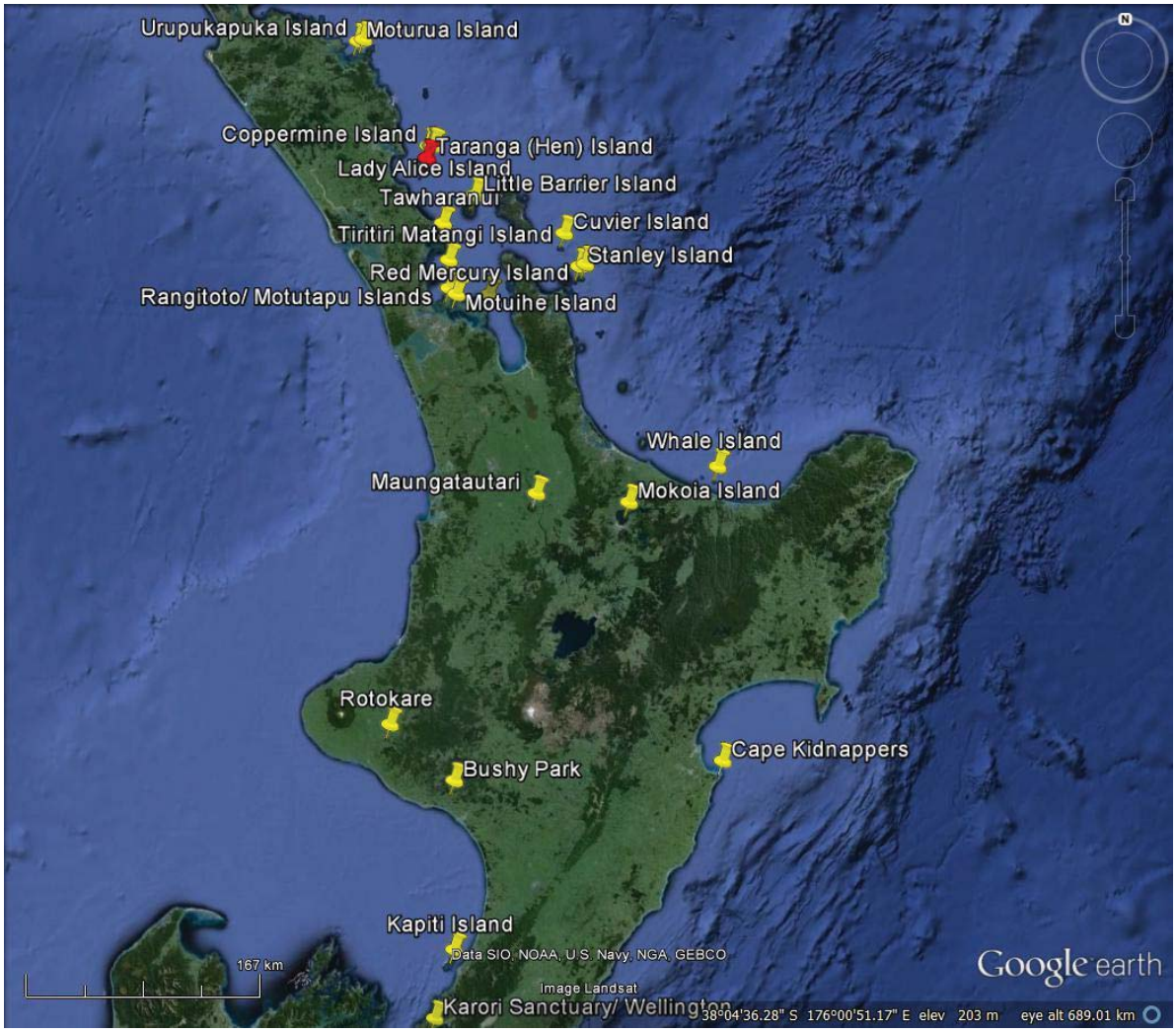


Table 3.1.: Summary of the status of North Island saddleback *Philesturnus carunculatus rufusater* populations (after Hooson and Jamieson, 2003; updated with data from Kevin Parker, pers. comm.)

Island	Size (ha)	Number released (year)	Population Estimates (year)
Hen Island	484	Original population	300 (1925)400 (1939) 560 (1968) 490 (1986) 500 (2000)
Bushy Park	100	40 (2006)	109 (2008)
Cape Sanctuary (Cape Kidnappers)	2500	120 (2013)	
Coppermine	80	Natural colonisation	20 (1979)
Cuvier Island	170	29 (1968)	1000 (1988)
Fanal Island	75	25 (1968) 29 (1985)	3 (1973) 5 (1987)
Kapiti Island	1965	9 (1925) 50 (1981) 25 (1981) 22 (1982) 50 (1982) 22 (1982) 50 (1983) 43 (1987) 39 (1988) 40 (1989)	Extinct by 1931 4 (1986) 38 (1991) 41 (1997) 65 (1998) 97 (2000)
Karori	225	2002	
Lady Alice Island	155	6 (1950)	300 (1986)

		21 (1971)	
Little Barrier Island	3083	9 (1925) 50 (1984) 42 (1986) 47 (1987) 49 (1988)	disappeared quickly 1500 (1995) 2500 (2000)
Maungatautari	3400	40 (2013)	
Mokoia Island	135	36 (1992)	140 (1994) 200 (1999)
Moturua Island	136	20 (2015) 20 (2015)	
Rangitoto/Motutapu Islands	3820	40 (2011) 20 (2012)	
Red Mercury Island	225	29 (1966)	400 (1996)
Rotokare	230	20 (2014) 40 (2014)	
Rotoroa Island	82	40 (2014)	
Stanley Island	100	24 (1977)	250 (1986-89)
Tawharanui		90 (2012)	160 (2014)
Tiritiri Matangi Island	197	24 (1984)	200 (1991) 500 (1997) 600 (2002)
Urupukapuka Island	208	20 (2015) 20 (2015)	
Whale Island	143	40 (1999)	22+ (2000) 60 (2001)
Whatupuke Island	102	23 (1964)	90-120 (1967) 500 (1996)

3.5 Wildlife health and translocations

Although wildlife translocations aim at the conservation of species, they may cause health problems either to the translocated individuals or to individuals/populations in the target area. The problem of acute and chronic stress and genetic makeup has already been mentioned above.

Infectious diseases can have a great impact on local populations and can cause a temporary or permanent decrease in abundance (Smith *et al.*, 2009). In the past 500 years, 833 animal species are known to have gone extinct, and in at least 3.7% of cases it is thought that infectious disease played at least a partial role in the disappearance (Smith *et al.*, 2006). Disease does not necessarily always cause death, but may also cause increased susceptibility to predation and/or further disease, a lowered reproduction rate, or a combination of these (Cunningham, 1996). Many endangered species survive in small and isolated populations. It has been a major conservation goal for decades to create linkages between such populations (either by spacial connections, so-called “corridors“, or conservation translocations) to create metapopulations. This is thought to provide necessary genetic flow which may help to prevent species extinction, for example in the case of a severe disease outbreak (Ballou, 1993).

The disease risk following translocations is twofold: translocated animals may either introduce pathogens into new areas, or acquire pathogens that are present in the target area. The greatest risk of disease transmission is posed by captive bred and raised animals, as captivity often results in unnatural and continuous stress and therefore might cause immune suppression and increased susceptibility to infection (Kock *et al*., 2010). Crowded captive situations may also enhance disease transmission in susceptible animals, as seen for example with coccidia in captive bred hihi (Schoener *et al.*, 2013). All of these reasons may contribute to the low success rate of translocations using captive-bred individuals (Mathews *et al.*, 2006). In the wild, normal selection will reduce the likelihood of pathogen persistence and therefore healthy individuals are rarely at risk. Despite this, there will never be a zero risk in translocating wild animals as they may instead become symptomless carriers of disease agents which may be translocated undetected (Dickens *et al*., 2010; Kock

et al., 2010). This means pathogens may be accidently introduced into wild populations by captive bred animals meant to restock depleted wild populations. Another possible problem encountered by captive breeding might be the adaptation of animals to captivity and handling by humans which might make it impossible for these individuals to survive in the wild. To mitigate this problem, hand puppets and handler disguises have been used for feeding and interacting with the chicks and fledglings of for example Californian Condors (*Gymnogyps californianus*) (Walters *et al.*, 2010, Utt *et al.*, 2008), Eurasian cranes (*Grus grusto*) (Cromie, 2015) and Takahe (Clout and Craig, 1995).

3.5.1 Pathogen pollution

"Pathogen pollution" was defined by Cunningham et al. (2003) as the anthropogenic movement of parasites (macro and microparasites including bacteria and viruses) outside their natural geographic or host species range, and their introduction to a new (or naïve) host species or environment. This definition already carries the implication that a parasite does not necessarily cause disease, although any parasite can develop into a pathogen given certain circumstances as described below. One of the most captivating historic examples of pathogen pollution has affected humans themselves, with the arrival of the Europeans in the Americas 500 years ago. Before this event, the Americas had been isolated from the rest of the world for around 10,000 years, and infectious diseases introduced by the new arrivals caused population declines of the native populations of up to 95% (Clark 2010). Other well-known examples of pathogen pollution include (1) the introduction of rinderpest into Africa with domestic cattle (*Bos taurus*), which infected the native ungulates and changed a whole ecosystem; (2) the introduction of chestnut blight into North America with the Japanese chestnut (*Castanea crenata*) which devastated the North American forests; and (3) the introduction of squirrel pox virus with grey squirrels (*Sciurus carolinensis*) into Europe, which plays an ongoing role in the decline of the native European red squirrel (*Sciurus vulgaris*) (reviewed in Cunningham *et al*., 2003). Similarly, after the arrival of Europeans in Hawaii many of the native birds became extinct, with avian pox and avian malaria hypothesised as playing a major role (Daszak and Cunningham, 1999).

Today, pathogen pollution is seen as one of the biggest threats to biodiversity, besides other man-made environmental changes, such as introductions of alien

predators/competitors, habitat destruction, overexploitation, biological and chemical waste pollution and climate change (Cunningham *et al*., 2003; Smith *et al*., 2006; Smith *et al.*, 2009). The most striking example and currently largest infectious disease threat to biodiversity (Kilpatrick *et al*., 2010) is chytridiomycosis which is causing the worldwide decline of amphibians (Appendix 2, Section 2.2). This disease is due to a pandemic emergence of the parasitic chytrid fungus *Batrachochytrium dendrobatidis* (Kriger and Hero, 2009, Kilpatrick *et al.*, 2010, Rodder *et al.*, 2010).

Another alarming example of pathogen pollution is the threat that *Toxoplasma gondii* is posing to marine mammals. This parasite was first noted in deaths in sea otters on the Californian coast and has been extensively studied by a team of researchers from the University of California/Davis (Miller *et al.*, 2002a, Miller *et al.*, 2002b, Conrad *et al.*, 2005, Dabritz *et al.*, 2007, Jessup *et al.*, 2007, Dabritz *et al.*, 2008 Shapiro *et al.*, 2010, Shapiro *et al.*, 2012). *Toxoplasma gondii* is a pathogen of wild and domestic felids, and its amount of infectious oocysts in the environment has become increasingly prevalent in the past decades due to an ever growing number of pet and feral cats (*Felis catus*). *Toxoplasma* oocysts are washed out to sea by run-off and waste water, where they are accumulated by shellfish and then ingested by marine mammals predating on these shellfish. In addition, *Toxoplasma* is also known to cause zoonoses and poses a threat to human health. This problem will only increase with the increasing number of domestic (and feral) cats in the US and around the world.

New Zealand has its own examples of pathogen pollution. In 1995, a herpes virus caused massive pilchard (sardines, family Clupeidae) mortalities in the North of the North Island (Hine, 1995). This was followed by an outbreak of a different herpes virus (OsHV-1) in Pacific oysters (*Crassostrea gigas*) in 2010 (Webb *et al.*, 2007, Martenot *et al.*, 2011, Hwang *et al.*, 2013, Castinel *et al.*, 2015). Both herpes virus outbreaks caused economic impacts for New Zealand fisheries. The amphibian disease chytridiomycosis is also present in New Zealand (Appendix 2, section 3), and may have been involved in the population decline of Archey's frogs (*Leiopelma archeyi*) in the late 1990s (Bell, 2010).

Pathogen pollution is also a potential risk for species re-introduction programmes aimed at species recovery, especially since translocations of endangered species

are becoming more and more important worldwide as a wildlife management tool. These reintroductions and translocations can pose a threat to pre-existing species through the introduction of pathogens and to the translocated individuals through exposure to pathogens already present in the release area and its inhabitants (Cunningham, 1996; IUCN guidelines 2013).

For a more in-depth review on pathogen pollution, see Appendix 2.

3.5.2 General rules for pathogen pollution in animal movements

According to Tompkins *et al.*, (2015), the main drivers for disease emergence in wildlife today are the exposure to domestic sources of infection and the human-mediated movement of both wild and domestic hosts into non-native regions. This makes pathogen pollution one of the most pressing conservation issues. Pathogen pollution with animal movements has been common in historic times. In these cases, the introduction of parasites was involuntary and the possible outbreak of disease was not considered when the animals where introduced. Historic examples of a severe ecological and economic impact include the introduction of rinderpest to Africa with domestic cattle, avian malaria to Hawaii in the 1920s, squirrel pox to Europe with American grey squirrels in the early 1900s and fungal disease with American crayfish to Europe in the 1850s. Back then, it was also not possible to test for these diseases, even if the infectious agent had been known at all. Another example is chytridiomycosis in amphibians, which may have been introduced with commercial trade worldwide since the 1950s, but has only been recognised since 1993 (Weldon *et al.*, 2004a). In all these cases, lack or complete absence of knowledge about the involved diseases, and disease spread in general, may have been instrumental in the severity of the impact they had on a variety of animal species.

Presently, the introduction of animals to new geographic regions is increasing with increased human travel and an ever expanding global economy, which may further amplify the risk of the introduction of pathogens to new environments and/or species (Daszak *et al.*, 2000).

When an animal is moved from one place to another, there are several possibilities for what might happen to its parasites. The parasite may be introduced with the host, but has no further impact on other, resident, species; the parasite spreads to other

species; or the parasite is lost during or shortly after translocation. The likelihood for a parasite to be introduced with its host is influenced by its transmission efficiency, namely the body size of the host (a large body is likely to host more parasites), host founder size population, type of parasite, and the sociality of the host (MacLeod *et al.*, 2010). Host species that colonize new regions often lose parasite species (MacLeod *et al*., 2010). MacLeod et al. (2010) tested the relative importance of different processes and mechanisms in causing parasite species loss, using population arrival and establishment data for New Zealand's introduced bird species and their ectoparasitic chewing lice species. Few lice failed to arrive in New Zealand with their hosts due to being missed by chance in the sample of hosts from the original population (missing the boat). Rather, most lice were absent because their hosts or the parasite themselves failed to establish populations in their new environment (sinking with the boat) (MacLeod *et al*., 2010).

Another example of this loss of parasites in introduced/invasive hosts is the case of the house sparrow (*Passer domesticus*) and it's infections with haemosporidian parasites. For several years, it has been known that parasites like *Haemoproteus passeris* are common in the native range of house sparrows, but have become absent in new regions of their range like northwestern Russia (Valkiūnas *et al.*, 2006b) and South America (Lima *et al.*, 2010). In an extensive study examining 1820 house sparrows in 58 locations on six continents (Marzal *et al.*, 2011b) found that these birds lost their native parasites especially in the Americas, Australia and New Zealand, where these parasites were only partially replaced by local generalist parasite fauna.

If a parasite is introduced with a domesticated animal, it may spread from its original "reservoir" host to native wildlife. This has been termed "spill-over" (Daszak *et al*., 2000). This may endanger native wildlife and even cause local extinction, as with the case of distemper, a disease of domestic dogs, which has "spilled-over" to African wild dogs (*Lycaon pictus*). A pathogen may also be transmitted to native animals, which then act as disease reservoirs for the original hosts, termed "spill-back" (Daszak *et al*., 2000). Such a "spill-back" event occurred in New Zealand, where bovine tuberculosis was transmitted to brushtail possums which from then on acted as potent reservoir for this disease.

3.5.3 Do these rules apply to animal translocations?

There is always the chance that translocated individuals are symptomless carriers of parasites. In cases such as avian malaria, the parasites cannot be diagnosed all year round in the blood of examined individuals, and therefore, there can never be an absolute guarantee that every pathogen will be detected upon translocation (Dickens *et al*., 2010, Kock *et al*., 2010). This may be a problem, because disease mediated extinctions become more likely when dispersal rates among small populations are artificially high, as in the case of conservation translocations, after which a pathogen would normally fade out in larger populations (Smith *et al.*, 2009).

In many cases of translocations, disease introduced with translocated individuals has been a problem, the most prominent examples are reviewed in Viggers et al. (1993), but I examine a few examples below.

The accidental introduction of blackhead disease in domestic turkeys (*Meleagris gallopavo*) to a remnant island population of heath hen (*Tympanchus cupido cupido*) resulted in the annihilation of this rare species (Viggers *et al*., 1993). Disease has also been introduced with individuals intended to reinforce a population; for example the release to the wild of captive reared orangutans (*Pongo borneo*) that were infected with tuberculosis. A similar fate was spared from wild golden lion tamarins (*Leontopithecus rosalia*) after a viral disease was found in time in captive reared animals intended for translocation (Viggers *et al*., 1993).

Disease may also arise in captive breeding programmes. This has been seen in the rearing of chicks of the endangered whooping crane (*Grus americana*), which used a different crane species as foster parents and subsequently transmitted a lineage of coccidia to the chicks. A similar problem arose with the use of rock pigeons (*Columba livia*) as foster parents for pink pigeons (*Columba mayeri*) which were infected with a herpes virus (Viggers *et al*., 1993). Another popular example of this is the introduction of chytrid fungus into wild populations of Mallorcan midwife toads (*A. muletensis*) by animals from a captive breeding facility (Walker *et al.*, 2008). Pathogens introduced with captive reared animals may also affect other native species, as with the introduction of whirling disease to rainbow trout (*Oncorhynchus mykiss*) after a release of captive reared brown trout (*Salmo trutta*) in Europe (Viggers *et al*., 1993). Humans can also threaten endangered closely related species

directly, for example tourists transmitting measles to wild mountain gorillas or poliovirus to chimpanzees in the Gombe National Park in Tanzania (Daszak *et al*., 2000). It has therefore become important to test animals for a certain disease before transfer, as in the case of Arabian Oryx (*Oryx leucoryx*) which has to be tested for bluetongue disease before a translocation can take place.

3.5.4 Recommendations to minimise pathogen pollution risk during translocations

While in the past animal translocations have often occurred and failed because of lack of required knowledge and experience of certain pathogens and their spread and biology, we can now access a wealth of knowledge about past translocation projects, learn from their mistakes and improve upon their experiences.

Although a vigilant diagnostic program for potential pathogens to minimize the transmission of pathogens to a naïve environment should be attempted, no screening program can be completely exhaustive, due to funding restraints and the limitations of diagnostic methods (Cunningham, 1996; Mathews, 2006). The benefits of the translocation project should also always be carefully weighed against possible adverse effects of accidental disease introduction before deciding whether or not such an intervention can be justified (Cunningham, 1996). It is also still in question as to whether the translocation of parasite-free individuals may pose a threat to biodiversity and endanger the survival of the species in the long term (Smith *et al*., 2009), a question which I will examine in more detail below.

To minimise the disease risks involved in translocation, the health screening of the animals for known diseases prior to translocation is recognised as good practice (IUCN guidelines 2013). Further, the following steps are suggested in the literature (Corn and Nettles, 2001, Kock *et al.*, 2010):

- Evaluation of health in the source population

- Quarantine (maintaining strict hygiene in keeping and handling)

- Physical examination and diagnostic testing, using not only microscopy, but also sensitive standard methods like PCR and serology (Lindenmayer, 2000, Parker *et al.*, 2006).

- Restrictions on translocations of animals from certain areas or populations (e.g. known carriers of a pathogen)

- Prophylactic treatment and/or vaccination against known diseases (on the topic of vaccination in conservation medicine, also see (Plumb *et al.*, 2007).

In addition, performing a pre-release risk assessment (e.g. the presence of local vectors and disease agents, history of disease prevalence and former incidence of diseases) of the target area, reducing stress by minimising handling and improving of captive facilities and performing post-release health monitoring have also been suggested (Mathews *et al.,* 2006; Kock *et al*., 2010).

A couple of decades ago, Cunningham (1996) had already suggested that animals for translocation should be kept in captivity for as short a time as possible and recent publications support this due to the negative impacts and potentially causing chronic stress to translocated individuals (Letty *et al*., 2007, Teixeira *et al*., 2007, Dickens *et al.*, 2009, Dickens *et al*., 2010). Negative impacts may or may not be seen shortly after translocation, so a long-term monitoring program needs to be in place to evaluate the success of a project (Richardson, *et al*. 2014), an approach that is also recommended by (Fischer and Lindenmayer, 2000).

Recent examples of project taking these points into account are the Great Crane project (reintroduction of Eurasian cranes to South West England) (Cromie, 2015) as well as translocations of Fisher's estuarine moth, the short-haired bumblebee, pool frog, cirl bunting (Vaughan-Higgins, 2015) which applied strict quarantine and biosecurity measures. In the great crane project (Cromie, 2015) a range of measures were used to mitigate risks from infectious disease, including:

- the design and building of the rearing facility;

- strict protocols for personnel and vehicle access to the facility;

- disinfection protocols within the facility;

- annual decontamination of the facility; and

- preventative medicine and reactive veterinary treatment.

In many cases, the baseline physiological data of endangered species is not known, so there is no possibility to compare actual health data of the animals in pre-release examinations (Mathews, 2006). There is still a lack of knowledge about many pathogens, their biology and threat level, and therefore gaining more information in this field should be a priority (Smith *et al*., 2009). Although there has been a huge influx of new information about wildlife health and diseases in the field of conservation translocations, there still is a massive need for more research to be done in this area.

3.5.5 Loss of parasites

3.5.5.1 Role of parasites in ecosystems

The introduction or acquisition of parasites during the translocation process is not the only problem that has to be taken into consideration. We are only beginning to take the loss of parasites into consideration, although this may be a problem of equal importance.

Although there is a short term advantage in keeping animals for translocation free of parasites, this is not desirable in the long term. The parasite burden within natural populations is important for the maintenance of genetic and other adaptations, and parasites that inhabit the host "ecosystem" are an important part of the biodiversity in the wild (Cunningham 1996).

For a long time, parasites have been ignored when looking at ecosystems, biodiversity and food webs, or were only seen as having a negative impact. Only recently, the vital role of parasites has been acknowledged (reviewed in Tompkins *et al.*, 2011). Indeed, parasites have the potential to alter food web topology (chain length, connectivity and robustness) and therefore may affect food web stability. In addition, many parasites are highly specialised, and therefore are very sensitive to the loss of their host species (Lafferty *et al*., 2008). Parasites, which have many complex life cycles, depend on a complex and functioning ecosystem-and decline with biodiversity loss. Not much is known in general on the diversity of parasites and studies in this subject have so far been neglected. Still, it can be assumed that parasites make up the unseen majority of species extinctions (Stork and Lyal 1993, Dunn *et al.*, 2009, Lafferty, 2012) and biodiversity loss may reduce parasite diversity more than previously thought (Lafferty, 2012). Hudson *et al*. (2006) even

hypothesised that a healthy ecosystem is one that is rich in parasites, with their effects on host population dynamics, interspecific competition, structure of food webs and biodiversity invaluable for the functioning of an ecosystem.

3.5.5.2 Translocation and conservation of native parasite biodiversity: Which parasites should we worry about?

Keeping the importance of parasites in ecosystems in mind, the loss of host-specific parasites from endangered species in captive breeding programmes as well as the strict indiscriminate parasite treatment of translocated animals poses a substantial threat to the conservation of biodiversity (Daszak *et al*., 2000). Furthermore, it has been found that threatened primates had fewer pathogens than their non-threatened counterparts (Altizer *et al.*, 2007). There have also been reports of host specific feather lice being lost, such as *Colpocephalum californici* of the Californian Condor during the captive breeding attempt to save the condor and *Rallicola (Aptericola) pilgrimi,* when the host, the little spotted kiwi *Apteryx owenii,* became extinct on the mainland (Buckley *et al.*, 2012, Rozsa and Vas, 2015). However, the actual list of such occurrences is most likely longer due to the lack of baseline data. Accordingly, an attempt to present a loss of co-extinct and endangered lice has been made by Rozsa and Vas (2015). The loss of parasites can also be seen in introductions of invasive species. On average, introduced populations had only half the parasites they had in their native range. Torchin et al. (2003) reported that on average, invasive species have 16 species of parasites in their native range. Of these, only three successfully accompanied animals into the introduced range and only four were newly acquired. This is due to the host population bottleneck which introduced species have to go through, as only a small subset of animals will be transferred. In addition, many parasites also possess complicated life cycles (Torchin *et al.*, 2003).

A loss of parasites may be positive for the individuals in the short term, but it may lead to a loss of genetic variation in immunity within the species and therefore make the species more susceptible to disease outbreaks in the long term (Altizer *et al.*, 2003, Smith *et al*., 2009, Sainsbury, 2015). In addition, in a review of different metapopulation models, Park (2012) suggested that even if disease is transmitted via animal translocation, the benefit of recolonizing habitats (which buffers against regional extinction) outweighs the costs associated with disease transmission. A study on avian translocation and disease by Parker *et al*. (2006) even suggested that

infectious disease was not a significant issue in bird translocations in New Zealand, although this study suffers from a low sample size and standard sensitive diagnostic methods like PCR were not applied. The extirpation of all parasites in animals destine for translocation should not be the main focus of health surveillance, since the effects of parasite losses on ecosystem function are still unclear (Sainsbury, 2015). Successful conservation programmes should maintain populations with intact evolutionary processes including their parasites (Altizer *et al.*, 2003). There are already management methods available to conserve parasites and at the same time safeguard the health of the hosts (Sainsbury, 2015). Examples can be found in the translocations of cirl buntings (*Emberiza cirlus*) and corncrakes (*Crex crex*) where birds destined for translocation were examined for coccidia; then parasites found were compared to those of free living populations. The treatment regime was adapted to prevent the eradication of the parasites (Sainsbury, 2015). Similar management decisions were made for hihi (Ewen *et al.*, 2012a) where coccidia were also regarded as native parasites. At the same time, infections with avian malaria parasites (*Plasmodium* spp.) previously caused confusions in a hihi translocation after birds were infected with a ubiquitous *Plasmodium* lineage (Ewen *et al.*, 2012a), as sufficient knowledge about the disease impact was unavailable and no plan was in place of what to do if birds tested positive. The consensus in managing translocated birds with *Plasmodium* infections in New Zealand now seems to be to translocate birds with low parasitaemia (chronically infected) or negative in PCR test, since *Plasmodium* lineages reported in New Zealand are currently either thought to be native or are cosmopolitan and ubiquitous around the country (Kate McInnes, 2015, pers. comm.). However, every conservation translocation has its own risk profile and should therefore be treated according to its own disease risk assessment.

To find the right balance between conserving host health during translocation and long term health by conserving parasites, the question has to be asked, which parasites will most likely cause problems and should be controlled/treated when found in animals for translocation? This question was reviewed by Rideout (2015) who listed the following characteristics of a troublesome parasite:

- microparasite (e.g virus or bacterium)

- alien to the host

- generalist with broad host range

- long incubation period

- long infectious period

- having a reservoir host

- vector-borne

- without effective treatment and or vaccination

Rideout (2015) suggested to control or eradicate parasites with several of these characteristics during a translocation programme.

Another possibility to conserve parasites would be to introduce native parasites to a population after it has been established and/or the initial stress of the translocation has passed (Isabel Castro, pers. comm.); although this option may not work for all parasites (i.e, parasites which cannot survive outside the host, cannot be cultured, have an unknown biology and life cycle, and which are very rare in the source host population).

3.5.6 Outlook into the future

The introduction of novel parasites can have potentially devastating effects on wildlife. Since it is not likely that the increasing trend of global travel and trade will change in the future, the problem of pathogen pollution is likely to become more and more apparent. As other anthropogenic environmental changes occur, such as habitat destruction, overexploitation, pollution and climate change, the spread of infectious agents by human activities will continue to contribute to the current global loss in biodiversity (Cunningham, 2003; Smith *et al.*, 2006; Smith *et al*., 2009). Predictions of the impact of climate change on extinction rates vary wildly, ranging between 0-54% (Urban, 2015). However, the results of a recent study (Urban, 2015) suggest that the extinction risk is accelerated by a future increase in temperature, with the risk highest for species in South America, Australia and New Zealand. The biggest danger is faced by endemic species, as well as by species in small ranges and by reptiles and amphibians. Climate change might cause some species to experience stress, as they will have to deal with conditions they are maladapted for. One example is the Tuatara (*Sphenodon punctatus*), which has adapted for cold

temperatures. Warmer temperatures may feed back to disease susceptibility (see above, Section 4.3.4 Stress). With climate change, it is estimated that vector-borne diseases like avian malaria will expand worldwide, either because the arthropod vectors show range expansion or because the pathogens themselves benefit from warmer temperatures for their development (Garamszegi, 2011, Loiseau *et al.*, 2012). For example, in 2011, *Plasmodium* parasite transmission was observed in the North American Arctic for the first time (Loiseau *et al.*, 2012). Other pathogens may also benefit from a warming planet; as many infectious agents are sensitive to temperature, rainfall and humidity (Harvell *et al.*, 2002) and therefore there may be an increase in pathogen development and survival rates, transmission and increased host susceptibility (Harvell *et al*., 2002). In addition, in the past few decades, there has been an emergence of new, previously unknown pathogens as well as new lineages of known infectious agents (Daszak *et al*., 2000). A range of factors is contributing to the emergence of diseases, most of them directly linked to human activities (Daszak *et al.*, 2000). All this puts enormous pressure on species and populations naïve to the newly appearing pathogens, and will most likely worsen the loss of biodiversity worldwide in the future.

In the future, it will be essential to carry out surveillance programs to detect pathogen or disease emergence, with local-scale impacts most likely more important than global impacts (Altizer *et al.*, 2013). This will assist in anticipating trends and therefore in identifying possibilities to mitigate impacts on wildlife and human health (Altizer *et al.*, 2013). It will also be important to risk match both the source and destination areas for parasites which are native (in order to conserve diversity) as well as being a possible problem (see above). This will help to avoid both introduction of new pathogens as well as to avoid the introduction of naïve hosts (Rideout, 2015). In particular, in places like New Zealand parasite surveys of target translocation areas should become essential, especially as New Zealand birds show a low genetic diversity and may be especially susceptible to novel parasites (Rideout, 2015, pers. comm.)

Chapter 4: A new real time PCR for the rapid diagnosis of the four most important lineages of avian malaria in New Zealand

4.1 Abstract

Wildlife rehabilitation and conservation translocations are important tools in wildlife conservation in New Zealand, but knowledge about disease screening of birds prior to translocations is still in its infancy. Molecular methods have become the most widespread tool for diagnosing avian malaria (*Plasmodium* spp.) infections. However, they can be time intensive, expensive and less specific in diagnosing mixed infections with more than one *Plasmodium* species or lineage. Thus, the aim of this study was to develop a new method for diagnosing and specifically identifying different avian malaria species/lineages, using the four most common and ubiquitous lineages of avian malaria in New Zealand for development. A two-step real-time PCR targeting the cytochrome b gene was able to detect four *Plasmodium* lineages; *P. elongatum* GRW6 and LINN1, *P. relictum* GRW4 and *P. vaughani* SYAT05 as well as combinations of these in the same sample. The detection limit of the assay was determined to be equivalent to 5 parasites in a comparison using quantified serial dilutions of cloned control DNA. An additional qPCR with a high resolution melt (HRM) was developed to discern *P. elongatum* GRW6 from *P. relictum* GRW4. The qPCR assays were then tested using tissue samples containing *Plasmodium* meronts from three naturally infected dead birds. This test determined that all three birds died due to infection with *P. elongatum* GRW6. Thus, these qPCR assays have demonstrated that they would be cost effective rapid screening tools for the detection of *Plasmodium* infection in native birds suffering from acute infection with clinical symptoms as well as in birds that have been found dead in New Zealand.

Keywords

Avian Malaria, disease screening, high resolution melt (HRM), New Zealand, *Plasmodium*, Real-time PCR

4.2 Introduction

Conservation translocations to preserve endangered species are becoming more significant worldwide as more restoration areas become available and captive breeding programmes for wildlife reintroductions become more successful. In New Zealand, reintroductions and translocations have become the preferred methods in species recovery programmes (Cunningham, 1996, Mathews *et al.*, 2006) and advances in pest control have enabled the creation of large mainland islands that are either predator free or intensively controlled (Craig *et al.*, 2000). In addition, wildlife rehabilitation centres like Wildbase at Massey University, Palmerston North treat injured and sick endangered native birds for re-release back to the wild.

Disease screening for both wildlife translocations and rehabilitation in birds has not been standardized in New Zealand, and often there are no protocols in place on how to deal with infections if they are found. For example, birds infected with ubiquitous avian malaria parasites (*Plasmodium* spp.) have previously disrupted translocations of saddleback (*Philesturnus carunculatus rufusater*) (Thorne, 2007) and hihi (*Nothiomystis cincta*) (Ewen *et al.*, 2012a) when no protocol was in place to manage or assess the impact of such a finding. Currently, the consensus in managing translocated birds with *Plasmodium* infections in New Zealand is to translocate birds with low parasitaemia or negative in a PCR test, since *Plasmodium* lineages reported in New Zealand are currently either thought to be native or are cosmopolitan and ubiquitous around the country (Kate McInnes, 2015, pers. comm.). However, each translocation needs to be viewed independently because the risks of disease transmission depend on different factors each time.

To date, avian malaria parasites belonging to 17 lineages have been found in 37 different bird species in New Zealand (Tompkins *et al*., 2008, McKenna, 2010, Tompkins *et al*., 2010, Castro *et al*., 2011, Howe et al., 2012, Baron et al,. 2014; Schoener *et al*., 2014, Hunter, 2015). The most common lineages infecting endemic/native New Zealand bird species, and also introduced passerines, are *Plasmodium* (*Huffia*) *elongatum* lineages GRW06 and LINN1 and *P.* (*Novyella*) *vaughani* lineage SYAT05; with *P. elongatum* (GRW6) having the widest host range. Other lineages of *Plasmodium* detected in endemic species are *Plasmodium* (*Haemamoeba*) *relictum* lineages GRW4 and SGS1 (Castro *et al*., 2011, Ewen *et al*.,

2012b, Howe *et al*., 2012). Although all of these common *Plasmodium* lineages found in New Zealand appear to be subclinical or chronic infections, they can be pathogenic and able to cause mortalities particularly when birds are under certain conditions which increase stress, such as captive management or translocation events (Alley *et al.*, 2008, Tompkins *et al*., 2008, Alley *et al.*, 2010, Banda *et al.*, 2013, Dinhopl *et al.*, 2015).

Currently, health screening of birds for *Plasmodium* spp. infection relies on either the examination of blood smears or a standard nested PCR protocol (Hellgren *et al.*, 2004). While the examination of blood smears can be very accurate and can identify parasite species and even mixed infections, it requires an experienced operator and is time expensive. It should also be noted that the identification of parasite morphology is often difficult as *Plasmodium* spp. are known for their cryptic diversity (Palinauskas *et al.*, 2015). Sensitive conventional PCR assays with post-amplification sequencing can resolve these problems, but current PCR assays can underestimate mixed infections, which are common in the wild. (Valkiūnas *et al.*, 2006a; Chapter 5). Both molecular methods and microscopy can potentially miss low level parasitaemia (Jarvi *et al.*, 2002, Fallon *et al.*, 2003b) because of varying assay sensitivity and examiner experience. Thus, the combined use of both microscopy and PCR is recommended (Valkiūnas *et al.*, 2006a).

Recent advances in PCR technology and availability of real time PCR (qPCR) and HRM (High Resolution Melt) machines have now provided a potential third option for the rapid diagnosis of *Plasmodium* spp. infections. These assays are in general faster, cheaper and more sensitive than conventional PCR (Madigan, 2015), because they require less material (smaller reaction volumes) and forgo a sequencing step. In addition, qPCR assays can be developed to detect multiple parasite species in one sample, preventing the need for post amplification sequencing and potential amplification bias.

To date, two genes have been examined for creating a qPCR assay for the detection of avian *Plasmodium* spp. infections. Previously published protocols by Friedl and Groscurth (2012) using the plastid-like large subunit ribosomal-RNA (LSU-rRNA) gene and Bell et al. (2015) using a conserved region of the mitochondrial ribosomal DNA (Friedl and Groscurth, 2012, Bell *et al*., 2015), have only been able to diagnose

the presence or absence of *Plasmodium* spp. in the blood and tissue of New Zealand birds. However, none of these methods were able to specifically identify different lineages or mixed infections. For over 10 years, the cytochrome b gene has been the reference gene used for avian malaria research with a wealth of information and sequencing data of a continuously growing number of new lineages and species available online. In published qPCR protocols using the cytochrome b gene (Bentz *et al.*, 2006, Njabo *et al.*, 2011), the emphasis has been on diagnosing infection without differentiating between different parasite species. Still, using a different protocol Knowles et al. (2011), were able to discern between two different species, *Plasmodium relictum* (lineages pSGS1 and pGRW11) and *P. circumflexum* (lineages pTURDUS1 and pBT7).

Thus, the aim of this study was to improve avian malaria screening by developing a qPCR technique that is able to detect and specifically identify infections and co-infections of the three most common lineages of avian malaria in New Zealand (*P. vaughani* SYAT05 and *P. elongatum* GRW6 and LINN1) as well as the potentially highly pathogenic *P. relictum* GRW4 lineage.

4.3 Methods

4.3.1 Establishing positive controls

To develop positive controls for the three most common avian *Plasmodium* lineages in New Zealand, blood samples from birds previously tested positive were used. These birds were one blackbird (*Turdus merula*), positive for *P. elongatum* LINN1 (homology 100%, GenBank # DQ847270), and two North Island saddleback (*Philesturnus rufusater*), one positive for *P. elongatum* GRW6 (homology 100%, GenBank # DQ368381) and one positive for *P. vaughani* SYAT05 (homology 100%, GenBank # DQ847271). All birds were caught at Bushy Park/Wanganui in February 2013 during a study examining avian malaria (Chapter 5). One sample of blood from a bird experimentally infected with *P. relictum* GRW4 (GenBank # AF254975) was provided by Carter Atkinson (USGS/Hawaii). DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions for nucleated blood. In order to confirm the parasite species, we used a nested PCR protocol for the amplification of the cytochrome b gene as previously described (Hellgren *et al.*, 2004, Castro *et al*., 2011), followed by automatic dye-

terminator cycle sequencing with BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc, California, USA) to confirm genomic sequence. Sequences were examined with Geneious^{TM} (Biomatters, Auckland, New Zealand) and compared in a BLAST search to previously published sequences available from the MalAvi database (Bensch *et al.*, 2009).

4.3.2 Amplification and cloning of cytochrome b

Due to the availability of sequence data for the cytochrome b gene, this gene was the most suitable target gene for the development of the qPCR assay. The whole cytochrome b gene (1101bp) was amplified by PCR from the positive control samples of *P. elongatum* LINN1, *P. relictum* GRW4 and *P. vaughani* SYAT05 using the primers CytbFnew and CytbR (Table 4.1). The PCR reaction mix of 50 µl volume contained 5 µl of template DNA (~ 50ng/µl)), 5 µl 10X PCR Buffer, 1.5 mM $MgCl_2$, 0.2mM of each dNTP , 0.2 µM of each primer, and 1 unit of Platinum® Taq polymerase (Invitrogen, California, USA). The cycling parameters were an initial denaturing at 94°C for 4 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes and a final extension step at 72°C for 10 minutes. The control for *P. elongatum* GRW6 was amplified with the first step of the nested PCR developed by (Hellgren *et al.*, 2004) using the primers HaemF and HaemR2 (Table 1) to amplify a 480bp sequence of the cytochrome b gene.

PCR amplicons were run on a 1% (w/v) ultra-pure agarose gel (Invitrogen) containing ethidium bromide and visualized under UV light on a transilluminator. All positive PCR amplicons were purified (PureLink PCR purification kit, Invitrogen) and subjected to sequencing as described above to confirm genomic sequence. Sequences were examined with Geneious^{TM} (Biomatters) and compared in a BLAST search to confirm correct target amplification.

Cloning of the PCR amplicons was performed using the pGEM T-easy vector kit (Promega, Madison, USA) according to instructions. For DNA extraction of bacterial clones, the PureLink® Quick Plasmid Miniprep Kit (Invitrogen) was used. DNA was eluted in 75µl and stored at -18°C until needed. Sequencing was again performed to confirm that the right insert had been cloned as described above. These clones were used as positive controls subsequently.

4.3.3 Assessment of primers for qPCR

Various primer pairs within the cytochrome gene, either previously published or generated for this study (Table 4.1 and Figure 4.1), were tested for their suitability to discriminate between the four target *Plasmodium* lineages. The primer pairs were chosen for testing if their expected amplicons product had between 50-250 base pairs and they showed a discriminatory DNA melting profile as assessed with Oligocalc (http://www.basic.northwestern.edu/biotools/oligocalc.html). All qPCR and HRM was performed on the Rotor-Gene 6000 platform (Corbett Life Science, Sydney, Australia). For the reactions, MeltDoctor™ HRM Master Mix (Applied Biosystems, Foster City, USA) was used according to instructions, with primer concentrations of 0.3 µM. Samples were run with 5µl of template (20 - 50ng/µl) per 20µl reaction volume.

In order to assess the usefulness of each primer pair combination, 1µl DNA (1ng/µl) from each of the cloned controls was added to the reactions. The thermocycling parameters for the primer pairs HRMF/HaemR2, CBQ/Plas56F and HaemF/PlasrevRT was 10 min at 95°C, then 35 cycles of 15s at 95°C and 10s at 55°C. The annealing temperature was changed to 61°C for Plas56F/PlasrevRT and 56°C for HRMF/HRM1.2R primer pairs. The PCR was run for 45 cycles for the primer pair Plas56F/PlasrevRT. All runs were then followed by a high resolution melt step of 65-75°C on 0.1°C increments pausing for two seconds per step.

To test the detection of mixed infections, 1:1 mixes of the different positive controls (1ng/µl) were prepared and tested with the primer pairs HRMF/HaemR2 and Plas56F/PlasrevRT.

4.3.4 Determination of qPCR detection limit

The detection limit of the qPCR with promising primer pair combinations was determined using seven duplicate 10-fold dilutions of DNA of the cloned controls. These were run as described above. All seven dilutions had a Cq-value (quantification cycle value) within the 35 (45 for Plas56F/PlasrevRT) thermo-cycles of qPCR and the standard curves were highly reproducible and had R^2-values of > 98%. The concentration of *Plasmodium* DNA in each assay control sample was calculated from the standard curves. To determine the detection limit of this method, the amount of parasite DNA had to be converted to a more meaningful measure for

the level of infection. The DNA standards consisted of clones of an 1101-bp fragment (LINN1, GRW4, SYAT05) or a 480bp fragment (GRW6) respectively of the cytochrome b gene. The total size of the inserted fragment in the vector, using the 3000bp Pgem vector, was 4101bp (3480bp respectively). This is, in the case of the 1101bp fragment, equivalent to 2665650 Daltons (assuming that the average weight of a base pair is 650 Daltons), corresponding to 4.42×10^{-6} pg (with one atomic mass unit/Dalton= $1.66053892 \times 10^{-12}$ pg). Thus, our standard 1 with 1ng/µl DNA corresponds to around 225,989,773 copies of the vector plus clone and the standard 6 with 10^{-5} ng/µl has about 225.99 copies of the gene. It was assumed that each *Plasmodium* parasite contained an average 50 copies of the cytochrome b gene. However, it should be noted that copy number has been estimated between 20-100 per parasite, with sexual stages having up to three times higher copy numbers than asexual stages (Learngaramkul *et al.*, 1999, Farrugia *et al.*, 2011, Putaporntip *et al.*, 2011). Therefore, for the clones of LINN1, GRW4 and SYAT05, 1ng of control DNA is approximately 2.26×10^{8} copies of vector plus insert and representative of 4.52×10^{6} parasites. For GRW6, 1ng of DNA is equivalent to 2.66×10^{8} copies of vector plus insert and representative to 5.32×10^{6} parasites.

4.3.5 Application of clinical samples

In order to assess the usefulness of the qPCR-HRM for detecting *Plasmodium* spp. in clinical samples, tissues were collected from three avian malaria infected birds that died in April 2015 at Wildbase Hospital at Massey University, Palmerston North. One was a North Island brown kiwi (*Apteryx mantelli*) and the other two were yellow eyed penguins (*Megadyptes antipodes*) which were submitted for post-mortem examination to Wildbase Pathology, where tissue samples were collected shortly after death. Histopathology on all three birds showed a high number of tissue meronts in heart tissue (kiwi, Figure 4.2a) as well as in lung, liver and brain tissue (penguins, Figure 4.2b and c) which are indicative of *Plasmodium* spp. infection. DNA was extracted from these tissues and analyzed with the qPCR and HRM protocols using the first primer pair HRMF/HaemR2 followed by Plas56F/PlasrevRT as described above.

Table 4.1: Cytochrome b gene primers used in this study; in bold primers that were used for the final HRM protocols

Primer name	Sequence	Reference
CytbF (forward)	ATGAACTTTTACTCTATTAATT	Musset et al., 2006
CytbFnew (forward)	GGATAATGAATTAARTGTGC	Designed in this study
CytbR (reverse)	AGCTCCCAAGCAAACATATAA	Musset et al., 2006
HaemNF1	CATATATTAAGAGAAITATGGAG	Hellgren et al., 2004
HaemNR3	ATAGAAAGATAAGAAATACCATTC	Hellgren et al., 2004
HaemF (forward)	ATGGTGCTTTCGATATATGCATG	Hellgren et al., 2004
HaemR2 (reverse)	GCATTATCTGGATGTGATAATGGT	Hellgren et al., 2004
Plas56F (forward)	GTCAAATGAGTTTCTGGGGTGC	Designed in this study
PlasrevRT (reverse)	GGTAGCACWAATCCTTTAGG	Designed in this study
CbQ (reverse)	GTCACTWACAAGATATCCACC	Designed in this study
HRMF (forward)	CAGCTYTAAAAATACCCTTYTATCCA	Njabo et al., 2011
HRM1.2R (reverse)	CCWGCWGTTTRTTAGGAATTGT	Njabo et al., 2011

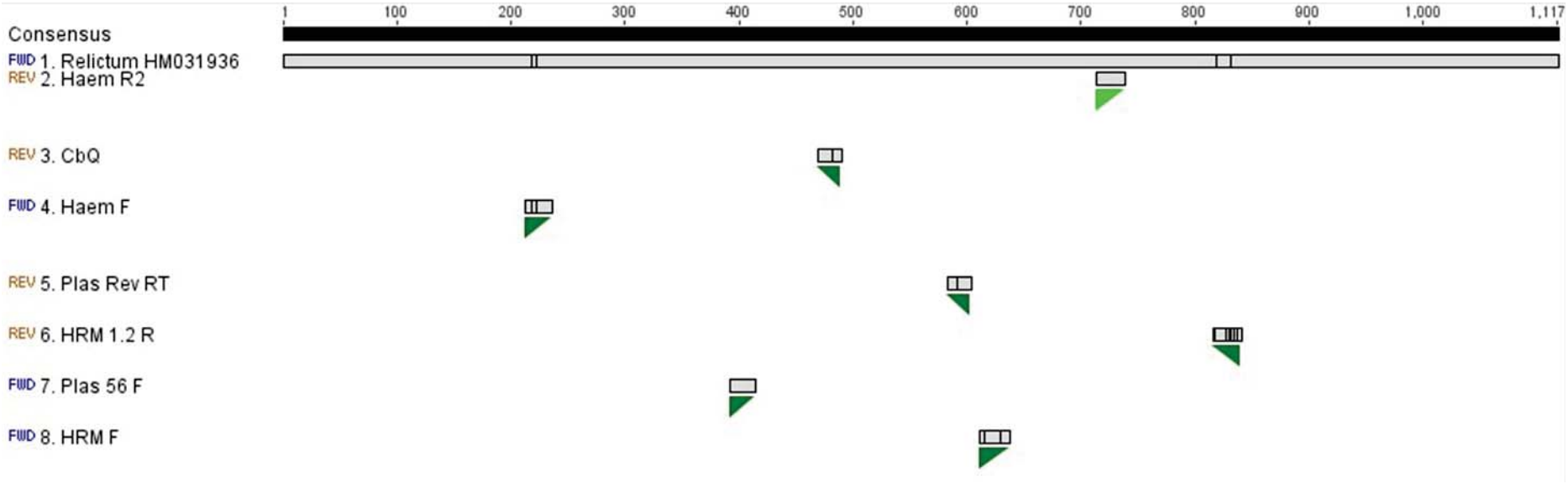


Figure 4.1: Complete cytochrome B gene aligned with the primers (grey rectangles) that were used in the experiment (Geneious version 6.1.7) (Kearse *et al.*, 2012). Green triangle open to the right= forward primer; green triangle open to the left= reverse primer.

Table 4.2: The four *Plasmodium* lineages examined in alignment showing the differences in the DNA sequences in a 127bp part of the cytochrome b gene flanked by the primers HRMF/HaemR2 (Table 4.1).

Consensus	CAGCTTTAAAAATACCCTTCTATCCAAATCTWTTAAGTCTTGATATTAAAGGATTTAATAATRTWYTAGTTTTATTYTTAKCWCAAAGTYTATTGGAATWTTACCATTATCACATCCAGATAATGC
Linn 1	----------A----------G-A----------T---T-T------T----------T----------
Relictum GRW4	----------T----------A-T----------C---G-A------C----------A----------
Elongatum	----------A----------G-A----------T---T-T------T----------T----------
Novyella SYAT 05	----------C----------A-T----------C---A-T------A----------T----------

Table 4.3: Expected melt temperature for the amplicons of each *Plasmodium* lineage used in this study for HRMF/HaemR2 using Oligocalc (Kibbe, 2007) (http://www.basic.northwestern.edu/biotools/oligocalc.html)

Lineage	Expected melt temperature
Linn 1	69.6°C
GRW6	70.6°C
GRW4	69.9°C
SYAT05	70.6°C

4.4 Results

4.4.1 Amplification and cloning of cytochrome b

The amplification of the whole cytochrome b gene was successful for all lineages except *P. elongatum* which failed to amplify using the CytbFnew and CytbR primers. Thus, a nested PCR as described by Hellgren et al. (2004) which uses the HeamF and HeamR2 primer pair to amplify a 480 base pair fragment was used for cloning. Subsequently, the use of the *P. elongatum* cloned DNA was restricted to primers that could amplify within the 480bp amplicons.

4.4.2 Assessment primers

Using the predictive software Oligocalc (Kibbe, 2007), the most promising primer pairs (HRMF/HaemR2 and Plas56F/PlasrevRT; Table 4.1) showed the best separation between the different melt peaks of the four examined *Plasmodium* lineages. Employing the HRM protocol with the primers HRMF and HaemR2, the melt analysis of the 127bp fragment was able to discriminate between the lineages LINN1 and SYAT05 from both GRW4 and GRW6. This primer set was not able to separate GRW4 from GRW6 (Figure 4.3), because of the melt curves dissociating within 0.2°C of each other and therefore they could not be consistently separated by the software, even under high resolution. Thus, a second qPCR-HRM step was needed, using the Plas56F/PlasrevRT primer pair (211bp fragment), that was able to separate *P. elongatum* GRW6 from *P. relictum* GRW4 (Figure 4.5, 4.8, 4.9).

Additional primer combinations (CbQ/Plas56F, HaemF/PlasRevRT and HRMF/HRM1.2R) were not able to discriminate between the different lineages and were removed from further analysis.

The detection limit for the qPCR using the HRMF/HaemR2 primer pair was approximately 5 parasites/µl from all four cloned control lineages, with detection of 5.32 parasites/µl for GRW6 and 4.5 parasites/µl for the remaining three (Figure 4.4). This is assuming an average cytochrome b copy number of 50 per parasite (Section 4.3.4). The detection limit for the Plas56F/PlasrevRT primer pair was higher at approximately 492 parasites/µl (Figure 4.5). In addition, mixed combinations of controls were prepared and tested with the primer pairs HRMF/HaemR2 and Plas56F/PlasrevRT. The best differentiation was found in the mix of LINN1 and SYAT05 lineages, using the HRMF/HaemR2 primer pair, which showed a double peak at the expected melt temperatures of both lineages (Figure 4.7). Both lineages were at the same concentration in the sample (1ng/µl), but lineage LINN1 showed preferential amplification.

4.4.3 Application to clinical samples

In the three clinical samples of deceased birds, it was possible to determine the lineage that had infected these birds. In DNA extracted from tissue, a qPCR melt peak indicating *P. elongatum* GRW6 was seen in all three cases using the primer pair HRMF/HaemR2 (Figure 4.10), with a confidence of 96.34% to 98.04% (Table 4.4). This result was confirmed by running all three samples on the second step of our HRM program using the primers Plas56F and PlasrevRT, which was able to differentiate between the lineages GRW4 and GRW6 (Figure 4.11).

Figure 4.2: *Plasmodium* spp. tissue merogony (arrow) in the heart muscle of a kiwi (A); Lung (B) and brain (C) of a yellow eyed penguin (*Megadyptes antipodes*)

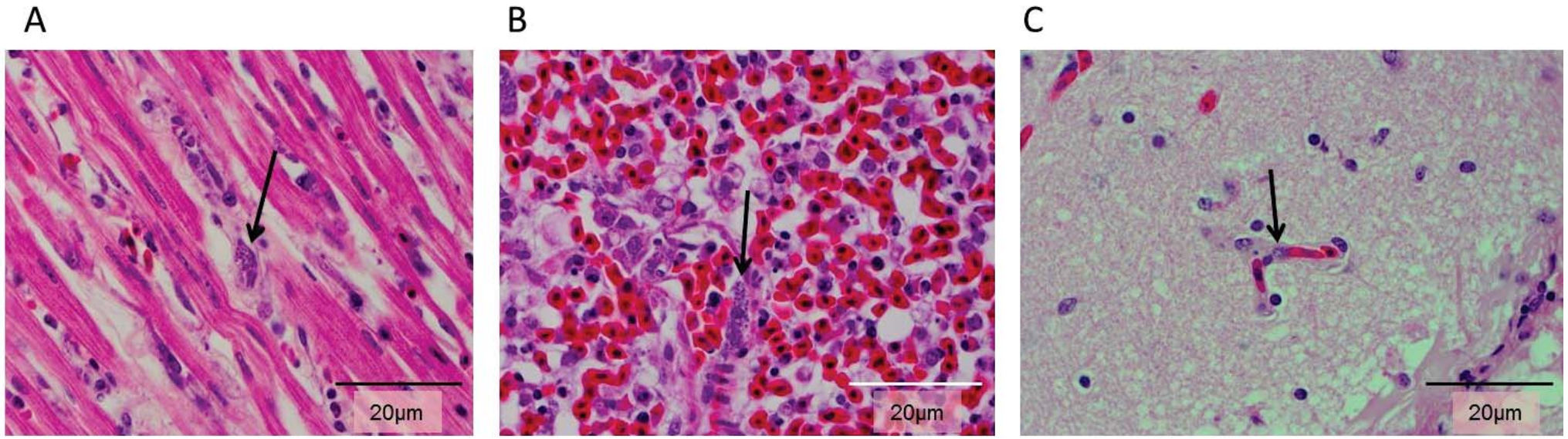


Figure 4.3: Melt of all four tested *Plasmodium* lineages (*P. elogatum* GRW4, *P. relictum* GRW6, Linn1 and *Novyella* SYAT05) using primers HRMF and HaemR2. X axis= temperature (°C); Y axis= derivative of fluorescence over temperature (dF/dT). Threshold= blue line.

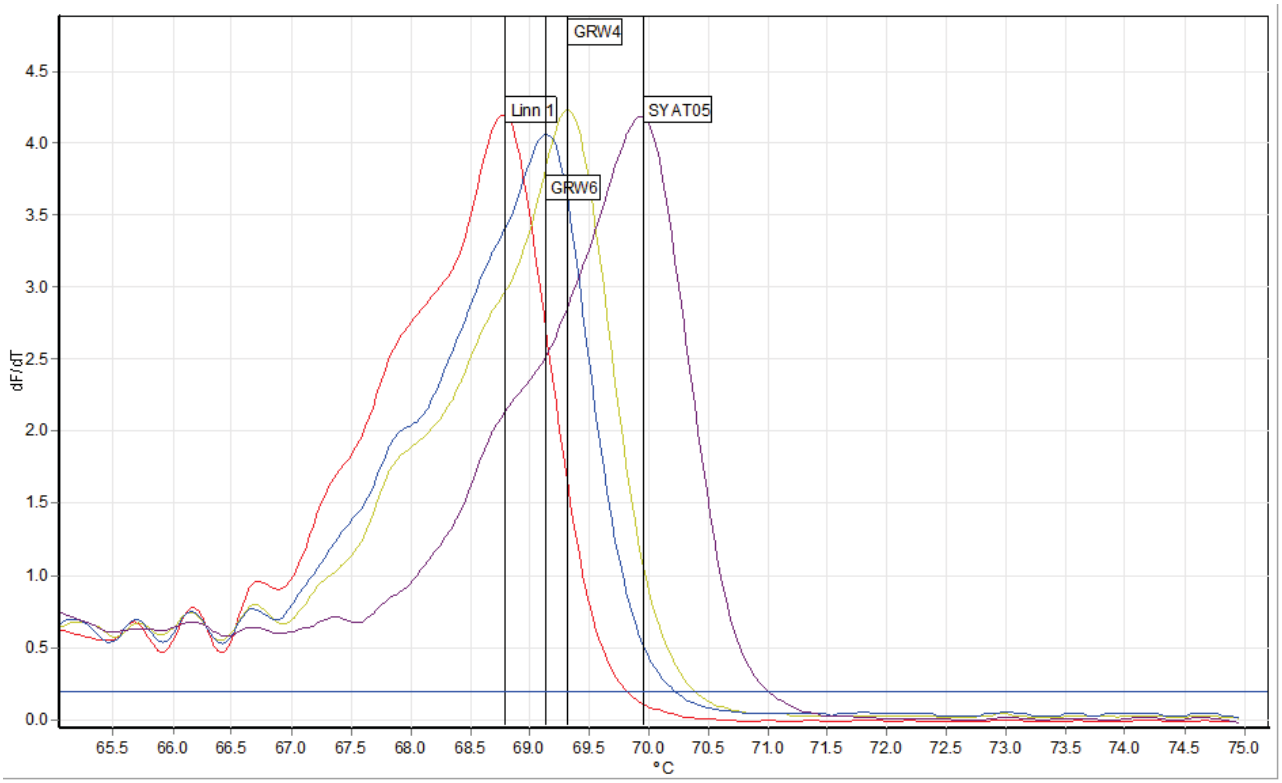


Figure 4.4: Seven duplicate 10-fold serial dilutions of a 1ng/µl standard starting at 0.1ng/µl and standard curve (insert) for *P. relictum* GRW4 using primers HRMF and HaemR2; detection limit is 10^{-5} ng/µl of DNA equivalent to ~5 parasites/µl when run for 35 cycles. X axis= number of cycle; y axis= normalised fluorescence; detection threshold= red line;

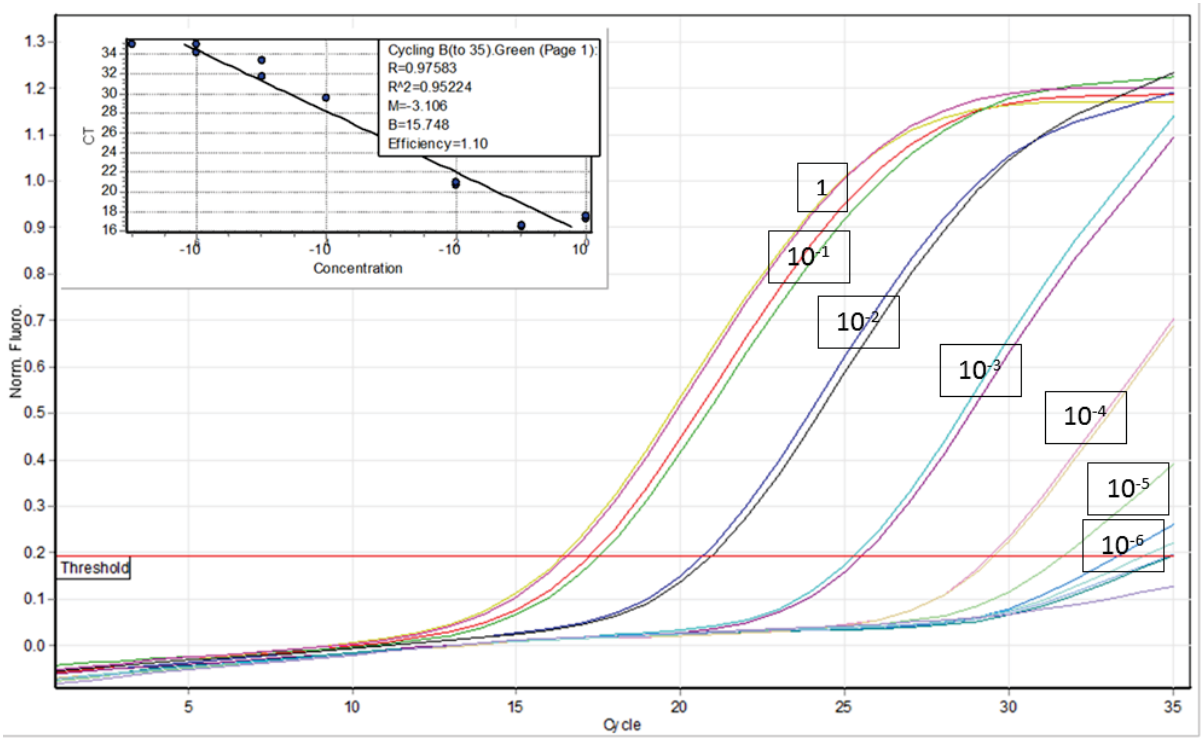


Figure 4.5: Seven duplicate 10-fold serial dilutions of a 1ng/µl standard starting at 0.1ng/µl and standard curve (insert) for *P. relictum* GRW4; detection limit is 10^{-4} ng/µl of DNA equivalent to ~500 parasites/µl when run for 45 cycles using primers Plas56F and PlasrevRT. X axis= number of cycle; y axis= normalised fluorescence; detection threshold= red line;

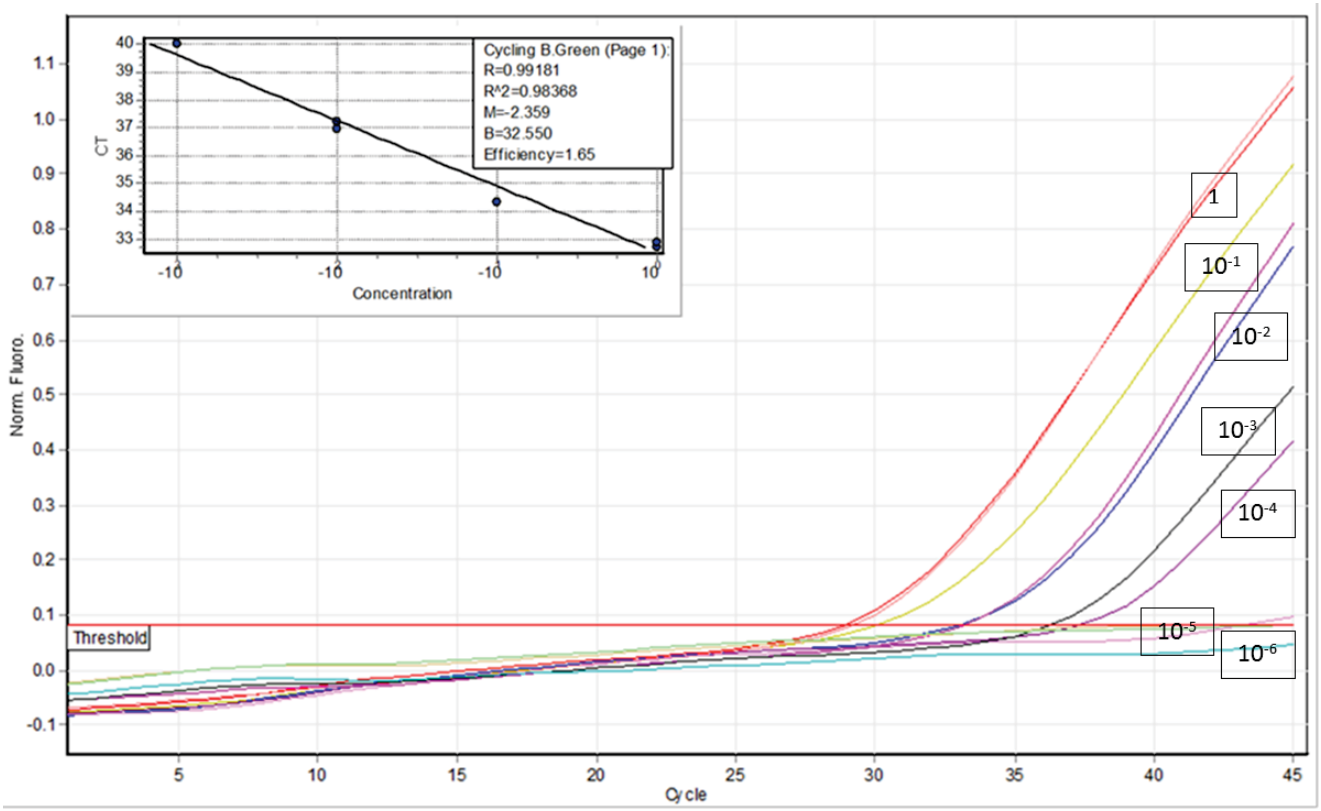


Figure 4.6: Assessing a "mixed" infection of 1:1 (1ng/µl) of *Plasmodium* lineages Linn1 and *Novyella* (SYAT05), showing a double peak at the melt temperatures of both lineages. X axis= temperature (°C); Y axis= derivative of fluorescence over temperature (dF/dT). Threshold= blue line.

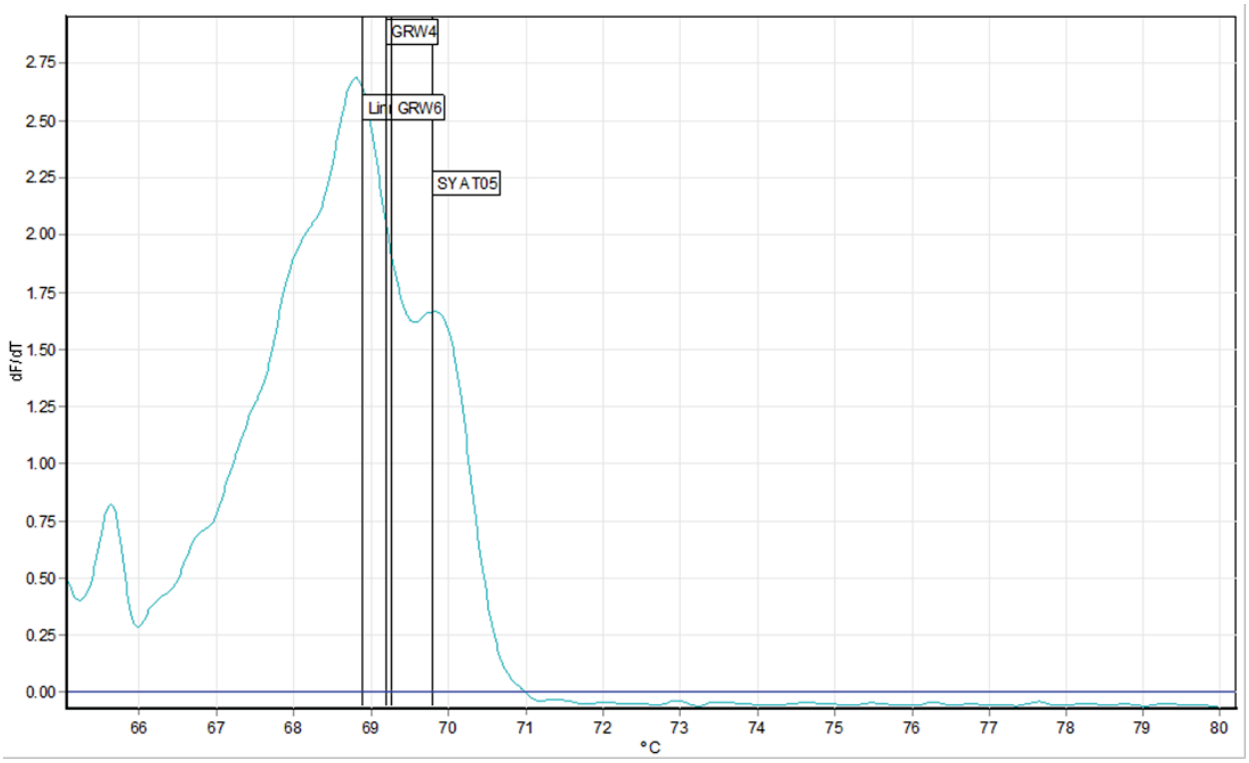


Figure 4.7: qPCR melt protocol using primer pair Plas 56F and Plas rev rt for discriminating between *P. elongatum* GRW6 (yellow) and *P. relictum* GRW4 (blue). X axis= temperature (°C); Y axis= derivative of fluorescence over temperature (dF/dT). Threshold= blue line.

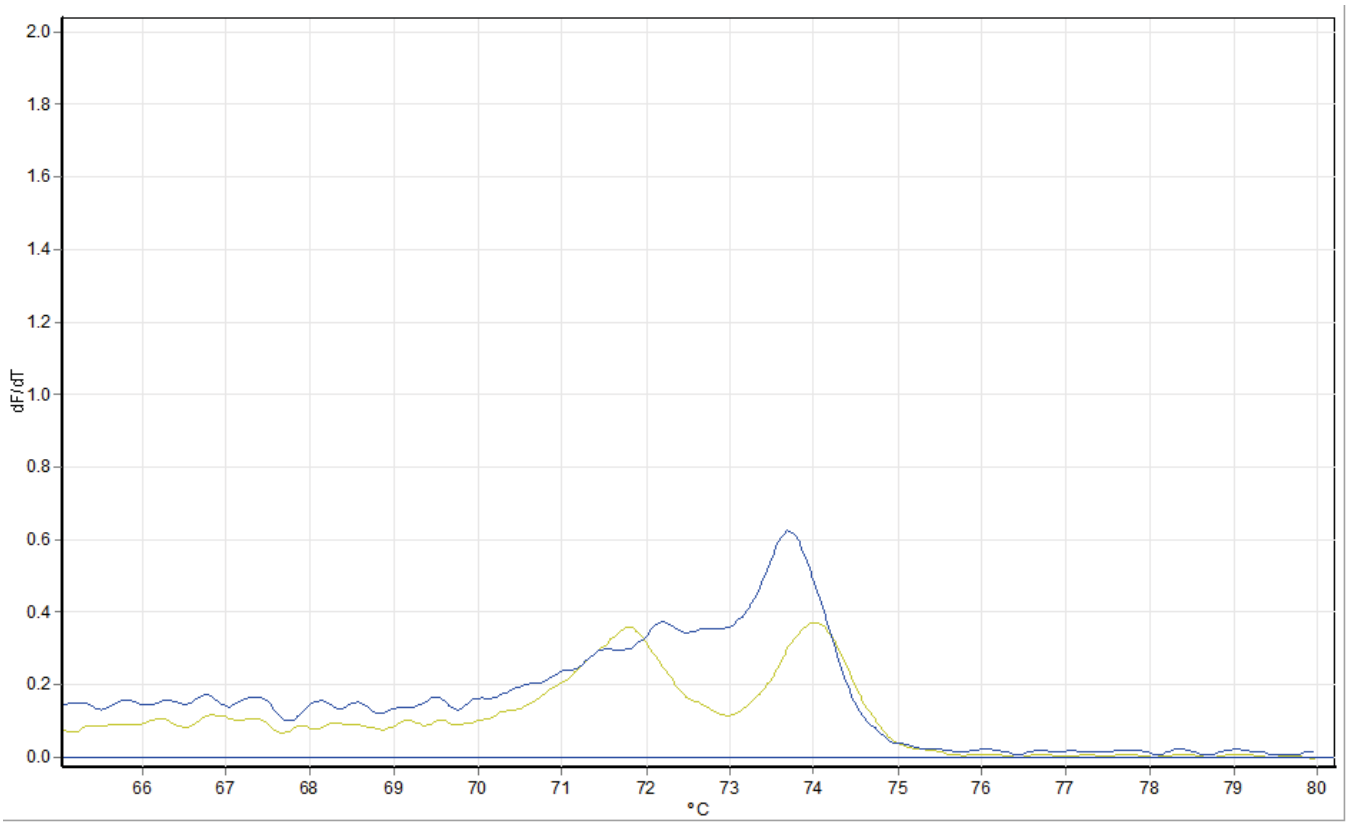


Figure 4.8: High resolution melt protocol using primer pair Plas 56F and Plas rev rt for discriminating between *P. elongatum* GRW6 ((yellow) and *P. relictum* GRW4 (blue); X axis= number of cycle; y axis= normalised fluorescence;

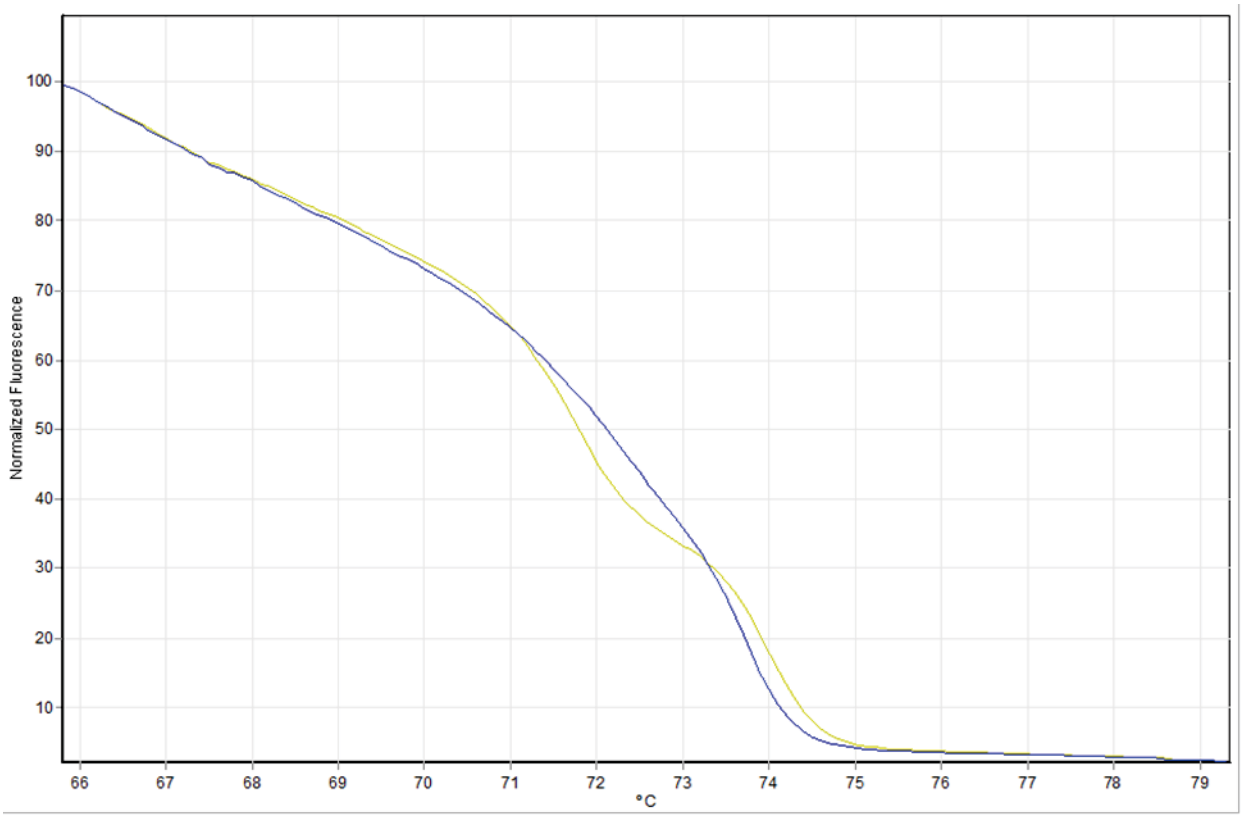


Figure 4.9: Clinical application; diagnosing the lineage infecting three deceased birds, North Island brown kiwi (purple) and two yellow eyed penguins (light blue and pink) using primers HRMF and HaemR2. X axis= temperature (°C); Y axis= derivative of fluorescence over temperature (dF/dT). Threshold= blue line.

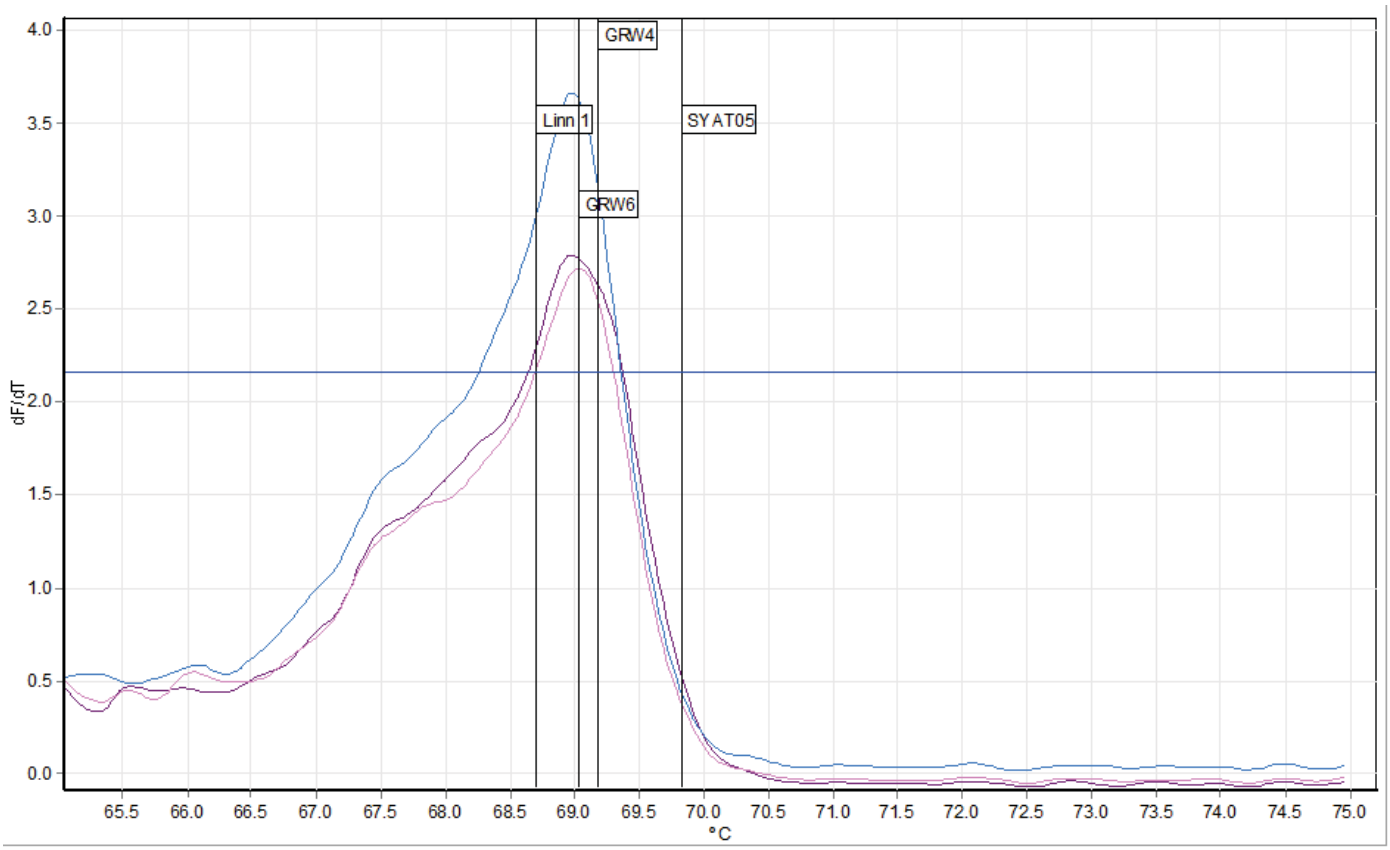


Table 4.4: Clinical application: using HRM to diagnose the lineage of *Plasmodium* infecting three deceased birds, a North Island brown kiwi and 2 yellow eyed penguins (YEP). Sample DNA was used in 1:10 dilution. No. = sample number; l, r, e, n= label of positive controls (l= LINN1, r= GRW4, e= GRW6, n= SYAT05);

No.	Color	Name	Genotype	Confidence %
4		Kiwi 28 1:10	GRW6	98.09
5		YEP 35 1:10	GRW6	96.19
6		YEP 36 1:10	GRW6	96.99
7		l	LINN 1	100.00
8		r	GRW4	100.00
9		e	GRW6	100.00
10		n	SYAT05	100.00

Figure 4.11: HRM using primer pair Plas 56F and PlasrevRTt of clinical samples Kiwi 28, YEP 35 and YEP 36; the samples are equivalent to the GRW6 baseline (green) compared to *P. relictum* GRW4 control (pink). X axis= number of cycle; y axis= normalised fluorescence;

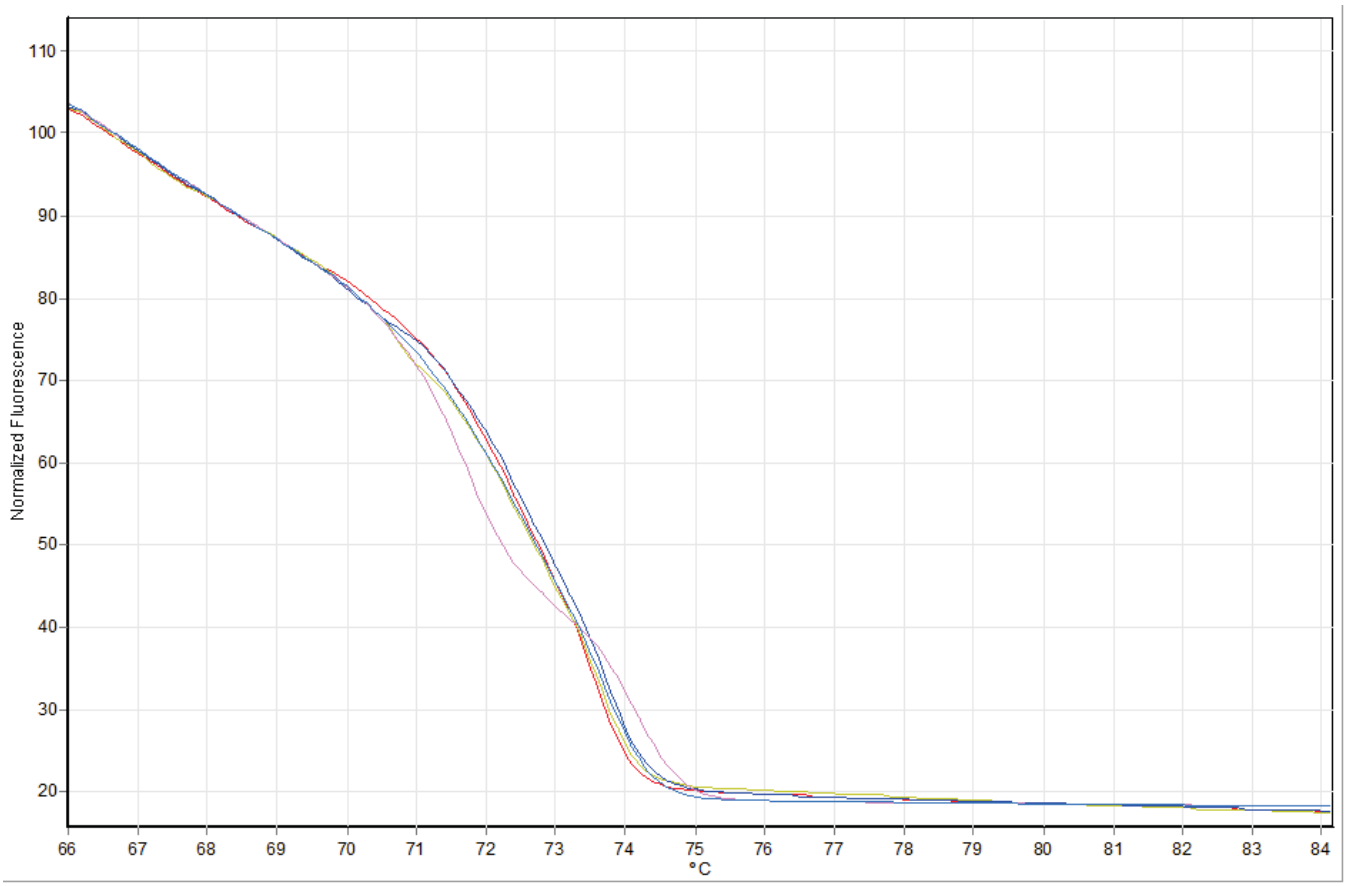
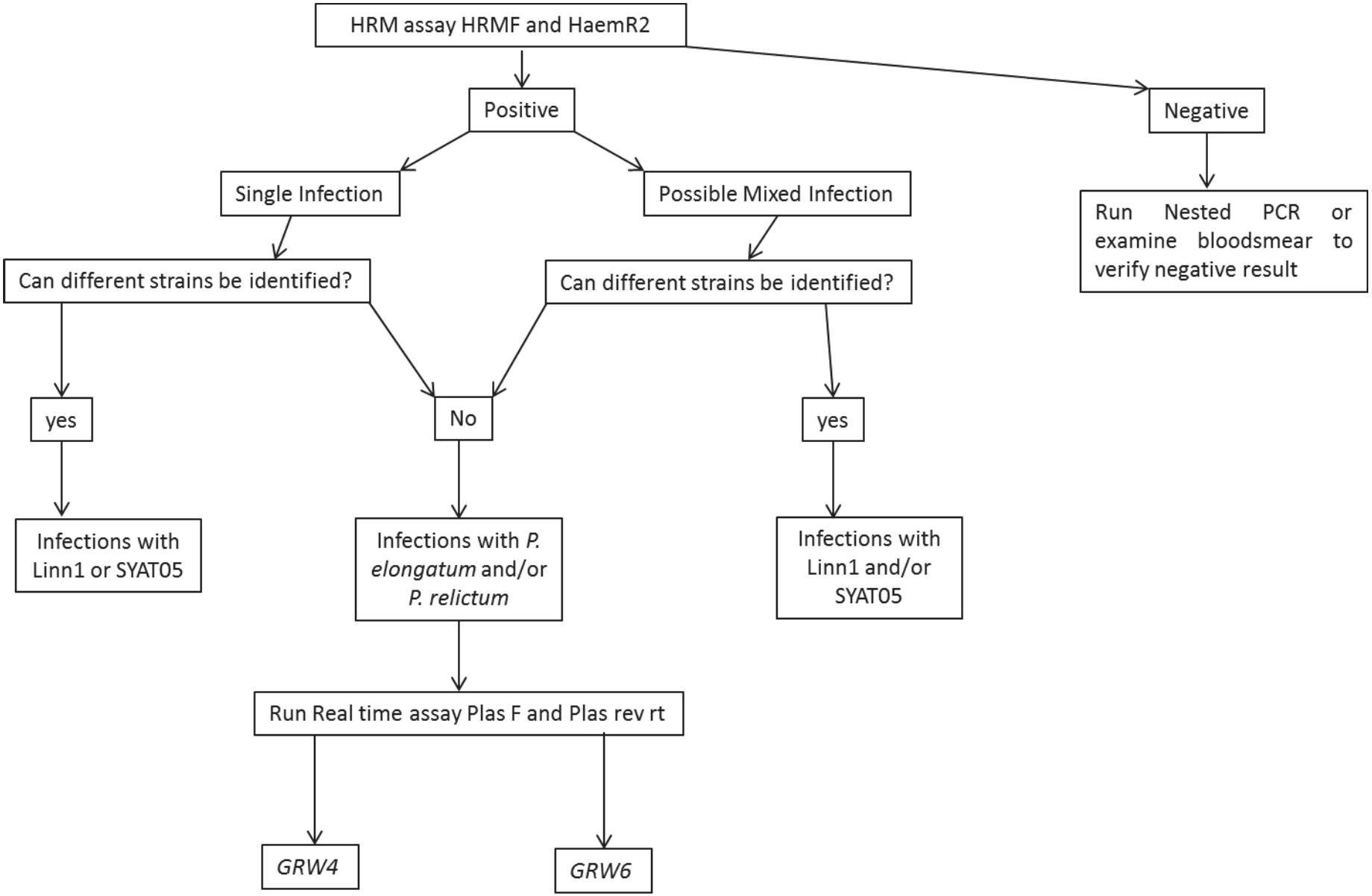


Figure 4.12: HRM assay flowchart



4.5 Discussion

The qPCR melt protocol using the primer pair HRMF and HaemR2 was able to discriminate between three of the main lineages present in New Zealand. An additional qPCR HRM (primers Plas56F/PlasrevRT) was able to be used as a confirmation assay between *Plasmodium* lineages GRW4 and GRW6 which were not reliably differentiated in the first assay. The differentiation of these lineages is of importance for New Zealand conservation, because although avian malaria parasites almost exclusively cause subclinical infections in areas with endemic infection (Bennett *et al*. 1993, Valkiūnas, 2005), disease and mortality remain a possibility as seen in the recent findings by Dinhopl et al. (2015). In particular, in captive situations and wildlife rehabilitation, mortalities have been observed in endangered New Zealand birds, like NI brown kiwi (Banda *et al*., 2013), mohua (Alley *et al.* 2008), great spotted kiwi (Howe *et al*., 2012) and a Fiordland crested penguin (Hunter, 2015). Indeed, worldwide, avian malaria has been widely recognised as one of the most significant infectious diseases in captive penguins and penguins in rehabilitation, with *P. relictum* and *P. elongatum* as the most important disease agents (Vanstreels *et al*, 2015, Parsons and Underhill, 2005, Jones and Shellam, 1999, Clarke and Kerry, 1993). Therefore, the finding of *P. elongatum* GRW6 during this study in a deceased brown kiwi as well as two yellow-eyed penguins is consistent with previous observations and it underlines the pathogenic nature of this parasite lineage for endangered birds in New Zealand.

The HRMF/HeamR2 assay shows a good sensitivity of approximately 5 parasites/µl extracted DNA. A higher sensitivity may be desirable for detecting low level parasitaemia of *Plasmodium* spp. in native birds. Compared to the standard nested PCR protocol (Hellgren *et al*., 2004), where 149 positives in bird blood samples of low level parasitaemias were detected (Chapter 5), it was only possible to detect 44 (33.33%) using the new assay. When compared to other published qPCR techniques, the assay presented here appeared less sensitive, although direct comparison is as difficult because other authors have not provided their detection limit. The assay developed by Njabo et al. (2011) was able to detect 17% more positives than the nested protocol by Hellgren et al. (2004). Friedl and Gruscurth (2012) could detect as few as 0.1 blood parasites per 100 blood cells, Knowles et al.

(2011) noted a significantly higher sensitivity in their qPCR assay compared to the nested protocol whereas Bell et al. (2015) found no difference in sensitivity of their protocol with the established nested PCR (Knowles *et al.*, 2011, Njabo *et al.*, 2011, Friedl and Groscurth, 2012, Bell *et al*., 2015). Only one (Knowles *et al.*, 2011) of these previously published assays was able to reliable discern between different *Plasmodium* species.

One important advantage of the assay presented here is the ability to detect co-infections with two or more different malaria parasites. This is important, because co-infections are common in wild birds (Jarvi *et al.*, 2002) and are present in up to 80% of all infected birds in Europe (Valkiūnas *et al.*, 2006a). There is some discrepancy in the literature about the effect of co-infections with different lineages of *Plasmodium*, although these are generally considered more virulent than infections with just a single lineage (Arriero and Moller, 2008, Marzal *et al.*, 2008, Palinauskas *et al.*, 2011), because different parasites simultaneously share the limited available physiological resources (Atkinson, 1991).

Still, due to the low sensitivity of the presented assay in detecting low level parasitaemia, microscopic examination of blood smears remains the most important method for diagnosing mixed infections in bird with low level parasitaemia. Microscopy is an inexpensive investigation method which is widely available and is able to reliably identify different haemosporidian parasite species and mixed infections (Valkiūnas *et al.*, 2008a). However, it requires training and experience, is time intensive with around 20 minutes of examination time per slide and has the potential to miss low-level parasitaemia (Valkiūnas *et al.*, 2008a). Therefore, both PCR and microscopy underestimate the prevalence of infection of blood parasites in naturally infected birds (Jarvi *et al.*, 2002, Valkiūnas *et al.*, 2008a). In addition, the identification of parasite morphology is often difficult, and avian malaria parasites are known for their cryptic diversity (where unrelated species may look similar). This can be made worse by the distortion of the cells on the smears due to air drying and fixation, environmental conditions at the sampling site or slight differences in the stains used (Perkins *et al.*, 2011). Furthermore, microscopy does not take into account potential differences between the skill and experience of individual technicians (Valkiūnas *et al*., 2008a).

Beginning with the further optimization of the presented assay, future work has to be performed to create new primer sites along the cytochrome b gene. For this, another primer pair for amplifying the whole gene has to be found for the NZ lineage of *P. elongatum*, since it was not possible to amplify this gene with the primers used by Musset et al. (2006) even after modification (self-designed primer CytbFnew; Table1). This restricted the primer design for assay development to only a 480 basepair sequence using the nested PCR designed by Hellgren et al. (2004) instead of having access to the whole cytochrome b gene (~1100bp). If the amplification of the whole cytochrome b gene is to succeed in all lineages, a first step would be a test of the real-time primer set that was used in a study by Knowles et al. (2011), where it was possible to discern the melt peaks of two different species, *P. relictum* (lineages pSGS1 and pGRW11) and *P. circumflexum* (lineages pTURDUS1 and pBT7). Another desirable step would be to include the detection of *P. relictum* SGS1 in this assay, a lineage very common in Europe (Dimitrov *et al.*, 2015) and which has been found in mixed infections in New Zealand (unpublished data). For this, the blood of a bird with a single SGS1 infection needs to be acquired and a clone standard established. The best choice of method for future studies would be the use of lineage specific probes for a TaqMan®- assay which would greatly improve the specificity of the real-time PCR. So far, the use of this approach is limited by available funding as specific probes are very costly. In addition, as Valkiūnas et al. (2014b) remark, the high diversity of avian haemosporidian parasites will make this task difficult.

The most important advantage of using the cytochome b gene for primer design is, that this gene has been used as a reference gene for avian haemosporidian parasites in recent years and therefore has the most sequence data of different haemosporidian species and lineages is available (Bensch *et al.*, 2009). However, the cytochrome b gene in malarial parasites has a high AT content (approximately 73%), which causes difficulties in designing effective primers (Fallon *et al.*, 2003b). Other genes are available that have previously been used in both avian and human *Plasmodium* research. The RNA region of the mitochondrial genome of avian malarial parasites is highly conserved, making it an excellent location to design PCR primers for detecting haemosporidian parasite infections (Fallon *et al.*, 2003b). Although, due to the low genetic variation in this region, differentiating between

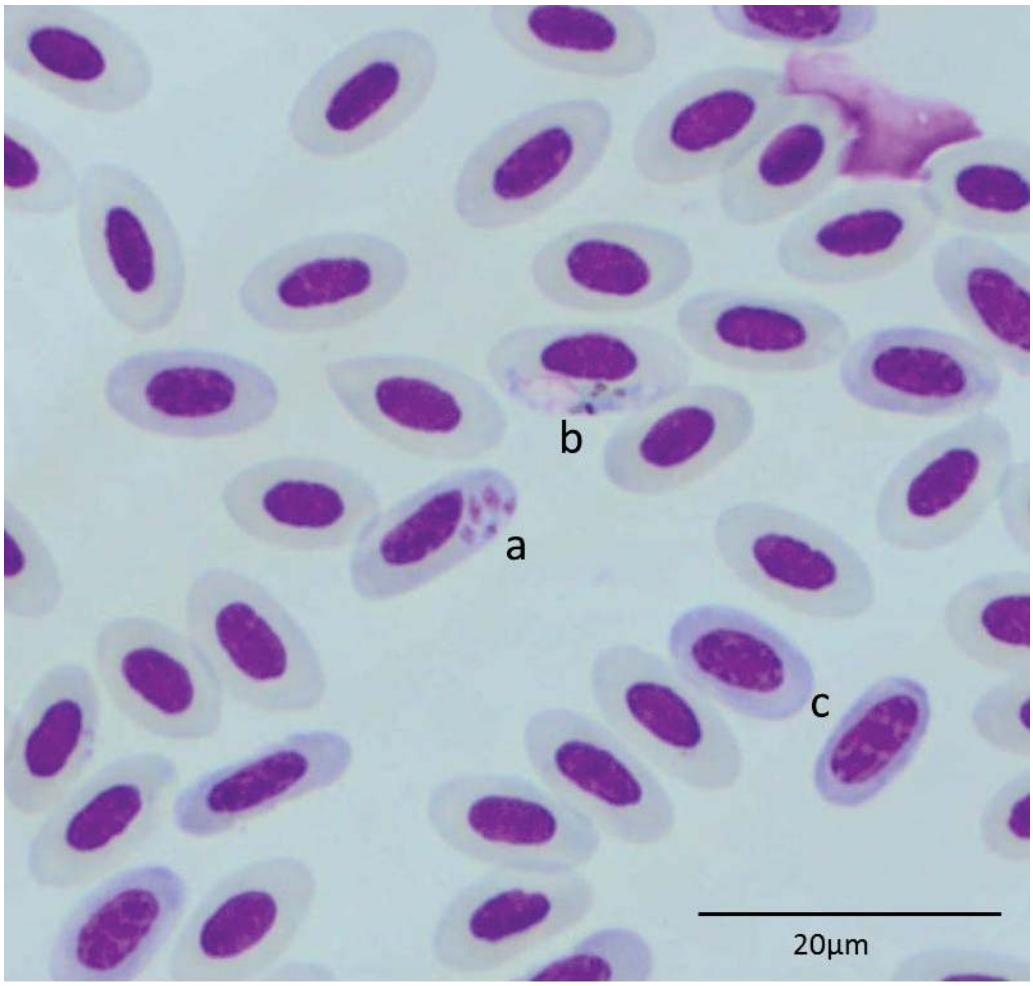
different species is difficult. The assay described by Fallon and Ricklefs (2003b), for example, was only able to detect *Haemoproteus* spp. and *Plasmodium* spp. that differed by up to 12% sequence divergence in the cytochrome b gene.

The 18S gene is commonly used in human malaria diagnostics, but assays using the 18S gene for avian malaria are rare and if existent, they are poorly optimized and the primers are not parasite specific (Richard *et al.*, 2002). The 18S gene also has a lower copy number, ranging on average in *P. falciparum* between 5 (Tran *et al.*, 2014) and 6 copies (Wampfler *et al.*, 2013) per parasite. In comparison, cytochrome b has an average of 50 copies (depending on the life cycle stage). Recently, Hellgren et al (2015) used the highly variable nuclear merozoite surface protein 1 (MSP1) gene for avian malaria detection, which is a gene linked to the invasion biology of the parasite. With this assay, Hellgren et al. (2015) were able to discern different alleles in several distinctive cytochrome b haplotypes of *P. relictum*. This made it possible to record the genetic variation occurring in different *P. relictum* populations and their geographical distribution. Using the MSP1 gene in a New Zealand setting would enable studies on the different populations of *Plasmodium* species and lineages in their geographical origins as well as on finding local patterns of virulence and host resistance.

One problem encountered with using these genes is the lack of available avian parasite sequences on Genebank and therefore the ability to design primers for NZ lineages to reliably amplify these genes. For future studies, full genome sequencing of the different *Plasmodium* lineages found in New Zealand has to be performed to be able to design primers for a new assay. Next generation sequencing techniques have already been performed on the cytochrome b gene of *P. relictum* GRW4 found on Hawaii (Jarvi *et al.*, 2013). With this, Jarvi et al. (2013) were able to document previously undetected levels of variation in a single *Plasmodium* lineage, suggesting the origins and evolution of this parasite on Hawaii may be more complicated than previously recognized. A similar approach in New Zealand would provide invaluable information about host parasite interactions and evolution and may also help answer the question as to whether the introduction of new *Plasmodium* lineages together with European birds in the late 1800's played a role in the disappearance of species endemic to New Zealand.

The new assay described here produced fast and accurate data that can be used in rapid disease screening for *Plasmodium* parasites in native New Zealand birds suffering from acute infection with clinical symptoms as well as in birds that have been found dead. It will be especially useful in a rehabilitation setting where the health of the birds is monitored on a regular basis (so that clinical symptoms are recognised early on) and where treatment is available. The advantage of this method compared to standard PCR and microscopy is the speed, lower cost and the ability to detect some mixed infections. However, there is the potential that preferential amplification of LINN1 in a sample with dissimilar numbers of different lineages might hide a mixed infection, because only one of them (LINN1) is amplified. However, this method did not prove sensitive enough to diagnose birds for translocation with low parasitaemia, which would be the majority of the infected population. Birds can now be tested for not only the genus *Plasmodium*, but also the most common lineages. This also applies to dead birds found in conservation reserves by visitors and during routine monitoring. Therefore, this method enables conservation managers to get an overview of *Plasmodium*, and if present, which lineage, plays a role in morbidity and mortality in monitored bird populations in captivity, at reserves that already have certain native bird species as well as at future translocation sites. In order to use this protocol most efficiently for this purpose most efficiently, a flowchart was created to help with step-by-step decisions (Figure 12).

Chapter 5: A bottleneck not just for the hosts: the impact of translocations of North Island saddleback on the diversity of their *Plasmodium* parasites



Plasmodium (novyella) vaughani **(genetic lineage SYAT05) meront (a) and gametocyte (b) in a blood smear from a blackbird (*Turdus merula*) from Auckland; note immature erythrocytes (c) with darker cytoplasm and less dense nuclear chromatin.**

5.1 Abstract

In this study, the North Island New Zealand saddleback (*Philesturnus rufusater*) and its infections with different species and lineages of *Plasmodium* spp. were used as a host-parasite model to examine the impact of wildlife translocations for species restoration on vector-borne pathogens. The North island saddleback is an excellent species to use in examining this issue as the current population was sourced from a single location through a series of well-documented translocations. Thus, the hypothesis of this study was that the highest diversity of *Plasmodium* lineages would be found in saddlebacks at the source location of a translocation sequence and that parasite diversity would diminish as translocations progressed. During the period 2012-2013, nine sampling trips were carried out at five sites in a saddleback translocation sequence and archived material from six different locations (previous and future translocation sites) was also examined. I also visited the site of a future translocation (Cape Kidnappers) as well as two sites close to conservation sites (Hen Island and Tiritiri Matangi Island) to examine parasite diversity and prevalence in introduced passerines. A total of 379 blood samples were tested by PCR, with 149 (39.3%) being positive for *Plasmodium* spp. Ten distinct lineages of *Plasmodium* parasites were identified. Fifty-nine of the samples (39.6%) had mixed infections with the most common lineages. Two lineages previously unknown to New Zealand were found. Of note was that 76.3% of North Island saddleback on Hen Island were positive for *Plasmodium* infection. As predicted, more parasite lineages were found on Hen Island, the first island in the translocation sequence, and generally lower diversity in subsequent translocation sites. However, a slightly elevated diversity was found at Titritiri Matangi Island, the third site in the translocation sequence and at Bushy Park, the fifth site. This finding may be explained by the fact that Tiritiri Matangi Island has been the focus of a restoration programme that has seen 18 new species translocated to the island in the past 40 years. In addition, Bushy Park is a mainland site located within pastoral land visited by many introduced bird species. Most of the *Plasmodium* lineages were common to island and mainland sites and are they are cosmopolitan lineages found worldwide. This suggests that parasites can move to new sites via translocations in what is known as “pathogen pollution”. One lineage, KOKAKO01, was found only on Hen Island and Tiritiri Matangi Island and may have been lost on the other sites due to host population bottlenecks after translocation.

Keywords

Haemosporidian parasites, New Zealand nature reserves, New Zealand passerines, pathogen pollution, *Plasmodium*, wildlife translocations

5.2 Introduction

The movement of plants and animals from one area to another has occurred throughout human history as a way to maintain important resources nearby. As a result of this practice humans have introduced a large number of plants and animals to new areas worldwide. Today the practice of re-introducing and re-enforcing populations of living organisms is used in conservation translocations for endangered species (IUCN/SSC, 2013). Globally, translocations of endangered species are becoming more important as more restoration areas become available and captive breeding programmes for wildlife reintroductions become more successful. Translocations have become one of the preferred methods used in species recovery programmes in New Zealand (NZ) (Cunningham, 1996, Mathews *et al.*, 2006), due to progress in pest control and the creation of large mainland islands that are either predator free or intensively controlled (Craig *et al.*, 2000).

The impact wildlife translocations can have on parasites which are carried by the translocated hosts is twofold. First, introductions, reintroductions and translocations can pose a threat to pre-existing fauna through the introduction of novel parasites and pathogens ("pathogen pollution"), and second, to the translocated animals themselves through exposure to parasites or pathogens already present in the release area and its inhabitants (Cunningham, 1996, IUCN/SSC, 2013). "Pathogen pollution" refers to the anthropogenic movement of parasites (including bacteria and viruses) outside their natural geographic or host species range, and their introduction to a new (or naïve) host species or environment (Cunningham *et al.*, 2003). One well known example is the extinction of many native birds on the Hawaiian Islands after the arrival of Europeans, with avian pox and malaria playing a major role (Daszak and Cunningham, 1999). The potential for Hawaiian birds to be infected with both malaria and pox had existed for centuries with the influx of migratory sea and shorebirds carrying such pathogens, but these diseases were not able to establish until the introduction of the necessary mosquito vectors (van Riper III *et al.*, 1986). Therefore, the immunological naïve status of Hawaiian bird populations prior

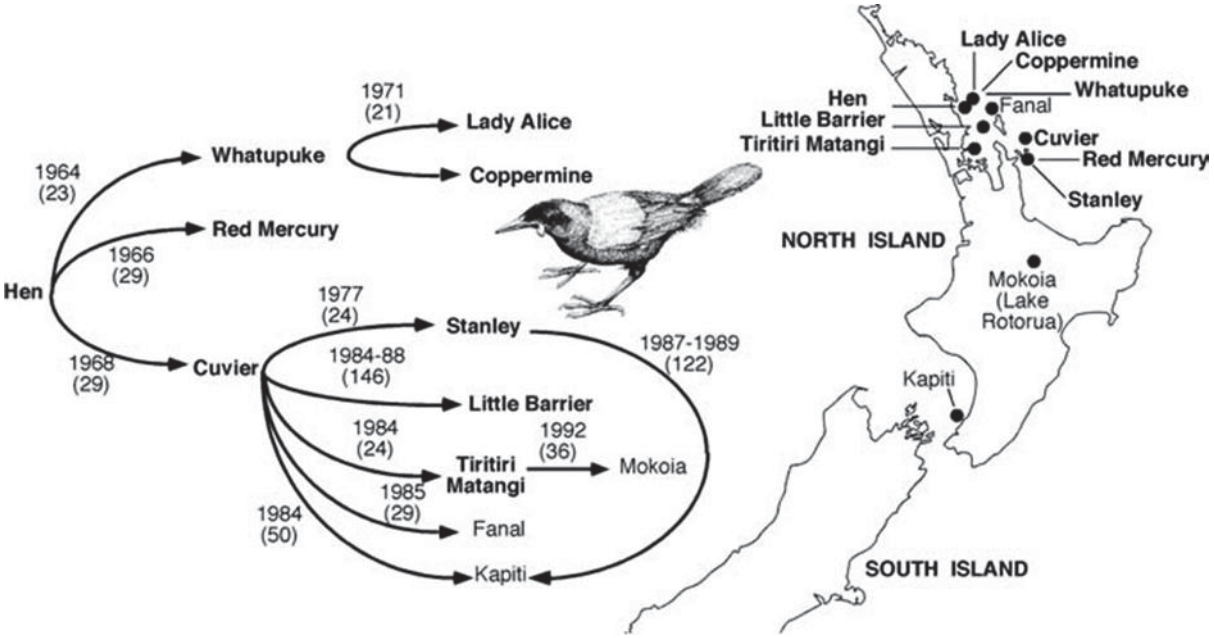
to their exposure following vector introduction is believed to have been a key cause of the subsequent disease impacts (van Riper III *et al*., 1986).

Translocations can also impact parasites in the opposite way and contribute to their extinction. The effects of wildlife translocations on endemic parasites has not been generally taken into consideration. Although, animals destined for translocation overseas are often kept as parasite free as possible (Sainsbury, 2015). Parasites are generally viewed as 'pathogenic' or potentially pathogenic and therefore as a burden to the host during a stressful situation such as a translocation. Worldwide, this practise has led to the extinction of at least two host specific feather lice, one of them in New Zealand (Rozsa and Vas, 2015, Buckley *et al.*, 2012). The number of species which has become extinct is most likely greater than recorded but due to incomplete lists of parasites living on or in hosts as well as lack of data collection during translocations it is impossible to know for sure. While there is a short-term advantage in keeping animals for translocation free of parasites, this is not desirable in the long term. Particularly, as the parasite burden in natural populations is important for the maintenance of genetic and other adaptations, and parasites that inhabit the host "ecosystem" are an important part of biodiversity in the wild (Cunningham 1996). Little is known about the diversity of parasites and studies on this subject have generally been neglected. Still, it can be assumed that parasites make up the unseen majority of species extinctions (Dunn *et al.*, 2009, Lafferty, 2012). In addition biodiversity loss may reduce parasite diversity more than previously thought (Lafferty, 2012). Hudson et al. (2006) even hypothesised that a healthy ecosystem is one that is rich in parasites, with their effects on host population dynamics, interspecific competition, structure of food webs and biodiversity invaluable for the functioning of an ecosystem. The negative effects of translocations on parasites include bottlenecks from the reduction of their populations as a consequence of the reduced number of hosts being moved, and extinction if the hosts by chance are not infected or die following their movement.

Conservation managers are currently hindered by a lack of information concerning such risks. The main objective of this study was to examine the impact of wildlife translocations on vector borne parasites using the North Island (NI) saddleback (*Philesturnus rufusater*) and its infections with different lineages of avian malaria

(*Plasmodium* spp.) as a model system. The NI saddleback is a range restricted endemic bird that belongs to the wattlebird family (Callaeidae) which is endemic to New Zealand. NI saddlebacks are vulnerable to the destruction of their natural habitat and are particularly sensitive to predation by introduced mammalian predators because of their roosting and nesting habits and poor flying abilities. Once widespread, these birds now only exist on predator-free offshore islands or in predator fenced mainland sites.

Figure 5.1: North Island saddleback translocations. All North Island saddleback populations in existence today originate in the population from Hen Island which survived away from mainland threats like habitat destruction and introduced mammalian predators. In a series of successive translocations the birds were re-introduced into newly created predator-free environments all around the North Island of New Zealand. This figure is from (Lambert *et al.*, 2005) and shows an incomplete translocation history up to the mid-nineties. Coppermine Island was colonised by saddleback naturally. The population on Fanal Island has since died out.



Avian malaria parasites of the genus *Plasmodium* are single-celled protozoans and belong to the order Haemosporidia, family Plasmodiidae (Chapter 2; Valkiūnas, 2005). In New Zealand, avian malaria parasites of 17 different lineages have been found in 37 different species including native and introduced birds (Chapter 2; Tompkins *et al*., 2008, McKenna, 2010, Tompkins *et al*., 2010, Castro *et al*., 2011, Howe *et al.*, 2012, Schoener *et al.*, 2014). This widespread presence together with low prevalence and parasitaemia has led to the suggestion that avian malaria has

been in NZ for a long time and that native bird species are possibly resistant to infection (Castro *et al*., 2011, Howe *et al*., 2012, Schoener et al., 2014). There are several *Plasmodium* lineages which appear to be specific to their endemic New Zealand hosts (Baillie *et al*., 2011, Howe *et al.*, 2012, Ewen *et al.*, 2012b). This proposition is also supported by the presence of endemic mosquitoes of genera known to vector *Plasmodium* (Derraik, 2004, Derraik and Slaney, 2007; Massey *et al*., 2007, Derraik *et al.,* 2008). Still, the most widespread and common lineages found in New Zealand today appear to be cosmopolitan (and have been widely distributed by human mediated bird introductions) and are particularly common in Europe (Chapter 2; Ewen *et al.*, 2012b, Schoener *et al.*, 2014). These *Plasmodium* lineages are carried by introduced birds like blackbirds (*Turdus merula*), which are known to be very susceptible to malaria infection and have shown a prevalence of up to 100% in Europe (Bentz *et al.*, 2006). Tompkins and Gleeson (2006) therefore suggested that blackbirds act as reservoirs for infection in New Zealand. These cosmopolitan lineages are also responsible for the recently reported malaria deaths in native New Zealand birds (Schoener *et al.*, 2014, Hunter, 2015, Dinhopl *et al.*, 2015). However, these fatalities appear to be connected to particular circumstances; for example they seem to occur most commonly in captivity such as New Zealand dotterel (*Charadrius obscurus*) chicks in 1996 (Tompkins *et al*., 2008), yellowhead/mohua (*Mohua ochrocephala*) in 2004 (Alley *et al*., 2008) and brown kiwi (*Apteryx mantelli*) in 2010/2011 (Banda *et al*., 2013). Although the NI saddleback is known to be a host to at least three different lineages of *Plasmodium* spp. (Castro *et al*., 2011), a recent disease outbreak caused deaths in SI saddlebacks in 2002 and 2007 (Hale *et al*., 2008, Alley *et al.,* 2010). These deaths were associated with environmental stress when the birds endured a period of severe drought.

The well-documented translocation history (Chapter 3; Figure 5.1) as well as the fact that all current populations of both saddleback species each originated from a single source population and that saddlebacks are hosts to a number of *Plasmodium* spp. makes this species a very good model for examining the impact of translocations on parasites. The translocations of both saddleback species were performed in a sequence (Figure 5.1 for NI saddleback). This particular history makes it possible to follow the steps of possible parasite translocations from the source populations to the new locations. In this study, I will also consider the introduction of European

passerine birds during the late 1800s therefore enabling me to examine the impact of animal movements on parasites taking both conservation translocations as well as species introductions from overseas into account.

This should enable me to examine the following predictions:

1. The most common *Plasmodium* lineages found in both NI saddleback and introduced birds will be widespread cosmopolitan ones, although it is possible to find endemic lineages in the sampled saddlebacks.

2. Parasite prevalence found in native and introduced birds are similar to the ones found in other areas like Europe and the Americas (Beadell *et al.*, 2004, Valkiūnas, 2005, Evans *et al.*, 2009) because New Zealand is an old landmass with potential vectors. In addition, many native passerine species in NZ belong to genetically ancient groups at the root of the evolutionary tree. The Australasian area has been a hotspot for avian evolution and diversification (Ericson *et al.*, 2003). These old NZ bird groups may have carried *Plasmodium* parasites for a long time. *Plasmodium* parasites have been found in fossil tertiary Dominican amber (Poinar Jr *et al*., 2005), supporting this idea.

3. Some parasite lineages have been moved around with bird translocations, therefore the same lineages can be found at different locations.

4. Parasite lineages have been lost due to host population bottlenecks, therefore the diversity is higher at the source location of saddlebacks and declines at subsequent translocation sites.

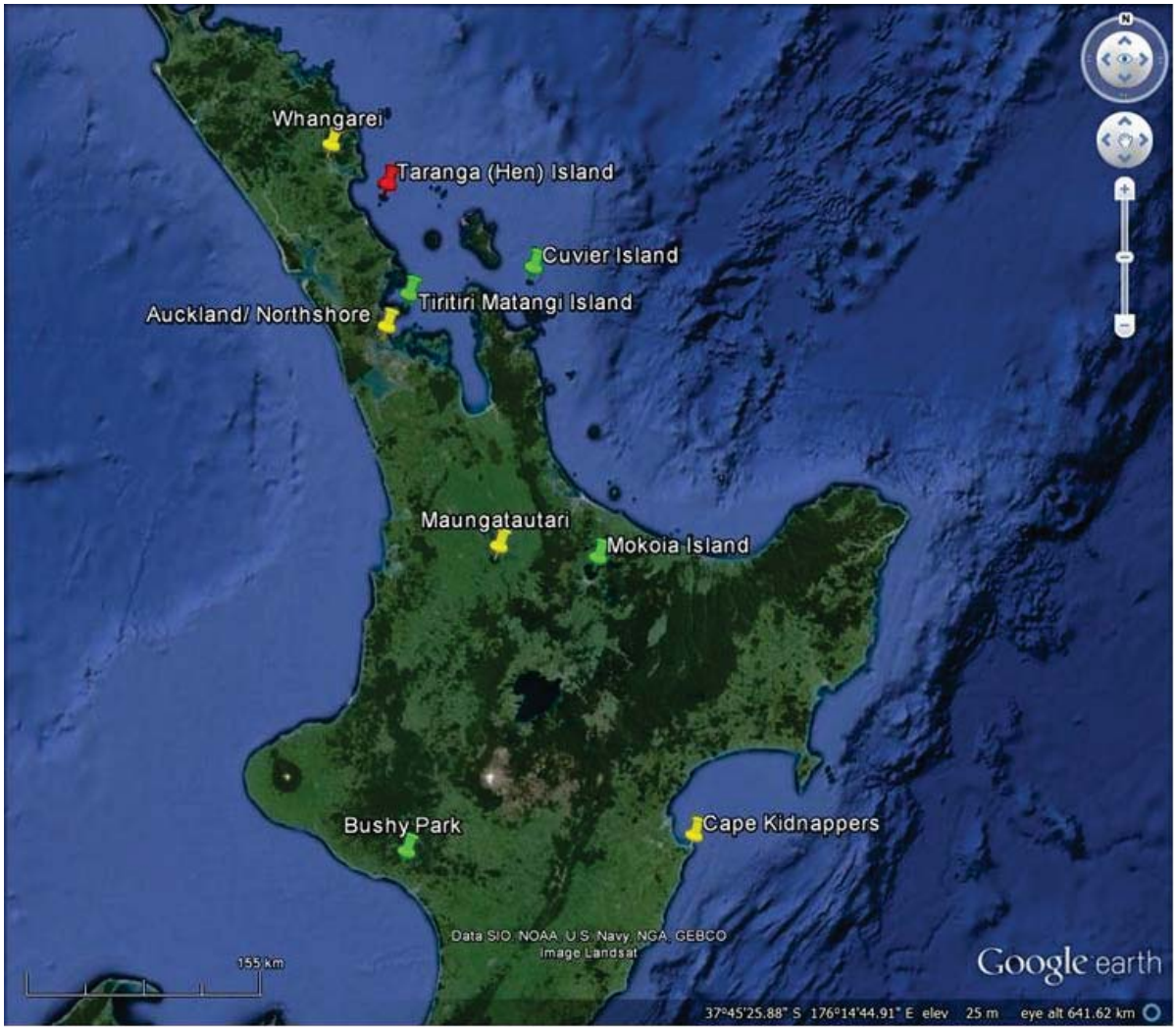
5. The number of parasite lineages is influenced by size of the examined island, therefore it is smaller on small offshore islands than on the mainland, according to island biogeography which states that the biodiversity in small areas is smaller than on bigger ones (islands vs. mainland) (MacArthur and Wilson, 1967).

In examining the predictions, care should be taken because of confounding factors. Factors influencing parasite diversity other than those in the above predictions include the possibility that migratory birds have brought parasites with them (Krizanaskiene *et al.*, 2006, Levin *et al.*, 2013), that parasite diversity positively correlates with host diversity (Valkinuas, 2005) and also the effect of latitude and

climate, since diversity is much higher in tropical “hotspot” areas (e.g. India, Australia, Southeast Asia) than in temperate regions (Clark *et al.*, 2014).

To address these predictions, I collected blood samples from NI saddlebacks as well as introduced *Turdus* species in one translocation sequence, Hen Island (initial source population),Cuvier Island, Tiritiri Matangi Island, Mokoia Island,Bushy Park; Figure 5.1). I also examined introduced birds at four different mainland sites, Cape Kidnappers, Maungatautari, Auckland, Northshore and Whangarei (Figure 5.2).

Figure 5.2: Map of the sampling sites visited during this study; Hen Island, origin of the source population of NI saddleback, has been marked in red, subsequent translocation sites in green and mainland sites (for introduced birds) in yellow



5.3 Material and methods

5.3.1 Capture and handling of the birds

Ideally, to test the hypotheses for this study, I needed to be able to examine the prevalence and lineages of *Plasmodium* present at each site before and after the translocations of saddlebacks and before other introduced birds were present. However, this was not possible as blood sampling has only recently become part of health screening for translocations and then only the birds that are being translocated are tested to detect disease that may affect the success of the translocation. Therefore, in the absence of such data we used samples from various origins and times to try to build the best picture possible (Figure 5.2). During the period 2012-2013, nine sampling trips were made during summer to catch saddleback as well as blackbirds and song thrushes (*Turdus philomelos*) from sites

of one saddleback translocation sequence (Hen Island- Cuvier Island-Tiritiri Matangi Island-Mokoia Island-Bushy Park). In addition, archived saddleback blood samples (collected in 2006/2007 for a previous project) from the target study sites in the translocation sequence described above were also examined. As a proxy of which *Plasmodium* were present before saddleback translocation, I tested bloods from three sources: (1) 19 birds (blackbirds, Australian magpies (*Cracticus tibicen*), silvereyes (*Zosterops lateralis*) and Eastern rosellas (*Platycercus eximius*)) at Maungatautari in 2007 prior to the translocation of saddleback in 2013; (2) *Turdus* spp. from Northshore, Auckland and Whangarei, two areas across the water from two of the saddleback translocation islands; and (3) Nine introduced finches and one dunnock (*Prunella modularis*) from Cape Kidnappers (saddlebacks were translocated there in 2013).

The birds were caught in mist nets using recordings of saddleback song and blackbird alarm calls as a lure. The bags for restraining the birds were used only once on each side and washed, disinfected and dried at the end of each day. The birds were carefully placed into these bags by hand. Hands were disinfected after handling each bird, to reduce the spread of transmissible diseases. Each bird was given a metal band for individual identification and to prevent resampling.

The health status of every bird was assessed by evaluating the body condition, looking for wounds and/or other lesions as well as colour of mucous membranes (eyes and mouth) and observing the behaviour and responsiveness of the bird in the hand. Unhealthy birds or those showing unusual stress were released without further manipulation. The birds were restrained using either blood-sampling cone or the hand. Blood samples were taken by venepuncture of the brachial vein directly into capillary tubes. The area of puncture was cleaned with 70% ethanol prior to pricking and pressure was applied to the puncture following blood extraction to stop the flow of blood. One drop of blood was used to prepare blood smears, and the remaining blood was placed into sampling tubes containing heparin. On Hen Island, where no cooling facilities were available, blood was collected in plastic microcentrifuge tubes containing 98% ethanol.

Saddlebacks, which are known to consume nectar, were given freshly prepared sugar water after handling and prior to release. Birds that were recaptured were

examined for health status as described above and released without further manipulation.

5.3.2 Preparation of blood smears

In this study, the blood smears were air-dried and fixed in absolute methanol at the end of the day of work in the field and stained after the field trip back at Massey University (Palmerston North, New Zealand) with Giemsa stain (diluted 1:10 for 45-60mins) (Applichem). Of the 230 blood smears collected, 179 were suitable for examination, as some were of poor quality as they were made in dusty or bad weather conditions. These were used to examine for infection status, parasite developmental stages, and morphologically identify any *Plasmodium* spp. present.

5.3.3 DNA extraction

DNA was extracted from blood samples using the DNeasy Blood and Tissue kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions for nucleated blood.

5.3.4 PCR and sequencing

For amplifying *Plasmodium* spp. DNA, each DNA sample was subjected to nested PCR amplifying a 480 base pair part of the cytochrome b-gene, described by (Hellgren *et al.*, 2004) without modification. After PCR, all the PCR products were run on a 1% (w/v) ultra-pure agarose gel (Invitrogen, California, USA) containing ethidium bromide and visualized under UV light on a transilluminator. All positive PCR amplicon samples were purified (PureLink PCR purification kit, Invitrogen, California, USA) and subjected to automatic dye-terminator cycle sequencing with BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc, California, USA) to confirm genomic sequence. The electropherograms resulting from sequencing were also checked for double nucleotide peaks to infer possible cases of mixed infections of two or more different parasite lineages.

5.3.5 Phylogenetics

Analysis of *Plasmodium* sequences of 410 base pairs obtained from saddlebacks, blackbirds and thrushes were compared to those of other published sequences available from Genbank and the MalAvi database (Bensch *et al.*, 2009). All sequences were trimmed to the same length using Geneious^{TM} (Biomatters,

Auckland, New Zealand) and aligned using Clustal W (Higgins et al. 1994). A phylogenetic tree was generated in MrBayes version 3.2 (Ronquist et al. 2011) using Bayesian phylogenetics. A general time-reversible model including invariable sites (GTR+I) was used. The Bayesian phylogeny was obtained using one cold and three hot Monte Carlo Markov chains, which were sampled every 1,000 generations over 5 million generations; 5,000 trees were generated. Of these trees, 25% were discarded as burn-in material. The remaining 3,750 trees were used to construct a majority consensus tree. The sequence divergence between and within the different lineages was calculated using a Jukes-Cantor model of substitution implemented by the program PAUP* 4.0 Beta version 10 (Swofford 2002).

5.3.5 Mixed infections

Samples that were positive using conventional PCR were re-examined using a newly developed two step qPCR/HRM protocol using the primer sets HRMF (Njabo et al. 2011) and HaemR2 (Hellgren *et al*., 2004) and Plas56F/PlasrevRT as described in Chapter 4. This method was able to discern between the most common *Plasmodium* lineages in New Zealand (*P. elongatum* GRW6, Linn1 and *P. vaughani* SYAT05) as well as *P. relictum* GRW4 and also mixed infections. In addition, sequencing results were examined for double peaks on the electropherogram which is an indication of mixed infection.

5.3.6 Statistical analysis

Linear regression was used to examine whether the number of lineages or the island/site size were good predictors of *Plasmodium* prevalence. Chi square was used to look for significant differences in infection in saddlebacks across the sites. Probability values ≤ 0.05 were considered significant. Tests were carried out in IBM SPSS statistics 23.

5.4 Results

Of 318 blood samples from NI saddlebacks and introduced birds, 149 (39.3%) were positive for *Plasmodium* spp. by PCR (Table 5.2). Ten distinct lineages of *Plasmodium* were found (Figure 5.6). Of the 179 useable blood smears, 114 (63.7%) were confirmed positives. On *Plasmodium* positive blood smears, I noted an increased incidence (up to 50%) of immature erythrocytes, although I did not

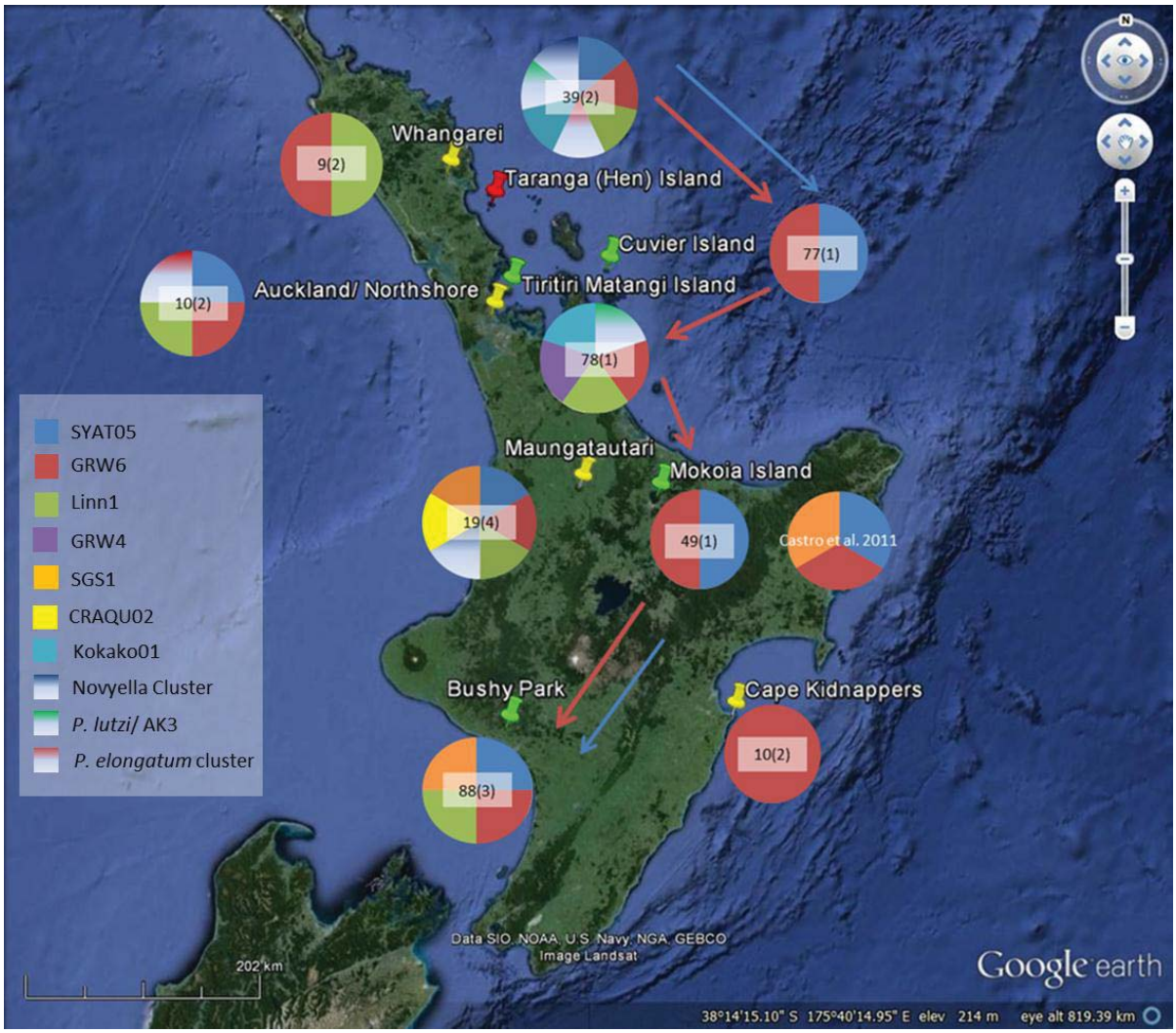
evaluate this finding in more detail. All positive birds showed low levels of parasitaemia by smear, and none of the birds caught during the sampling trips showed signs of ill health or were in poor condition. All results for this study are included in Appendix 6 as raw data tables.

Not all of the PCR-positive samples had corresponding useable blood smears, and on three birds, no PCR could be performed, because not enough blood had been collected. Of the 92 PCR positives with a corresponding blood smear, 83 (90.2%) were confirmed positives by microscopy. Nine PCR positives were negative on the blood smears (9.8%). 28 (24%) birds were positive in microscopy, but negative by PCR. Identification to species level could only be performed on the blood smears in a few cases (n= 31/114) where all life cycle stages were present. The most common species found were of the *Plasmodium* subgenus *Novyella* (n=17), where both *P. rouxi* (n=1) (Figure 5.4b) and *P. vaughani* (n=16) (Figure 5.4a) were found. *P. vaughani* comprises the genetic lineage SYAT05 (Zehtindjiev *et al.*, 2012, MalAvi Database). The second most common was *P. elongatum* (n=11) (Figure 5.4c+d) followed by *P. relictum* (n=7) (Figure 5.4e; Table 5.1). Four of those positives were mixes of *P. relictum* and *P. elongatum/rouxi*.

Table 5.1: Examined blood smears

Site	**Hen Island**	**Cuvier Island**	**Tiritiri Matangi Island**	**Bushy Park**	**Cape Kidnappers**	**Auckland and Whangarei**
No. of slides	29	42	42	88	10	19
No. useable slides	29	22	22	77	10	19
Positive	29 (100%)	15 (68.2%)	16 (72.8%)	34 (44.2%)	2 (20%)	18 (94.7%)
Identified species	4x *P. (novyella) vaughani, 3x P. elongatum, 1x P. relictum*		2x *P. elongatum*	1x *P. relictum*, 1x *P.elongatum*, 4x *P. (novyella) vaughani*	1x *P. elongatum*	5x *P. relictum*, 4x *P. elongatum*, 8x *P. (novyella) vaughani*, 1x *P. (novyella) rouxi;* 4 of those positives were mixes of *P. relictum* and *P. elongatum/rouxi*

Figure 5.3: Map of the North Island of New Zealand with sampling sites visited during this study; Hen Island, origin of the source population of NI saddleback, has been marked in red, subsequent translocation sites in green and mainland sites (for introduced birds) in yellow. The pie charts present the number of different *Plasmodium* lineages found at a given site each represented by a different colour. Arrows indicate the direction of the translocations and the arrow colour corresponds to the *Plasmodium* lineage (s) that was (were) translocated. Red = GRW6, Blue = SYAT05, Green = LINN1, Turquoise = KOKAKO01; Orange = SGS1, Purple = GRW4, Yellow = CRAQU02, Gradient blue = *Novyella* Cluster, Gradient green = *P. lutzi*/AK3, Gradient red = *P. elongatum* cluster. The numbers in the centre of the pie charts represent the number of individual birds sampled and the number of bird species in brackets.



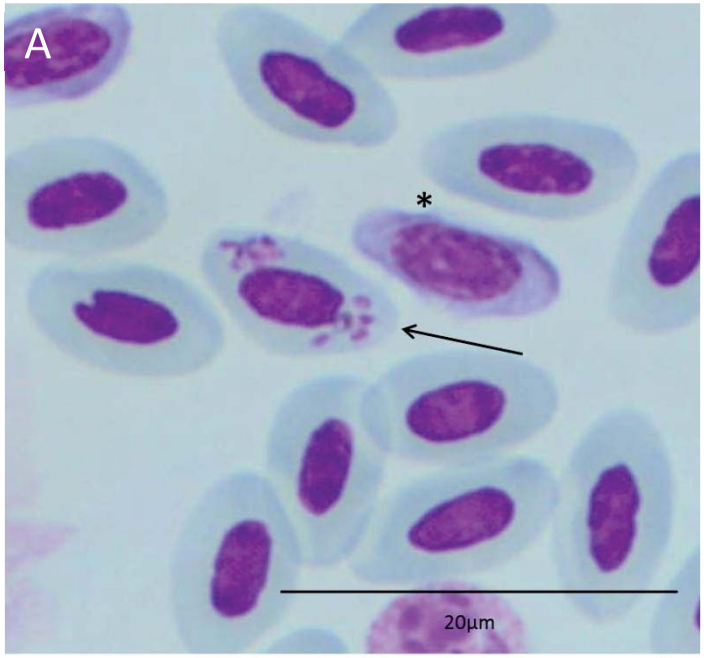


Figure 5.4a: *P. vaughani* meronts (arrow) in a blackbird showing only four merozoites (stained purple) and a residual body with a small vacuole; note immature erythrocyte (*)

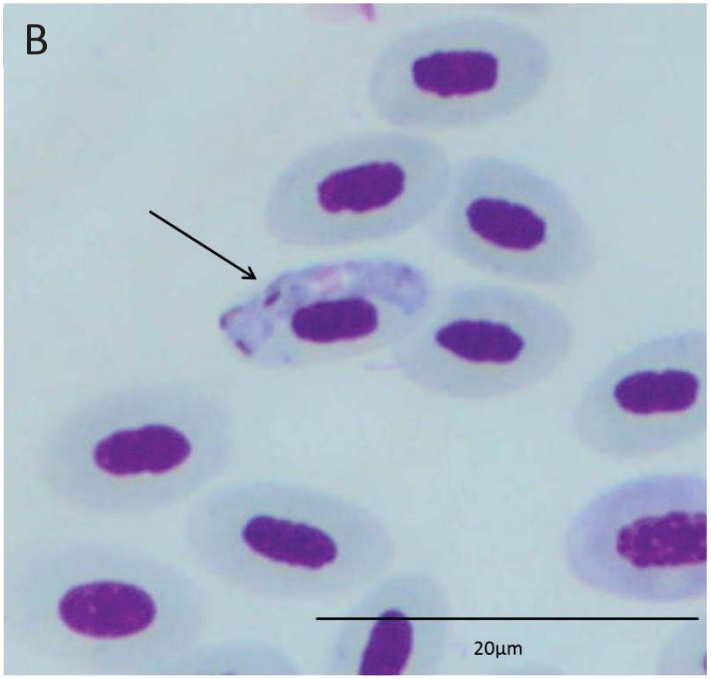


Figure 5.4b: *P. rouxi* gametocyte in a blackbird with the typical large pigment granules (arrow)

Figure 5.4c: *P. relictum* gametocyte (arrow) in a blackbird, showing round shape displacing the host cell nucleus

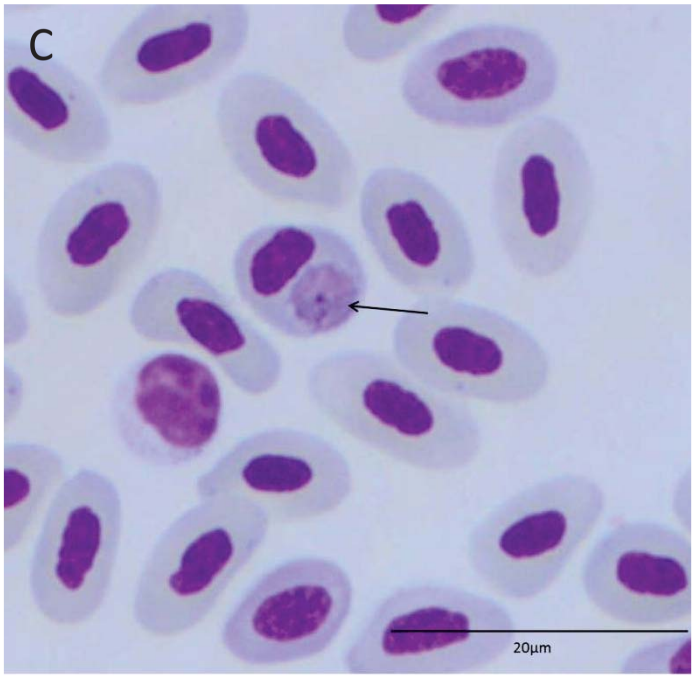
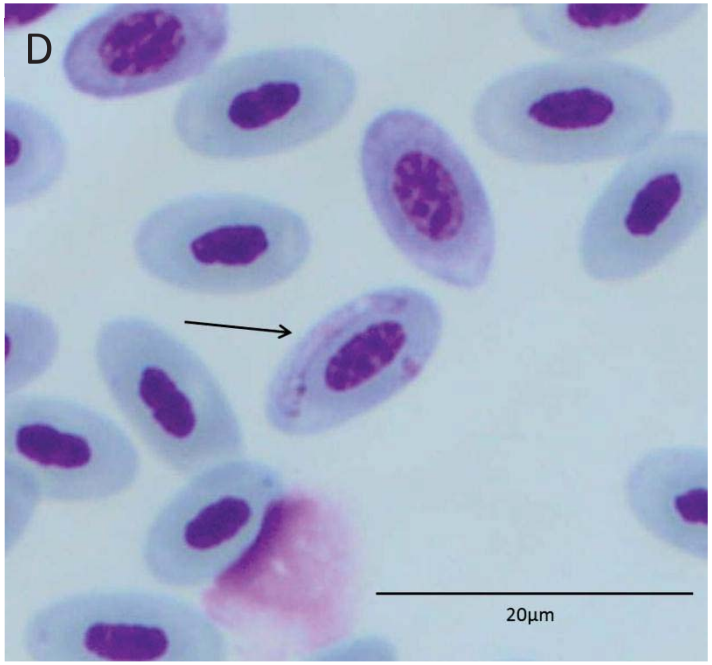


Figure 5.4d: *P. elongatum* gametocyte in a blackbird (arrow) with the typical elongated slender shape



5.4.1 Prevalence

There were significant differences in prevalence between the examined sites (Chi square = 51.8, df = 4; p ≤0.0001). The highest observed prevalence of infection in saddlebacks, 76.3% (n=38), was found in the source population (Hen Island). This prevalence included both archived (2006) and fresh samples from 2012; a higher prevalence of 85.2% (n=27) was observed when only the samples from 2012 were considered. The lowest prevalence was found in the samples from Mokoia Island, with 14.3% (n= 49). The prevalence in the samples I took from introduced blackbirds and song thrushes in Auckland and Whangarei, Northland was 100% in Whangarei (n=9) and 90% in Auckland (n=10) (Fig. 5.5 a-c). For the islands, both the number of lineages (linear regression F = 18.1; df = 3; p = 0.05) and island size (F = 205.9; df = 3; p = 0.005) were good predictor of prevalence.

Mainland sites appear to have a higher *Plasmodium* prevalence when compared to islands (Figure 5.5a), although this was not tested statistically because unfortunately we caught very few hosts species and did not catch the same numbers and hosts species at each site. When prevalence and number of lineages are plotted against the time since translocation (Figure 5.5b), no relationship is apparent.

Figure 5.5a: Comparison of number of lineages (blue diamonds) and parasite prevalence (red bars) in saddlebacks and introduced birds on both mainland and offshore island sampling sites. Offshore islands are arranged by size, from small to large (x-axis).

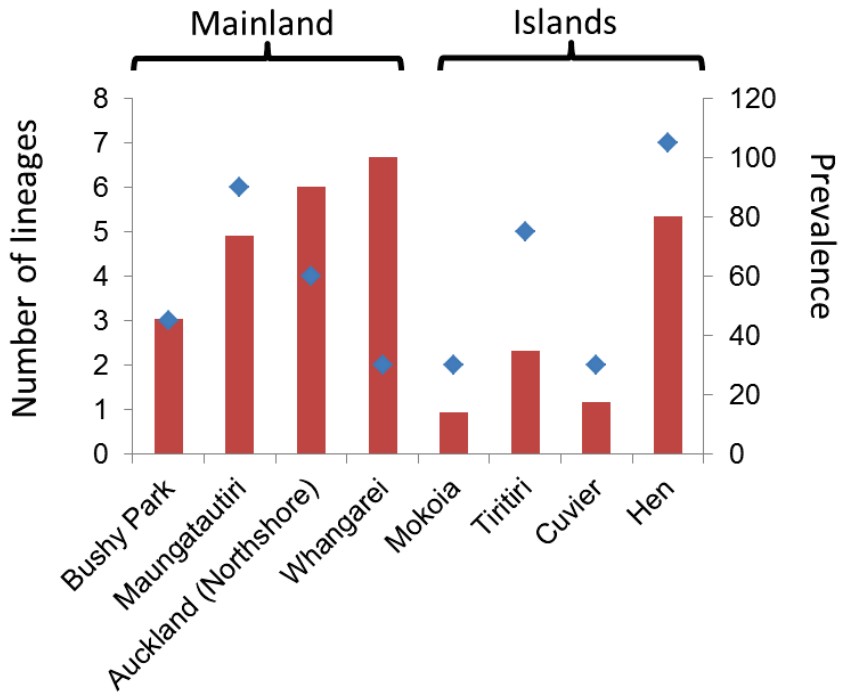


Figure 5.5b: Comparison number of years since translocation (green bars) and parasite prevalence (red squares). The sampling sites are arranged by number of bird species sampled: 1) only one species (North Island saddleback) was sampled, 2) two species were sampled 3) three species were sampled, 4) four species were sampled.

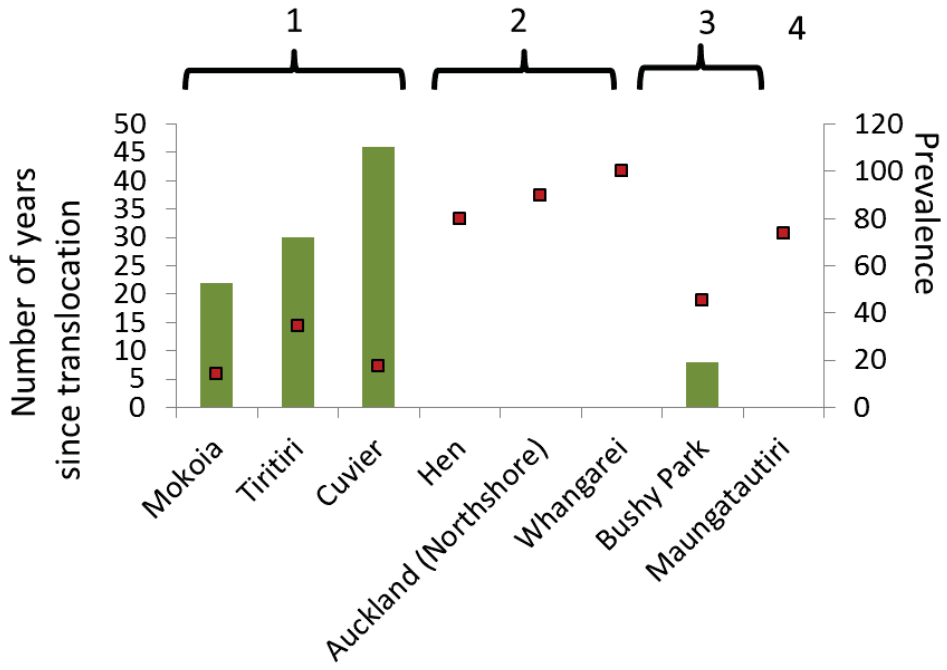
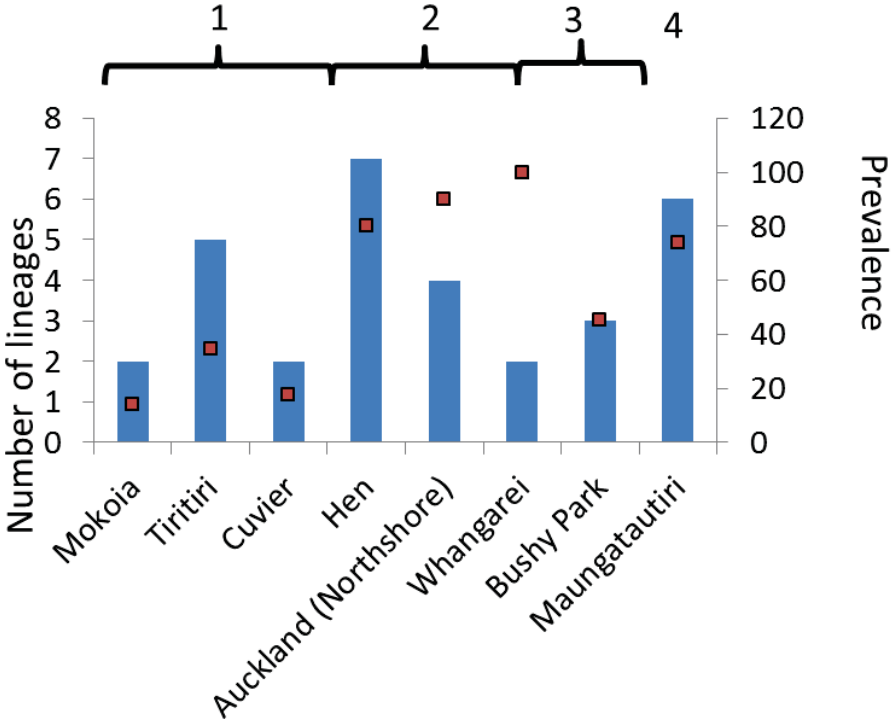


Figure 5.5c: Comparison of number of lineages (blue bars) and prevalence (red squares). The sampling sites are arranged by number of bird species sampled: 1) only one species (North Island saddleback) was sampled, 2) two species were sampled 3) three species were sampled, 4) four species were sampled.



5.4.2 Phylogenetic analysis

Of 149 positive samples, 134 yielded enough DNA to perform sequencing and of those 123 (82.6%) delivered conclusive sequencing results. The most common *Plasmodium* lineages found were the cosmopolitan *P. elongatum* (GRW6) with 46.96% of samples testing positive for this lineage and *Plasmodium* subgenus *Novyella* (SYAT05) with 28.7%. Another common lineage was of *Plasmodium elongatum* (LINN1) with 12.17%. Two lineages previously unknown to New Zealand were also found.

The BLAST search revealed that one isolate, “3406”, had a 96% sequence identity to the common *Novyella* lineage “SYAT05” and the related isolate “204289” showed 98% sequence identity to the same lineage. Two isolates, “204239 Hen” and “204236 Hen” had a sequence identity of 98% with the common *P. elongatum* “GRW6”.

Three isolates, “Hen 232”, “Tiri 5” and “Tiri 21” have a 99% sequence identity with two unspecified *Plasmodium* lineages described both from Alaska, AK3 (JQ026526) and Colombia (KF537312), which are lineages formerly unknown in New Zealand. Two magpies from Maungatautari (Maunga 12+13) had a 98% sequence identity with the *Plasmodium sp*. lineage CRAQU02 (Genebank JQ905579), previously found in a black butcherbird (*Cracticus quoyi*) from Northern Australia.

A phylogenetic tree constructed using Bayesian analysis shows 30 sequences from this study compared with *Plasmodium* lineages known in New Zealand and around the world (Figure 5.6). This resulted in the isolates “3406 Auck” and “204289 Hen” clustering with members of the common SYAT05 lineage. “3406 Auck” had a sequence divergence of 3.9% from SYAT05, while “204289 Hen” showed a sequence divergence of 2.2%. The isolates “204239 Hen” and “204236 Hen” clustered with *P. elongatum* GRW6, with a sequence divergence of 2.2% for both. The isolates “Hen 232”, “Tiri 5” and “Tiri 21” clustered with two unspecified *Plasmodium* lineages from Alaska (“AK3”/Genebank JQ026526) and Colombia (*P. lutzi* KC138226). While both “Tiri 5” and “Tiri 21” had a sequence divergence of 0.7 % from AK3, “204232 Hen” had 1.2%. These isolates are therefore very closely related to each other. Isolates “Maunga 12” and “Maunga 13” from two Magpies at Maungatautari showed a sequence divergence from lineage CRAQU02 of 2%.

The highest number of lineages was found in the source population of saddlebacks on Hen Island (7), followed by Tiritiri Matangi Island (5) and Bushy Park (4). Cuvier Island and Mokoia Island had the lowest diversity with only two different lineages found (Table 2; Figure 3 and Figure 5a). The examined mainland sites (Mokoia Island, Auckland, Whangarei, Bushy Park and Cape Kidnappers) had lower *Plasmodium* diversity than Hen and Tiritiri Matangi Island, with four lineages at Auckland, three at Bushy Park, two for both Whangarei and Mokoia Island and one at Cape Kidnappers. High lineage diversity was also found at Maungatautari (6) (Figure 5.3).

5.4 3 Mixed infections

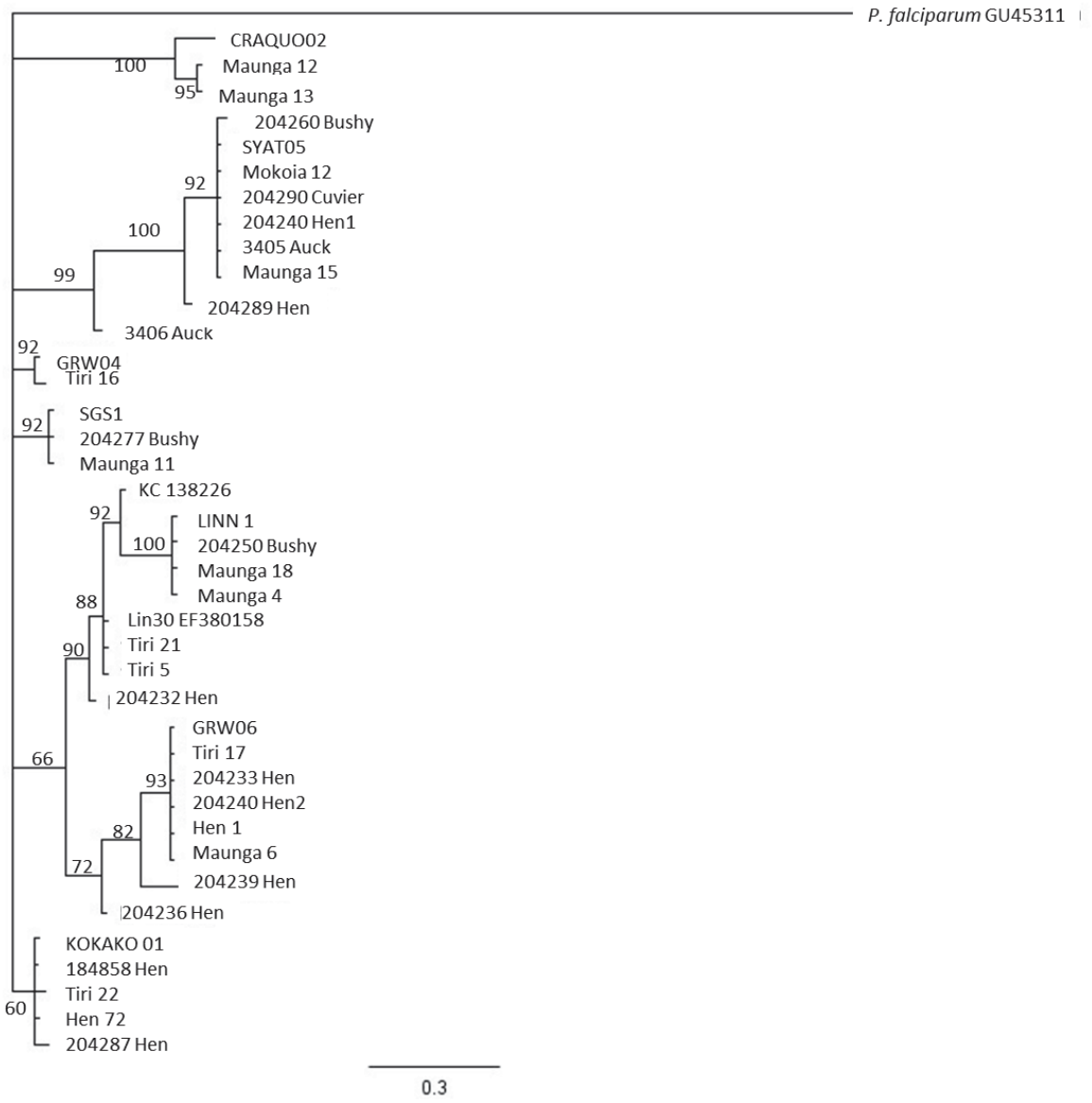
I found evidence of mixed infections in 59 (39.6%) of all positive samples (n=149). Thirty-six of these mixed infections were detected after sequencing as they formed different disassociation curves on the electropherograms. The qPCR (Chapter 4) detected 54 (36.2%) *Plasmodium* positives with 24 (16.1%) showing mixed infections. Only four samples showed mixed infections in both qPCR and on the electropherogram. These mixed infections involved the most common *Plasmodium* lineages in New Zealand, *P. elongatum* GRW6, Linn1 and SYAT05 in all combinations. Mixed infections were also seen in the collected blood smears (Table 5.1).

Of the 106 saddlebacks tested positive for *Plasmodium* spp., 37 (34.9%) carried mixed infections. In introduced birds, 19 (44.2%) of 43 positives carried mixed infections.

5.4 4 Conversion of infection

The samples taken from Bushy Park were collected on two occasions in two consecutive years. Seven saddlebacks were re-caught after one year, with four birds positive for *Plasmodium* by PCR in 2012 and three birds in 2013. One bird converted from positive to negative, one from negative to positive and two birds were positive in both years

Figure 5.6: Phylogenetic analysis and comparison of 30 *Plasmodium* isolates from North Island saddleback, blackbirds and Australian magpies and previously published *Plasmodium* sequences. Bayesian and Neighbour-joining (NJ) phylogeny of mitochondrial cytochrome *b* gene from *Plasmodium falciparum* was used as an out-group. Names of the lineages, as listed on the MalAvi database, and Genbank accession numbers of the sequences are given. The branch lengths are drawn proportionally to the amount of changes (scale bar is shown).



	Hen Island	Whangarei (near Hen)	Cuvier Island	Tiritiri Matangi Island	Auckland (near Tiri)	Mokoia Island	Bushy Park	Cape Kidnappers	Maungatautari	Total
Saddlebacks	38	-	77	78	-	49	76	-	-	**318**
Infections in saddlebacks	29 (76.3%)	-	12 (15.6%)	29 (37.2%)	-	7 (14.3%)	29 (38.2%)	-	-	**106 (33.3%)**
Number of different lineages found in saddleback	7	-	2	5	-	2	3	-	-	
Birds total	39	9	77	78	10	49	88	10	19	**379**
Infections total (PCR)	30 (76.9%)	9 (100%)	12 (15.6%)	27 (34.6%)	9 (90%)	7 (14.3%)	40 (52.6%)	1 (10%)	14 (73.7%)	**149 (39.3%)**
Number of conclusive Sequences	29	9	7	23	8	2	24	1	12	**115 (75.7%)**
Number of different lineages found in all examined bird species	7	2	2	5	4	2	4	1	6	
SYAT05	8	8	1	-	4	1	9	-	2	**33 (28.7%)**
Other within *Novyella* cluster	1	-	-	-	-	-	-	-	2	**3 (2.6%)**
***P. elongatum* GRW6**	13	-	6	18	1	2; also in a dead blackbird	8	1	5	**54 (47%)**
Other within *P. elongatum* cluster	1	-	-	-	1	-	-	-		**1 (0.9%)**
***Plasmodium* spp. (LINN1)**	1	1	-	1	2	-	6 (LINN1)	-	3	**14 (12.2%)**

Other (within *Plasmodium* spp. cluster)	1	-	-	2	-	-	-	-		**3 (2.6%)**
***Relictum* cluster**	-	-	-	1 (GRW4)	-	-	1 (SGS1)	-	2 (1x SGS1)	**4 (3.5%)**
Other within *P. relictum* cluster	-	-	-	-	-	-	-	-		**1 (0.9%)**
KOKAKO 01 (in *P. relictum* cluster)	4	-	-	1	-	-	-	-		**5 (4.4%)**
Mixed Infections	12	6	4	9	5	4	12	-	7	**59 (24 with new method/ 36 by sequencing) (39.6%)**

Table 5.2: Results from blood sampling trips to the saddleback translocation sites from the sequence Hen Island-Cuvier Island-Tiritiri Matangi Island-Mokoia Island-Bushy Park-Cape Kidnappers as well as Maungatautari and two mainland sites (Whangarei and Auckland) close to the islands all located in the North Island of New Zealand. The two 'near' sites were used to compare prevalence and *Plasmodium* diversity of introduced birds with close-by saddleback populations.

5.5 Discussion

5.5.1 Lineage diversity, prevalence and mixed infections

There were statistically significant differences in lineage diversity and parasite prevalence between the different sites. Previous studies in the Lesser Antilles (Apanius *et al*., 2000, Fallon *et al*., 2003a, Ricklefs *et al*., 2011) have shown that while there is a significant difference in haemosporidian parasite diversity between different species, the parasite prevalence did not vary significantly between different islands. While these results from the Lesser Antilles might appear applicable to my findings, they are difficult to compare with the situation in New Zealand. The host-parasites relationships in the Lesser Antilles have evolved over a long period of time on larger islands, while the translocation history of endangered bird species on small offshore islands in New Zealand has only been performed for the last few decades. I was also examining a much smaller range of bird host species.

As predicted, the most common *Plasmodium* lineages found in this study were the cosmopolitan species *P. elongatum* with two lineages, GRW6 and Linn1 and *Plasmodium* (*Novyella*) *vaughani*, SYAT05 (Baillie *et al*., 2011, Howe *et al.*, 2012, Ewen *et al.*, 2012b). Previous studies in saddlebacks have found especially *P. elongatum* (GRW6 and LINN1) (Castro *et al*., 2011,Howe *et al*., 2012) and in a previous study on Mokoia Island (Castro *et al*., 2011), two additional lineages (*P. relictum* SGS1 and a lineage from a *Culex* mosquito (GQ471953)) were found in saddlebacks. These findings indicated that the *Plasmodium* diversity varies by season and year of sampling. My study reports for the first time *Plasmodium sp*. (SYAT05) in NI saddlebacks. The detection of these lineages in saddlebacks is interesting, because *Plasmodium* species of the subgenera *Novyella* (SYAT05) and *Huffia* (*P. elongatum* GRW6) appear to have a more limited host spectrum than *Haemamoeba* (e.g. *P. relictum* SGS1) (Dimitrov *et al.*, 2015), and SYAT05 has been found mainly in blackbirds in New Zealand (Schoener *et al*., 2014, Sijbranda *et al*., 2016).

The finding of these cosmopolitan *Plasmodium* spp. lineages begs the question of whether generalist cosmopolitan parasites are indeed dominating the New Zealand diversity, or whether true diversity is not detected by standard methods. There is a discrepancy between the *Plasmodium* parasite community seen in Europe and

introduced European birds in New Zealand. In Europe, the lineage *P. relictum* SGS1 is the most prevalent in sampled passerine birds (Dimitrov *et al*., 2015), however in New Zealand (Castro *et al*., 2011) it appears to exist in only low prevalence. During examination of my blood smears, I found a higher incidence of *P. relictum*, possibly SGS1 (Table 5.1), than was detected with PCR. None of the *P. relictum* found on smears from Hen Island and Auckland and Whangarei were detected by PCR. One possible explanation for the differences in findings between the blood smears and PCR is that when SGS1 is present in mixed infections the other parasite is preferentially amplified using standard nested PCR. This problem has been recognised in avian malaria research in the past years as PCR sometimes fails to amplify DNA of a clearly visible and even predominant parasite in blood samples (Zehtindjiev *et al.*, 2012). This might also explain why Sijbranda et al. (2016) did not find any *P. relictum* in their study of introduced and native bird species in New Zealand, using only PCR methods. The low prevalence of this common lineage could also be explained if SGS1 was not introduced in large numbers to the Pacific area or if it is not as well adapted to this region either because of resistance in the birds or because of unsuitable vector species. These hypotheses are not mutually exclusive. Indeed, SGS1 has been found in Australia, but has been so far absent from the Pacific Islands (MalAvi Database: http://mbio-serv2.mbioekol.lu.se/Malavi/) suggesting a possible synergy between the various hypotheses.

Similarly, on the blood smears both *P. vaughani* and *P. rouxi* of the subgenus *Novyella* can be seen, but only the lineage SYAT05 (*P. vaughani*) could be detected by PCR. The most likely explanation is that most of the *Plasmodium* infections seen are mixed infections and one of the parasites gets preferentially amplified by the PCR. Therefore, microscopy remains essential for avian malaria studies, and needs to be used in concert with molecular methods, to ensure that the true *Plasmodium* diversity is not overlooked. Based on my findings, it is very likely that during this and other studies previously performed in New Zealand, that the true *Plasmodium* diversity was underestimated.

Among the less common lineages found in this study, I amplified a *P. relictum*, a lineage from the subgenus *Novyella* and a *P. elongatum* lineage that were different to the established published ones and are potentially new. I also was able to identify

two lineages formerly unknown to New Zealand, one of which with its closest relatives found in passerine birds in Colombia (*P. lutzi* KC138226 (Mantilla *et al.*, 2013) and Alaska (AK3 JQ026526 (Loiseau *et al.*, 2012) and the other, CRAQU02, in an Australian magpie, with the closest relative found in a black butcherbird from Australia (JQ905579) (Ewen *et al*., 2012b). The lineage CRAQU02 was most likely brought to New Zealand by the Australian magpies that were introduced by the Acclimatisation Society in the 1860s. This lineage has so far not been found in birds other than the two magpies and the ramifications of this introduction are unknown.

The KOKAKO01 lineage found in saddleback on Hen Island (n=4) as well as Tiritiri Matangi Island (n=1) has previously only been identified in a closely related bird species, the kokako (*Callaeas cinerea*) (2, Ewen *et al*. 2012b). It is possible that the KOKAKO01 lineage is native to New Zealand and a parasite of the wattlebird-family (Callaeidae) which includes saddleback and kokako as well as the extinct huia (*Heteralocha acutirostris*). To date, only two kokako, one each from Tiritiri Matangi Island (Ewen *et al*., 2012b) and Boundary Stream Mainland Island, Hawkes Bay (Schoener *et al.*, 2014), have been reported to carry this parasite. Thus, this finding may suggest introduction of this parasite occured as saddlebacks from Hen Island were translocated to Tiritiri Matangi Island via Cuvier Island prior to kokako being translocated to Tiritiri Matangi Island. Another possibility is that the parasite was introduced to Tiritiri Matangi Island when kokako were translocated there.

Factors such as: number of hosts translocated and established; area size; number of reservoir host species; vector habitat and presence; time since translocation and parasite species composition are all expected to affect the final numbers of parasite groups present at a site and may explain some of my results. In general, the number of parasite lineages on small offshore islands was smaller than on the mainland sites, with the exception of the source population on Hen Island (Figure 3). This may confirm that biodiversity on small islands is smaller than on bigger ones (mainland); even if parasites manage to be transferred to a new (small) island, the diversity of vectors and small bird population sizes will limit the number of species that can be supported.

Tiritiri Matangi Island had a greater diversity of *Plasmodium* than was expected being a translocation site and being smaller than Hen Island. However, this Island is

a restoration site and has received many birds of 18 new species which have been translocated there in the past 40 years from several locations, and it is likely that these bird species have contributed to the greater diversity of *Plasmodium* found there. To a lesser degree, this might also be true for Bushy Park. Bushy Park was the only mainland reserve site in the examined translocation sequence and the NI saddleback there may have acquired parasites via spillover from introduced birds living in the surrounding pastoral land. The likelihood of contact of saddleback with introduced birds is a likely factor influencing the diversity of *Plasmodium* parasites at a site. The two sites with the lowest diversity of parasites, Cuvier and Mokoia Island, also had a low presence of introduced *Turdus* species during the time of sampling. In the case of Cuvier Island, the remoteness and distance from the mainland is most likely a contributing factor, while the low number of blackbirds on Mokoia Island might be connected to the ongoing replacement of remains of open grassy areas that blackbirds favoured, with regenerating native bush. It is also possible that the density of introduced *Turdus* species has decreased on these islands following the establishment of saddlebacks (Diamond and Veitch, 1981), which use a similar niche, therefore displacing the most important *Plasmodium* reservoir species. Another likely influence is the availability of habitat for vectors. Mokoia Island for example, although surrounded by fresh water, is very dry, and lacks appropriate habitat for mosquito larvae. Supporting this lack of habitat, in my study on mosquito vectors (Chapter 6), I did not collect any mosquitoes in the very dry summer of 2013/14, although some were caught the following year (Chapter 6). Ricklefs et al. (2011) remarked that on one of their studied islands in the Lesser Antilles, *Plasmodium* was relatively less common which most likely was due to the absence of suitable vectors, although it was not possible to draw definite conclusions without more detailed information on vector abundance and feeding patterns. Higher lineage diversity was also found at Maungatautari (6 lineages) which was most likely due to more bird species being examined there; it is also a mainland site, and the biggest conservation site by area included in this study, characteristics which are also associated to higher lineage numbers (Figure 5.3).

There are two further explanations for the differences in lineage diversity between sites which should be considered. All samples were collected with heparinised capillary tubes, but the samples on Hen Island, where the highest diversity was

found, were stored in 98% ethanol, not in tubes containing heparin, which has been shown to be a PCR inhibitor (Beutler *et al.*, 1990). It is possible that a higher diversity could have been found at the other sites if ethanol had been used instead as a storage medium. Another possible explanation for diversity differences is a North-South gradient. *Plasmodium* diversity is generally higher in tropical "hotspot" areas (e.g. India, Australia, Southeast Asia) than in temperate regions (Clark *et al*., 2014). This correlates to environmental factors such as vector presence, which diminishes in New Zealand from North to South (Tompkins and Gleeson, 2006).

In general, this study supports the finding by Hellgren et al. (2009) that parasites with the ability to successfully infect a wide variety of bird host species (like *P. elongatum*) also have the ability to be the most prevalent in a single host species. The high prevalence of *Plasmodium* infection in saddleback in the populations on Hen Island (76.9%), Bushy Park (52.6%) and Tiritiri Matangi Island (34.6%) found in this study corresponded with my prediction that the prevalence is similar to other areas like Europe and the Americas (Evans *et al.*, 2009, Beadell *et al.*, 2004, Valkiūnas, 2005). Previously, malaria prevalence in New Zealand native birds was thought to be low (Baillie *et al*., 2011, Castro *et al*., 2011, Howe *et al.*, 2012, Ewen *et al.*, 2012b). Sijbranda et al. (2016) reported a *Plasmodium* spp. prevalence for introduced bird species of 80.5%, for native species of 19% and for endemic New Zealand bird species of 3.5%. A previous study in saddleback on Mokoia Island found a low prevalence of up to 10.6% depending on the season (Castro *et al*., 2011) and a similar study on bellbirds (*Anthornis melanura*) found a prevalence of 13.4% (Baillie *et al*., 2011). A high prevalence in the source population at Hen Island might indeed be evidence for a long co-evolutionary history of the examined birds with these parasites. The prevalence of *Plasmodium* in introduced *Turdus* species is equal to records in Europe, with up to 100% in Whangarei (Table 2; Bentz *et al.*, 2006). The differences in parasite prevalence most likely are connected to vector abundance, which at small geographical and ecological scales (like the islands examined in this study) appears to cause variation in prevalence regardless of the bird host species (Scheuerlein and Ricklefs 2004).

I also found mixed infections in my samples, finding a total of 50 (32.9%) birds infected by more than one *Plasmodium* lineage when using both electropherograms and a newly developed qPCR method (Chapter 4) as well as blood smears. This

finding can only be an estimate, due to the apparent lower sensitivity of the newly developed qPCR compared to the standard nested PCR. Finding mixed infections using standard nested PCR and sequencing results is unreliable and greatly underestimates mixed infections, which are common in the wild, because they may preferentially amplify the DNA of one parasite over another (Valkiūnas *et al*., 2006, 2014). Compared to the prevalence indicated by this study, the prevalence of mixed infections in Europe, when diagnosed by microscopy by an experienced parasitologist, has been found to be as high as 80% (Valkiūnas *et al.*, 2006a). Therefore microscopy remains the gold standard for diagnosing mixed infections of *Plasmodium* species in bird blood, until species-specific primers can be developed (Valkiūnas *et al.*, 2014b).

Co-infections with two or more malaria parasite species are common in wild birds (Jarvi *et al.*, 2002). It is possible to find several haemosporidian parasite lineages in both a population and the individual (Hellgren *et al.*, 2004). Beadell et al. (2004) found mixed infections in 29 of 428 individuals (6.8%) in their study on the prevalence of two avian blood parasite genera (*Plasmodium* and *Haemoproteus*) in the Australo-Papuan region. In a recent study in New Zealand, double infections with *P. relictum* and *P. rouxi* were found in introduced blackbirds (Gudex-Cross, 2011), a finding that corresponds to what I found in the blackbird blood smears from Auckland and Whangarei in this study (Table 1), but which could not be replicated by PCR. There is some discrepancy in the literature about the effect of co-infections with different lineages of *Plasmodium,* although these are generally considered more virulent than infections with just a single lineage (Arriero and Moller, 2008, Marzal *et al.*, 2008, Palinauskas *et al.*, 2011), because different parasites simultaneously share limited available physiological resources (Atkinson, 1991). Palinauskas et al. (2011) found heavy parasitaemia (over 35% and up to 90% during peaks) in three species of experimentally infected passerines, but did not note any significant effects in body mass. In contrast, Marzal et al. (2008) found a negative additive cost in body condition in individuals from a natural population of house martins experimentally infected by two different *Plasmodium* lineages. In a study in house martins (*Delichon urbicum*), individuals infected with two different parasite species were in poorer condition and had a lower body mass than uninfected or single-infected individuals. These individuals also suffered from a higher intensity of chewing lice in the feathers

(Marzal *et al.*, 2008). It is therefore likely that the particular combinations of *Plasmodium* spp. as well as host species determine the magnitude of the measurable health effects of mixed infections and further study in this area is needed.

5.5.2 Loss of parasites

The most important finding of this study is that *Plasmodium* diversity in saddlebacks was higher on Hen Island, when compared to the other sites (Figure 5.3). This suggests that *Plasmodium* as a genus has suffered a severe bottleneck following saddleback translocations with lineages being lost. This supports the prediction that with small number of hosts being translocated there is a chance that parasites are lost either through not being present in the translocated hosts or lost after translocation from lack of transmission (MacLeod *et al.*, 2010). It also suggests that *Plasmodium* species/lineages were lost when saddlebacks became extinct in the mainland and confined to Hen Island. It is very likely that the same process has happened to the *Plasmodium* parasites of birds introduced 150 years ago by the acclimatisation societies, because *Turdus* spp. and their parasites brought over from Europe went through a bottleneck of their own and parasite lineages may have either not been transported at all or lost after arrival to New Zealand. This would explain the discrepancy between *Plasmodium* community composition between Europe and New Zealand.

This loss of parasites due to introduction or translocation to new areas has been noted in other studies. A study comparing *Plasmodium* diversity in introduced populations of species worldwide with their native populations, found that on average introduced ones only carry half the number of parasites (Torchin *et al.*, 2003). A study by Marzal et al. in 2011 examined 1820 house sparrows (*Passer domesticus*) worldwide. They looked at the diversity, loss and gain of malaria parasites in this globally invasive bird. The diversity of parasites varied by the geographical region where the sparrows were sampled, and a lower diversity and prevalence was found in newly colonized regions (e.g. the Americas). In New Zealand, 47 sparrows were tested for this study, with 21.3% being infected with *Plasmodium* spp., compared to 31.6% worldwide. The diversity was also diminished, with 30 lineages found worldwide, but only three of them found in New Zealand sparrows, namely GRW04, *P. relictum* SGS1 and GRW06.

These findings can be seen as examples of the Enemy Release Hypothesis which postulates that a host has a better chance to successfully establish in a new environment, if it loses some of its parasites (Valkiūnas *et al.*, 2006b, Lima *et al.*, 2010, Marzal *et al.*, 2011a). This may also have been the case during the translocations of NI saddlebacks, where a loss of parasites in the population might have provided an advantage for the birds in the short term by making it easier to survive translocation and establishment in a new site. In the case of New Zealand native birds, experimental studies would be needed to understand the relationship between hosts and parasites and how the loss of the parasite may improve the hosts' chances of establishment after translocation. This has been attempted before in study for a Master's thesis by Thorne (2007) using coccidia in translocated saddlebacks as a model, but results were inconclusive because long term quarantine had a negative impact on first year survival of all birds used in the study. During Thorne's (2007) work, *Plasmodium* spp. was also detected in four birds, which were kept captive until blood samples were negative in PCR, and were then released.

In the long term, however, it may be possible that the loss of parasites has a negative impact on the saddleback populations and/or the ecosystems where they are translocated to. Parasites perform vital roles in ecosystems; they have been termed "the ultimate missing links" in food webs (Lafferty *et al.*, 2008). They can have a role organizing community structure (Poulin, 1999, Mouritsen and Poulin, 2005) and in increasing food-web complexity (Lafferty *et al.*, 2008, Dunne *et al.*, 2013) and possibly also food-web stability (Lafferty *et al*., 2008). They also can play a role in increasing ecosystem biodiversity (Mouritsen and Poulin, 2005). In 2006, Hudson et al. (2006) even considered a healthy ecosystem one that is rich in parasite species.

5.5.3 Implications for wildlife health in New Zealand

Avian malaria parasites almost exclusively cause subclinical infections in areas with endemic infection (Bennett *et al.*, 1993); Valkiūnas 2005), but disease and mortality remain a possibility as seen in the recent findings by Dinhopl et al. (2015). In the first large scale study looking at causes of mortality in wild birds, these authors found that 14.6% of 233 birds found dead in Austria had died of severe avian malaria infection (Dinhopl *et al.*, 2015). This is interesting from a New Zealand perspective as the three avian malaria lineages that were found during the Austrian study are also the

most common ones found in New Zealand (namely GRW6, SYAT05 and Linn1) (Dinhopl, Nedorost et al. 2015). In addition, deaths in native birds in New Zealand have been caused by the lineages GRW4, Linn1 and GRW6; the latter two seem to have a bigger impact (Schoener, *et al*. 2014). Today, it is impossible to say if parasite introductions through the Acclimatisation Societies' introduction programmes in the 1860s had an impact on the saddleback (and other native bird) communities back then and contributed to the disappearance of these birds from the mainland of New Zealand. In a study in Australia, evidence has been found that invasive Indian mynas (*Acridotheres tristis*) carry a large number of exotic *Plasmodium* lineages potentially lethal to native bird species (Clark *et al*., 2015). When examining the blood smears for parasites, the level of parasitaemia appeared to be very low in saddleback in most cases, with few parasites visible on the whole slide. When comparing parasite stages in blood smears from saddlebacks with introduced birds in this study, another important difference was the absence of advanced life cycle stages (gametocytes) in virtually all saddlebacks. If the parasites were affecting both introduced and native birds the same way, the parasitaemia intensity would be expected to be similar in both cases (G. Valkiūnas, 2015, pers. com.). There are two possibilities to explain this pattern. Firstly, the saddlebacks may be resistant to the infection and so only a few parasites develop in the blood. Secondly, it is also possible that saddlebacks experience lethal abortive infections, in which the initial merogony occurs (in the tissues of organs where initial sporogonic stages develop) but the parasites do not complete their life cycles due to the death of the host. In this case, no gametocytes in the blood are formed, this was observed for example in Galapagos, where *Plasmodium* was recently introduced (Levin *et al*., 2013). Abortive development often causes severe pathology and potential mortality in avian hosts (Valkiūnas *et al.*, 2014b). It is possible that these birds were hiding and have not been sampled, since there is a sampling bias towards birds with low parasitemia when mist netting is used as the main capture method. However, during my study and other intensive long-term saddleback studies on Mokoia Island, where approximately half of the population were colour banded for individual identification, no saddleback mortalities or birds showing clinical signs of a severe pathological infection have been observed. This is in contrast to intensive studies done on hihi (*Notiomystis cincta*) where birds suffering aspergillosis were detected both when sick and dead (Alley *et al.*, 1999). So far, no solution has been found to improve capture

rates of heavily infected birds (Valkiūnas, 2005). To examine this question in more detail, experimental infections and detailed pathological and histological examinations of all organs and tissues, including bone marrow, will be necessary (G. Valkiūnas, 2015, pers. com.). In addition, blood should be tested for haematocrit levels to determine if the birds are suffering from anaemia due to a highly pathogenic malaria infection. On my blood smears I noted an increased incidence of immature erythrocytes, which might indicate ongoing pathology. However, the populations of North Island saddleback at all island and mainland sites in New Zealand are growing and no increased mortalities have been noted by research teams, DoC rangers or members of the public.

Still, chronic and subclinical infections with avian malaria (Atkinson and van Riper III 1991) may cause some permanent costs to the host (Atkinson, 1991, Bensch *et al.*, 2007). For example, Asghar *et al.* (2011) found that chronic infections can affect both the arrival date at the breeding ground and the number of fledged offspring in migratory birds (Asghar *et al.*, 2011). Kilpatrick *et al.* (2006) observed that chronically infected adult Hawaiian amakihi had a 17% lower survival rate compared to uninfected birds. Chronically infected female blue tits (*Cyanistes caeruleus*) treated with the anti-malarial drug Malorone, had higher hatching success, higher provisioning rates and a higher fledging success than untreated females (Knowles *et al.*, 2010). In a nine year study, chronic avian malaria caused significant fitness costs to hosts, which varied depending on the infecting lineage; blue tits infected with *P. relictum* had higher survival rate but were less likely to breed successfully than those infected with *P. circumflexum* (Lachish *et al.*, 2011). A recent study found that chronic infections might cause an even bigger hidden cost. Ashgar et al. 2015 found that while low-level chronic avian malaria infections did not have a direct short term cost for the birds, it reduced the life span as well as the lifetime number and quality of the offspring in great reed warblers. This process was mediated by higher degradation of telomeres in infected birds (Asghar *et al.*, 2015). Only the future will tell what impact findings like these have for the conservation of rare endemic birds in New Zealand.

When looking at studies from New Zealand's neighbouring Island nations in the Pacific region, an interesting picture begins to emerge. Diverse indigenous *Plasmodium* spp. communities are common in the Pacific region and have been

found in American Samoa, the Cook Islands (Ishtiaq *et al.*, 2006) and the Australo-Papuan region (Beadell *et al.*, 2004). More recently, Australia and Papua New Guinea have appeared as a diversity "hotspot" for avian haemosporidian parasites, with 30 different lineages of *Plasmodium* found (Clark *et al.*, 2014). It is interesting that New Zealand, with a much larger land area than for example Vanuatu, has a much lower unique *Plasmodium* diversity. This may be due to the general lower diversity in temperate regions (Clark *et al.*, 2014) but it might also be a result of New Zealand's native biodiversity loss. Around 50% of New Zealand's native avifauna has been lost, and many other species have gone through severe population bottlenecks. The extinction of a host will also result in extinction of its parasites, especially if these parasites are host specific. In the present study, I have shown that population bottlenecks, like the saddleback translocation events, can result in a loss of *Plasmodium* diversity, an event that very likely has also happened to these parasites in New Zealand's past. In addition, generalist parasite lineages have been introduced with European birds and are now the most widespread and common ones found, maybe because they have displaced and outcompeted the specialist ones. This is likely, since many endemic New Zealand birds are rare and exist only in a few protected areas, while introduced European birds, reservoirs for introduced *Plasmodium* lineages, thrive and massively outnumber them. This begs the question of whether in the future of New Zealand translocations, one should worry as much about the parasites as about the bird hosts, after all, they too are part of New Zealand's biodiversity.

Chapter 6: New insight into avian malaria vectors in New Zealand

A CO_2 baited light trap used in this study set up in the forest

6.1 Abstract

Insect vectors, namely mosquitoes (Culicidae), are compulsory for malaria parasites (*Plasmodium* spp.) to complete their life cycle. Despite this, little is known about the roles of the different mosquito species in New Zealand in the avian malaria life cycle. In this study, I used a high resolution melt qPCR and nested PCR to determine *Plasmodium* spp. occurrence, and the diversity of mitochondrial cytochrome b gene sequences, in wild-caught mosquitoes sampled across nine sites on the North Island of New Zealand. I collected a total of 788 un-engorged mosquitoes of six species as well as five hippoboscid flies. The most commonly caught species were the introduced *Aedes notoscriptus* and the native *Culex pervigilans*. Four different *Plasmodium* lineages were found, among them the three most frequently detected in New Zealand (SYAT05, Linn1, GRW6) and *Plasmodium relictum* GRW4. The minimum infection rate (MIR) of the different positive mosquito species was 3.2% for *A. notoscriptus*, 7.8% for *C. pervigilans*, 2.3% for *Opifex fuscus* and 9.1% for *Culex quinquefasciatus*. The mosquitoes were pooled and their thoraxes and abdomens were examined separately. The hypothesis being that mosquitoes which have parasites present in the salivary glands (thorax) were more likely to be vectors than those with the parasites only in their abdomen. *A. notoscriptus* and *C. pervigilans* were positive for malaria DNA in the thorax samples, lending more weight to their role as avian malaria vectors. One pool of the collected hippoboscid flies also contained the DNA of two different *Plasmodium* lineages in both thorax and abdomen. This is the first detection of avian *Plasmodium* DNA from mosquito thoraxes and hippoboscid flies in New Zealand.

Keywords

Aedes, Avian Malaria, *Culex*, Hippoboscid, Mosquito, New Zealand, *Opifex*, *Plasmodium*.

6.2 Introduction

Avian malaria parasites of the genus *Plasmodium* are common in birds worldwide. So far, avian malaria parasites of 22 different lineages have been found in 39 different bird species in New Zealand (Chapter 5; Tompkins *et al*., 2008, McKenna, 2010, Tompkins *et al*., 2010, Castro *et al.*, 2011, Howe *et al.*, 2012, Baron *et al.*, 2014, Schoener *et al.*, 2014, Hunter, 2015). Recent outbreaks of avian malaria in endangered New Zealand birds causing fatalities include an outbreak in captive New Zealand dotterel (*Charadrius obscurus*) chicks in 1996 (Tompkins *et al*., 2008), in yellowhead/ mohua (*Mohua ochrocephala*) in 2004 (Alley *et al.*, 2008), in South Island saddlebacks (*Philesturnus carunculatus carunculatus*) in the summers of 2002 and 2007 (Alley *et al.*, 2010) in brown kiwi (*Apteryx mantelli*) in 2010/2011 (Banda *et al.*, 2013) and the death of several yellow eyed (*Megadyptes antipodes*) and Fjordland crested penguins (*Eudyptes pachyrhynchus*) in 2015 (Hunter, 2015). The most frequently detected lineages infecting native New Zealand bird species and introduced passerines are *Plasmodium (Huffia) elongatum* lineage GRW06 with the widest host range, *Plasmodium elongatum* Linn1 and *P. (Novyella) vaughani* lineage SYAT05. Other lineages of *Plasmodium* detected in endemic species are *Plasmodium relictum* (lineages GRW4 and SGS1) (Chapter 2 and 5) (Castro *et al*., 2011, Howe *et al.*, 2012, Ewen *et al.*, 2012b, Schoener *et al.*, 2014). Deaths in native New Zealand birds have so far been caused by the lineages GRW6, Linn1 and GRW4 (Chapter 2) (Schoener *et al.*, 2014) and *P. relictum* GRW4 was also widely implicated as causative agent for the extinction of a range of endemic birds on Hawaii.

Competent insect vectors are compulsory for malaria parasites to complete their life cycle and therefore should not be overlooked when studying *Plasmodium* spp. Following several cycles of merogony (asexual reproduction) in the vertebrate host, the *Plasmodium* sexual stages start to develop in mature erythrocytes, namely gametocytes or gamonts which develop into macro (female) and microgametes (male) in the insect. These sexual stages are acquired by the vector by feeding on vertebrate blood (Valkiūnas, 2005). After sexual reproduction and one period of asexual reproduction (sporogony), sporozoites travel through the haemocele of the vector and penetrate the salivary glands in the thorax of the mosquito. From there, they are transmitted by the insects to the avian host during feeding on its blood

(Atkinson, 1991, Ritchie *et al*., 1994, Gabrisch and Zwart, 2001, Valkiūnas *et al.*, 2002).

For this study, I examined the thorax and abdomen of mosquitoes separately, because not all mosquitos are competent vectors for all *Plasmodium* lineages and therefore abort the parasite lifecycle before the production of sporozoites. Parasites found in the abdomen will be assumed to be from a recent blood meal while parasites in the thorax will be assumed to have completed the invertebrate host part of their life cycle and to be capable of infecting the vertebrate host.

Different *Plasmodium* parasites can be transmitted by a range of mosquito species. On Hawaii, a culicine mosquito, *Culex quinquefasciatus,* is the main, but not sole, vector for *Plasmodium relictum*, with two other mosquitoes, *Aedes albopictus* and *Wyeomyia mitchellii* also possible vectors (LaPointe *et al.*, 2005).

Mosquitoes as vectors in New Zealand have not been studied in detail. New Zealand possesses 12 species of indigenous mosquitoes as well as four introduced species (Derraik, 2004) (Table 6.1).

So far, it is mostly unknown which of these species are vectors of avian malaria in New Zealand (Derraik, 2004, Tompkins and Gleeson, 2006, Derraik and Slaney, 2007), but possible vectors include *Culex quinquefasciatus,* an exotic mosquito that has spread rapidly in NZ (Tompkins *et al*., 2006), *Culex pervigilans* and *Ochlerotatus (Aedes) australis* (Derraik and Slaney, 2007). The only mosquito that has been found to carry *Plasmodium* DNA in New Zealand is *Culex pervigilans* (the only species tested to date), a native mosquito (Massey *et al.,* 2007), although this finding was only in a single engorged female. Therefore its vector status cannot be determined. In an outbreak of avian malaria in mohua in Orana Park, Christchurch, *Culex pervigilans* was identified as the most likely vector which showed a peak population in midsummer (February) (Derraik *et al.*, 2008).

For this study I predict the following outcomes:
1. Mosquito larval habitat is underutilized in New Zealand, therefore I expect to catch a lower number of adult mosquitoes and fewer species than other studies have done in other parts of the world.

2. The most commonly caught North Island mosquito species will also be caught in this study.

3. Evidence will support that *C. quinquefasciatus*, which is a confirmed avian malaria vector in other parts of the world, and *C. pervigilans* are potential vectors of *Plasmodium*. Other mosquitoes in the genera *Culex* and *Aedes,* if caught, are also possible also vectors for *Plasmodium*.

4. I expect a similar *Plasmodium* prevalence and minimum infection rate (MIR) in New Zealand mosquito species (especially in introduced ones) as elsewhere.

5. The most common New Zealand avian *Plasmodium* lineages found in bird blood will also be found in collected mosquito vectors.

6. The prevalence of infections will be higher in examined mosquito abdomens than in thoraxes because not all mosquitoes will be competent vectors.

Table 6.1: List of established mosquito species in New Zealand, their origins and associated pathogens (modified after (Derraik, 2004)

Origin	Species	Associated pathogens
Endemic	*Coquillettidia (Austromansonia) tenuipalpis* (Edwards)	unknown
	Coquillettidia (Coquillettidia) iracunda (Walker)	unknown
	Culex (Culex) asteliae (Belkin)	unknown
	Culex (Culex) pervigilans (Bergroth)	WH
	Culex (Culex) rotoruae (Belkin)	unknown
	Culiseta (Climacura) novaezealandiae (Pillai)	unknown
	Culiseta (Climacura) tonnoiri (Edwards)	WH
	Maorigoeldia argyropus (Walker)	unknown
	Ochlerotatus (Nothoskusea) chatamicus (Dumbleton)	unknown
	Aedes (Ochlerotatus) antipodeus (Edwards)	unknown
	Ochlerotatus (Ochlerotatus) subalbirostris (Klein & Marks)	unknown
	Opifex fuscus (Hutton)	unknown
Exotic	*Culex (Culex) quinquefasciatus* (Say)	AM AP BF CH JE KUN LF MVE RR SIN SLE WNV
	Aedes notoscriptus (Skuse)	BF CH DEN JE RR RVF
	Ochlerotatus (Halaedes) australis (Erichson)	CH DEN RR WH
	Ochlerotatus (Ochlerotatus) camptorhynchus (Thomson)	BF KOK RR SIN

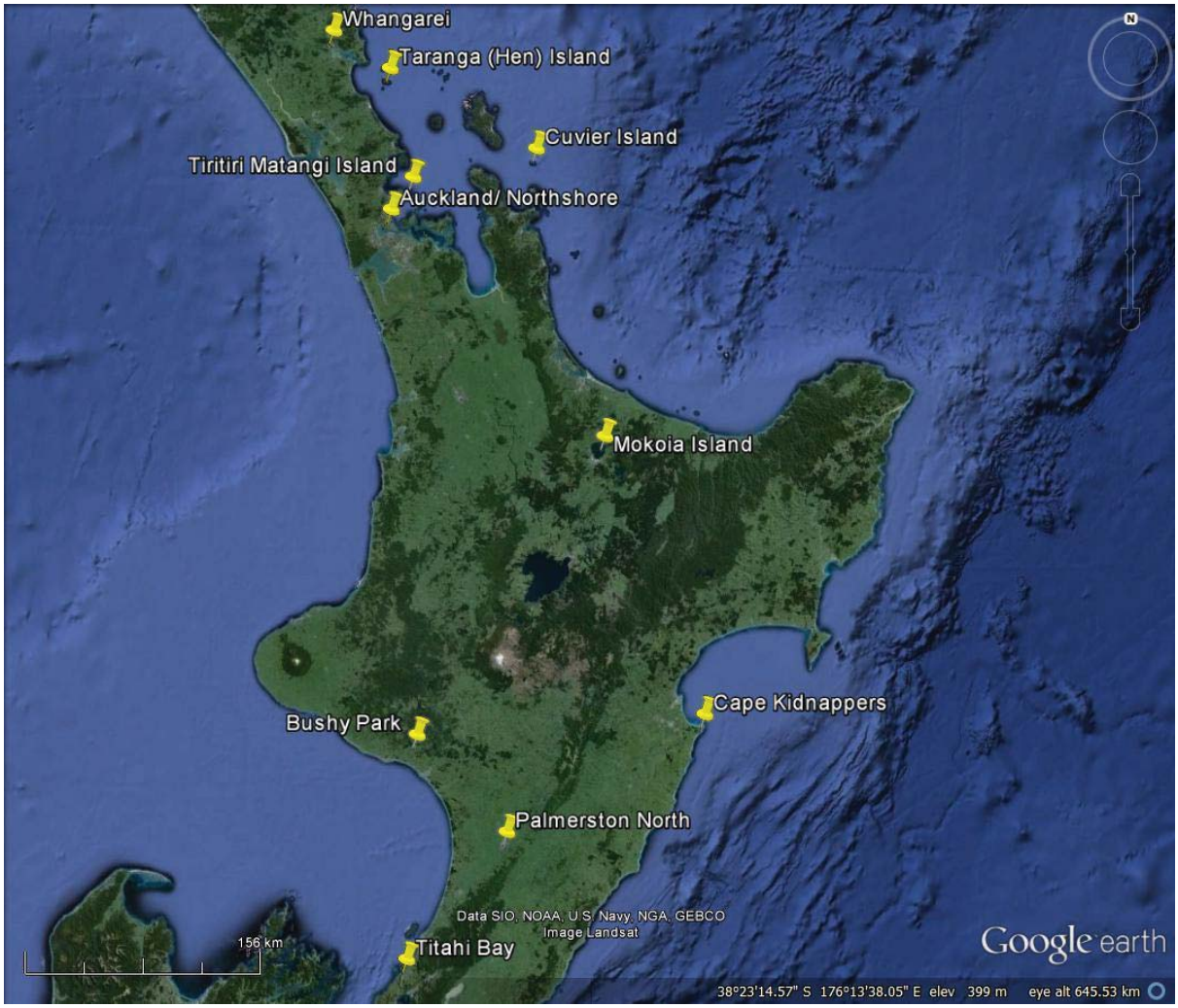
Note:
Arboviruses: BF (Barmah Forest), DEN (dengue), JE (Japanese encephalitis), KOK (Kok obera), KUN (Kunjin), MVE (Murray Valley encephalitis), RR (Ross River), RVF (Rift Valley fever), SIN (Sindbis), SLE (St. Louis encephalitis), WH (Whataroa), WNV (West Nile). Other pathogens: AM (*Plasmodium relictum*, avian malaria), AP (avian pox viruses), CH (*Dirofilaria immitis*, canine heartworm), LF (*Wuchereria bancrofti*, lymphatic filariasis).

6.3 Material and methods

During the period 2012-2014, thirteen sampling trips were carried out to catch mosquitoes all around the North Island of New Zealand (Figure 6.1). This sampling was done at the following sites: Hen Island, Cuvier Island, Tiritiri Matangi Island, Mokoia Island and Bushy Park. Additional sampling sites were Cape Kidnappers, Titahi Bay, Porirua (for *Opifex fuscus*) and the cities of Auckland, Northshore, Whangarei and Palmerston North.

During the summer 2012/2013, dry weather with drought conditions in large parts of the North Island had a negative impact on the number of mosquitoes caught. Sampling was therefore repeated in January/February 2014 (summer) at Mokoia Island, Bushy Park, Titahi Bay and Palmerston North because these sites were easy to access. On Hen Island, mosquitoes were sampled in the forest around the campsite at Dragonmouth Cove; on Cuvier Island, the sampling area was around the historic human settlement which has a small stream and a limited number of artificial fresh water containers. On Mokoia Island sampling was done along the walking paths in the forest as well as around the campsite and public toilets and on Tiritiri Matangi Island, sampling was done in the forest surrounding the human settlement. At the mainland sites in Bushy Park, the traps were placed in the forest along the walking tracks including a swampy pond area. And at Cape Kidnappers, the sampling was done at the area designated as the release site for the translocated North Island saddleback. In Palmerston North, sampling was performed at an urban site with bushy vegetation and a small swampy area. At Titahi Bay, *Opifex fuscus* was collected with insect aspirators after being attracted by volunteers.

Figure 6.1: Map of the sampling sites visited during this study



6.3.1 Trapping of mosquitoes

For the trapping of mosquitoes, eight modified CO_2 (using dry ice) baited CDC light traps using a UV-diode as light source, as well as insect aspirators (for the collection on vegetation and around human encampment) were used. The traps were placed at about eye height in trees or shrub. Since most of the mosquito species present in the North Island of New Zealand appear to be crepuscular and/or nocturnal feeders (Derraik, 2005), the traps were set approximately 2 h before sunset (7pm) and emptied approximately 2 h after sunrise (7am) for a total of 12 trapping hours per sampling bout. In Titahi Bay, only *Opifex fuscus* were collected during one afternoon (March 2013) with insect aspirators. Only female mosquitoes were kept for identification and analyses. The mosquitoes were killed by freezing at -20°C and stored in 80% ethanol until processing.

6.3.2 DNA extraction

Mosquitoes were identified under a stereomicroscope, using the identification keys by Snell (2005). Single individuals as well as mosquito samples pooled by species, location and date were dissected in 0.9% saline into abdomen and thorax. Instruments were wiped with 75% ethanol between pools and sterilized with the flame of a Bunsen burner. Not all mosquitoes could be identified to species and genus level, because they had been stored in ethanol and in some cases their distinguishing features like scales had washed off. These mosquitoes were pooled separately. The heads were discarded to avoid introducing PCR inhibitors, while ensuring that the salivary glands were not removed (Massey *et al.*, 2007, Ejiri *et al.*, 2009, Kim *et al.*, 2009a, Kim *et al.*, 2009b). The pools varied in size from 3-20 mosquitoes. The pool size was equal for all species, if enough individuals were caught. The insects were then homogenized with the aid of plastic micropestules (Njabo *et al.*, 2009, Njabo *et al.*, 2011). The DNA from abdomen and thorax was extracted separately from each other using the DNeasy Blood and Tissue kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. To help dissolve the hard exoskeleton, 30 µl of 100 mg/mL dithiothreitol were added to the digestion buffer (Cooper, 1994) and total DNA was eluted in the final step with 200 µl elution buffer (Njabo *et al.*, 2009, Njabo *et al.*, 2011).

6.3.3 PCR and sequencing

The samples were first submitted to a real-time PCR according to (Friedl and Groscurth, 2012) amplifying an 85bp segment of the LSU-rRNA. There was considerable unspecific amplification, possibly due to mosquito genomic DNA, which was not described previously by Friedl and Groscurth (2012) when using bird blood. Therefore, this method had to be abandoned.

For amplifying the DNA of *Plasmodium* spp., each DNA sample was then subjected to nested PCR, as described by (Hellgren *et al.*, 2004) without modification. After PCR, all the PCR products were run on a 1% (w/v) ultra-pure agarose gel (Invitrogen, California, USA) containing ethidium bromide and were visualized under UV light on a transilluminator. All positive PCR amplicon samples were purified (PureLink PCR purification kit, Invitrogen, California, USA) and subjected to

automatic dye-terminator cycle sequencing using the BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc, California, USA) to confirm genomic sequence. The electropherograms resulting from sequencing were also checked for double nucleotide peaks to infer possible cases of mixed infections of two or more different parasite lineages.

6.3.4 Minimum infection rate

To evaluate the infection rate in the collected mosquitoes, the minimum infection rate (MIR) of each mosquito species was calculated. If a mosquito pool was positive for *Plasmodium* in the PCR, it was assumed that the pool contained at least one positive individual. Therefore, MIR (percentage) was calculated as follows:

MIR (%) = Number of PCR positive pools/total number of analysed mosquitoes x 100 (White *et al.*, 2006, Ventim *et al.*, 2012).

6.3.5 Presence of several different lineages in one sample

Samples that were positive using conventional PCR were re-examined using a newly developed two step qPCR/ HRM protocol using the primer sets HRMF (Njabo *et al*. 2011) and HaemR2 (Hellgren *et al.*, 2004) and Plas56F/PlasrevRT as described in Chapter 4. This method is able to discern between the most common *Plasmodium* lineages in New Zealand (*P. elongatum* GRW6, Linn1 and SYAT05) as well as *P. relictum* GRW4. The assay is able to detect parasite DNA concentrations of down to 10^{-5} ng/µl for all four lineages which is equivalent to 5 parasite units (containing 50 copies of the cytochrome b gene each). This assay is not as sensitive as the standard nested PCR and therefore can only give an indication of possible presence of several different lineages.

6.3.6 Statistical analysis

A chi-square-test was used to test for significant differences in parasite diversity and prevalence per site. Regression analysis was used to examine whether mosquito number or number of mosquito species were good predictors of prevalence and *Plasmodium* diversity. Chi square was used to look for significant differences in prevalence and *Plasmodium* diversity in both thorax and abdomen pools across the sites. For all tests, a P-value of ≤.0.05 was considered significant. Owing to low

sample sizes of certain mosquito species, we did not test for differences in the parasite prevalence among mosquito species. Tests were carried out in IBM SPSS statistics 23.

Table 6.2: Dipterans sampled during this study

			Mosquito Species								
Location	**Sampling Date**	***Aedes notoscriptus***	***Aedes antipodeus***	***Culex pervigilans***	***Opifex fuscus***	***Culex quinquefasciatus***	***Culex astelidae***	**unidentified Culex; C Rotoruae??**	**Unidentified**	**Hippoboscid**	**Number of individual insects**
Cape Kidnappers	Feb-13 (late summer)	1	-	5	-	-	-	-	-	-	**6**
Whangarei Mair Park	Feb-13 (late summer)	9	-	-	-	-	-	-	-	-	**9**
Auckland Northshore	Feb-13 (late summer)	21	-	-	-	-	-	-	-	-	**21**
Titahi Bay	Mar-13 (autumn)		-	-	24	-	-	-	-	-	**24**
Cuvier Island	Apr-13 (autumn)	20	-	-	19	-	-	-	-	5	**39**
Hen Island	Nov-12 (spring)	3	8	-	-	-	1	-	-	-	**12**
Mokoia Island	Feb-14 (late summer)	20	4	-	-	3	-	2	-	-	**31**
Mokoia Island	Mar-12 (autumn)	20	9	29	-	1	-	-	-	-	**59**
Bushy Park	Feb-14 (late summer)	8	9	1	-	-	-	-	-	-	**18**
Bushy Park	Feb-13 (late summer)	12	4	61	-	-	-	-	22	-	**77**
Palmerston North	Jan-14 (summer)	442	-	6	-	7	-	-	17	-	**472**
Species numbers total	**-**	**556**	**34**	**102**	**43**	**11**	**1**	**2**	**39**	**5**	**793**

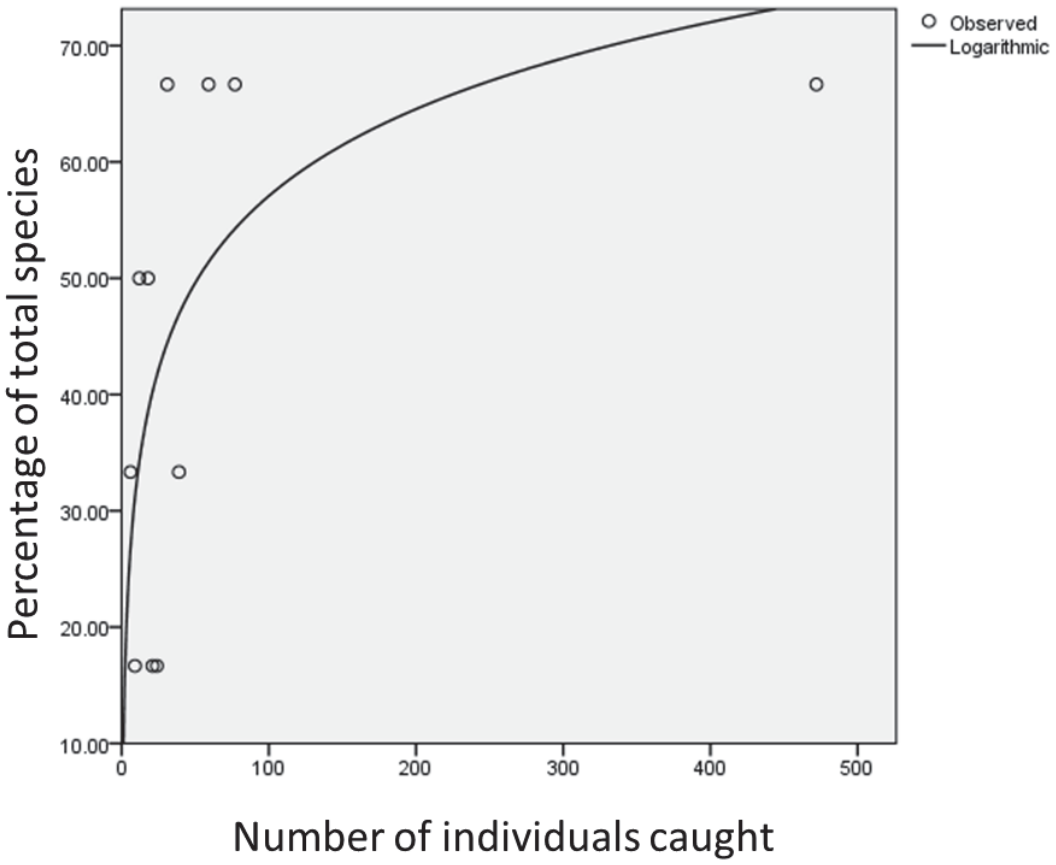
6.4 Results

During the sampling period between November 2012 to February 2014, a total of 788 mosquitoes of six species were caught at nine sites (Table 6.2). All mosquitoes were unengorged. No mosquitoes were caught on Tiritiri Matangi Island. In addition, five hippoboscid flies that were found on North Island saddleback (*Philesturnus carunculatus rufusater*) during a translocation were caught on Cuvier Island in April 2013. The hippoboscid flies were not identified. All results for this study are included in Appendix 6 as raw data tables.

6.4.1 Mosquito fauna

None of the factors examined were good predictors of prevalence or *Plasmodium* diversity, although the number of mosquito pools (used as a proxy of total mosquitos caught) was close to significance ($F=4.7$; $df = 1$; $p = 0.067$). However, there were significant correlations between the number of mosquito pools and the number of mosquito species (Spearman's Rho = 0.794; n = 9; p = 0.011) and *Plasmodium* diversity (Spearman's Rho = 0.8; n = 9; p = 0.014) suggesting that, the more individuals were sampled, the higher the chance of sampling a higher number of species (Figure 6.2). There was a significant difference in the number of individuals and species caught per site, but only if Palmerston North (with by far the highest number of collected samples) was included in the data analysis. There was no statistical difference between sites if the data from Palmerston North was removed from the analysis. The most common mosquito species captured was *A. notoscriptus* with a total of 556 individuals, which was found at all sites where CO_2 baited traps were used (Table 6.2). *Aedes notoscriptus* was most common in urban areas and dominated the collected community there, with 100% of the caught mosquitoes in Whangarei and Auckland as well as 93.6% in Palmerston North being this species (Table 6.2). The native *C. pervigilans* was the second most common mosquito found, with 102 individuals. This mosquito was more prominent in nature reserves, and predominated in March 2012 on Mokoia Island (49.2%) and in February 2012 in Bushy Park (79.2%). Other species found were *Aedes* (*Ochlerotatus*) *antipodeus*, *C. astelidae*, *C. quinquefasciatus* and *Opifex fuscus* (Table 6.2).

Figure 6.2: Percentage of total mosquito species caught (n=6) in correlation to individuals caught for each site



6.4.2 Potential avian malaria vectors

A total of 44 (29.33%) out of 150 (75 each of abdomen and thorax) mosquito pools were positive for *Plasmodium* spp. using nested PCR. Of these, 26 (59.1%) abdomen and 18 (40.9%) thorax pools tested positive. There was a significant difference in the percentage of positive pools between the different sites (x^2=1, df=10, P<0.05), but no significant difference was found when differentiating between positive thorax and abdomen pools. Avian malaria parasites were found in four different mosquito species as well as in the collected hippoboscid flies (Table 6.3). The MIR of the infected species was highest for *C. pervigilans* (12.8%), followed by *C. quinquefasciatus* (9.1%),*A. notoscriptus* (4%) and *Opifex fuscus* (2.3%) (Table 6.4). Of these, only *C. pervigilans*, *A. notoscriptus* and the hippobiscid flies showed parasites in thorax as well as abdomen.

6.4.3 *Plasmodium* lineages

A total of 32 (72.7%) of the positive pools could be sequenced with conclusive results (Tables 6.5 and 6.6). More than half (53.1%) carried more than one *Plasmodium* lineage (Table 6.5). Four different *Plasmodium* lineages were found, namely Linn1 (62.5%), SYAT05 (50%), GRW6 (28.1%) and in one case (3.1%) GRW4 (Table 6.5). There was no significant difference in lineage diversity between sites (x^2=7.27, df=10, P>0.05). Both *A. notoscriptus* and *C. pervigilans* carried the malaria lineages Linn1, SYAT05 and GRW6 in both abdomen and thorax, a single pool (abdomen) of *A. notoscriptus* was found to carry GRW4 (Table 6.5). Although the hippoboscids carried Linn1, SYAT05 and GRW6, only Linn1 and GRW6 were found in one thorax pool (Table 6.4).

The lineages Linn1, SYAT05 and GRW6 were widespread and found on all positive sites around the North Island, except for Cape Kidnappers (only SYAT05 and GRW6) and Hen Island (SYAT05) (Table 6.6). *Plasmodium relictum* GRW4 was only found in Palmerston North.

When diagnosing mosquito pools containing more than one lineage, 17 (53.1%) were detected as they formed different disassociation curves on the electropherograms after sequencing. The qPCR/HRM protocol found 24 (16%) of the 150 tested pools positive for *Plasmodium*, with 11 (45.8%) showing more than one lineage. Only four of these cases with multiple lineages were picked up by both methods simultaneously.

Table 6.3: *Plasmodium* spp. found in collected mosquito pools (a= abdomen; t= thorax)

Location	Sampling Date	Number of total mosquito pools (one each of abdomen and thorax)	*Aedes notoscriptus*	*Aedes antipodeus*	*Culex pervigilans*	*Opifex fuscus*	*Culex quinquefasciatus*	*Culex astelidae*	unidentified Culex; C. Rotoruae??	Unidentified	Hippoboscid	Number of positive pools
Cape Kidnappers	Feb-13 (late summer)	4	1(t)	-	1(a)	-	-	-	-	-	-	**2 (50%)**
Whangarei Mair Park	Feb-13 (late summer)	2	-	-	-	-	-	-	-	-	-	**0**
Auckland Northshore	Feb-13 (late summer)	2	-	-	-	-	-	-	-	-	-	**0**
Titahi Bay	Mar-13 (autumn)	4	-	-	-	1 (a)	-	-	-	-	-	**1 (25%)**
Cuvier Island	Apr-13 (autumn)	10	-	-	-	-	-	-	-	-	1(a); 1 (t)	**2 (20%)**
Hen Island	Nov-12 (spring)	6	1 (a)	-	-	-	-	-	-	-	-	**1 (16.7%)**
Mokoia Island	Feb-14 (late summer)	10	-	-	-	-	-	-	1(t)	-	-	**1 (10)%)**
Mokoia Island	Mar-12 (autumn)	10	-	-	2 (a); 2 (t)	-	-	-	-	-	-	**4 (40%)**
Bushy Park	Feb-14 (late summer)	8	-	-	1 (t)	-	-	-	-	-	-	**1 (12.5%)**
Bushy Park	Feb-13 (late summer)	14	1 (a); 1 (t)	-	3(a); 1(t)	-	-	-	-	2 (a)	-	**8 (57.1%)**
Palmerston North	Jan-14 (summer)	80	10(a); 8(t)	-	2(a); 1(t)	-	1 (a)	-	-	1(a); 1(t)	-	**24 (30%)**
Individuals numbers total		**150**	**22 (1.5%)** (12(a); 10(t))	**0**	**13 (0.9%)** (8(a); 5(t))	**1 (0.7%)** (a)	**1 (0.7%)** (a)	**0**	**1 (0.7%)** (t)	**4 (2.7%)** (3(a); 1(t))	**2 (1.3%)** (1(a); 1(t))	**44** (26(a); 18(t))= **29.3%**

Table 6.4: Minimum infection rate (MIR) for collected mosquitoes

Mosquito species	Number of mosquitoes	Positive mosquito pools (not differentiated into abdomen or thorax)	Minimum infection rate (MIR; %)
Aedes notoscriptus	556	22	**4**
Culex pervigilans	102	13	**12.8**
Opifex fuscus	43	1	**2.3**
Culex quinquefasciatus	11	1	**9.1**
Hippoboscid	5	2	**40**

Table 6.5: *Plasmodium* lineages found in collected mosquito pools (a=abdomen; t=thorax); there were multiple mixed infections with two or more lineages in one mosquito pool

						Plasmodium Lineages				
Mosquito species	Number of mosquitoes	Number of total mosquito pools (one each of abdomen and thorax)	Positive for *Plasmodium* spp.	Number successfully sequenced	Number of *Plasmodium* linages	LINN1	GRW6	SYAT05	GRW4	Number of pools with more than one lineage (s=by sequence; q=by qPCR)
Aedes notoscriptus	556	90	**22** 12(a); 10(t)	17	4	9 (6a;3t)	1(t)	10 (4a;6t)	1(a)	8 (s); 7(q)
Aedes antipodeus	34	10	0	0	0	-	-	-	-	0
Culex pervigilans	102	22	**13** 8(a); 5(t)	9	3	7 (5a;2t)	6 (4a;2t)	3(2a;1t)	-	6 (s); 3(q)
Opifex fuscus	43	6	1 (a)	0	0 (no sequence)	-	-	-	-	0
Culex quinquefasciatus	11	6	1 (a)	1	2 (multiple lineages))	1(a)	1(a)	-	-	1(s)
Culex astelidae	1	2	0	0	0	-	-	-	-	0
unidentified Culex; C. Rotoruae??	2	2	1 (t)	1	1	-	-	1(t)	-	0
Unidentified	39	8	**4** 1(a); 3(t)	2	2	1(a)	-	1(a)	-	1(q)
Hippoboscid	5	4	**2** 1(a); 1(t)	2	3 (multiple lineages)	2(1a;1t)	1(t)	1(a)	-	2 (s)
Totals	**793**	**150**	**44 (29.3%)** (26(a); 18(t))	**32**	**4**	**20 (62.5%)** (14a; 6t)	**9 (28.1%)** (5a; 4t)	**16 (50%)** (8a; 8t)	**1 (3.1%)** (a)	**17 (s); 11(q)**

Table 6.6*: Plasmodium* lineages found at sites (a=abdomen; t=thorax); there were multiple mixed infections with two or more lineages in one mosquito pool

				Plasmodium Lineages				
Location	**Number of sequenced mosquito pools**	**Number successfully sequenced**	**Number of *Plasmodium* lineages**	**LINN1**	**GRW6**	**SYAT05**	**GRW4**	**Number of mixed infection pools (s=by sequence; q=by qPCR)**
Cape Kidnappers	2	2	2	-	1 (t)	1 (a)	-	1(s); 1(q)
Titahi Bay	1	0 (no sequence)	0 (no sequence)	-	-	-	-	0
Cuvier Island	2	2	3 (multiple lineages))	2(1a;1t)	1(t)	1(a)	-	2(s)
Hen Island	1	1	1	-	-	1(a)	-	0
Mokoia Island	5	3	3	2(1a;1t)	2(1a;1t)	4(2a;3t)	-	2(s)
Bushy Park	9	7	3	5(4a;1t)	2(a)	1(a)	-	2(s);2(q)
Palmerston North	24	17	4	11(8a;3t)	3(2a;1t)	8(2a;6t)	1(a)	10(s); 7(q)
Total	**44**	**32**	**4**	**20 (62.5%)** (14a; 6t)	**9 (28.1%)** (5a; 4t)	**16 (50%)** (8a; 8t)	**1 (3.1%)** (a)	**17 (s); 11(q)**

6.5 Discussion

6.5.1 Mosquito fauna

During the summer 2012/2013, dry weather with drought conditions in large parts of the North Island had a negative impact on the number of mosquitoes caught and I therefore did not catch any mosquitoes from one of my sites, Tiritiri Matangi Island. In addition, an additional sampling trip to Mokoia Island in the very dry month of February 2013 also did not yield any mosquitoes. Other similar studies have caught between 40-100 individuals per sampling period per site in favourable climatic conditions (Lalubin *et al.*, 2013, Okanga *et al.*, 2013, Fryxell *et al.*, 2014). Therefore, the low number of mosquitoes caught in this study is most likely due to the dry climate. For that reason it is difficult to draw conclusions on whether underutilized mosquito larval habitat in New Zealand has indeed an impact on the number of adult mosquitoes and mosquito species in general in the environment.

Very few mosquitoes were collected on the offshore islands in this study (Hen Island, Cuvier Island, and Tiritiri Matangi Island) during the sampling period. These sites require permits, financial effort and extended logistics, and trips have to be arranged months in advance. As a result, a second trip there was not feasible. Without doubt the composition of mosquito species on these islands is of interest, as species there carry avian malaria and are considered competent vectors. My study provided only a glimpse into this, and further studies on these islands would have to be conducted in more favourable weather conditions.

As expected, the most common mosquito species (Gudex-Cross, 2015), were also caught during my study, namely *A. notoscriptus*, *C. quiquefasicatus*, *A. antipodeus*, *C. astelidae* and *C. pervigilans* with the addition of *O. fuscus* in two coastal areas (Titahi Bay and the historic settlement area on Cuvier Island). The most common two species collected in this study were the native *C. pervigilans* and the introduced *A. notoscriptus*, which are presently the two most common mosquitoes in the country (Derraik, 2004, Gudex-Cross, 2015). I also confirmed the findings made by Gudex-Cross (2015), that native mosquitoes outnumbered introduced ones at conservation sites. The introduced species *C. quinquefasciatus* and *A. notoscriptus* are known to prefer habitats modified by humans and are therefore often found in urban and semi-urban areas (Derraik, 2004, Knoeckel *et al.*, 2013, Molina-Cruz *et al.*, 2013) and

were collected in this study predominantly in urban areas (Auckland, Whangarei and Palmerston North). By far the biggest sample size during this study was of *A. notoscriptus*, where most individuals were collected during two days of sampling in an urban swampy bush area in Palmerston North. This site apparently provided ideal breeding conditions for this species. The same introduced species was also collected predominantly on the remote Cuvier Island, but there the sampling area was around the historic human settlement which has a small stream and a number of artificial fresh water containers. *A. notoscriptus* was also the most abundant introduced species recorded by (Gudex-Cross, 2015) during a study in the northern North Island.

At the sites where several sampling trips had been conducted in consecutive years, differences in mosquito prevalence were observed. On Mokoia Island, in March 2012 most individuals collected were of the introduced species *A. notoscriptus*, while in February 2012 no mosquitoes were caught and in February 2014, most collected individuals were of the native *C. pervigilan*s. In Bushy Park, *A. notoscriptus* was predominant in February 2013, while one year later in February 2014, more individuals of the native *C. pervigilans* were collected. Most likely these differences were due to seasonal, within season and/or yearly variations. Seasonal and yearly variations in mosquito abundance have been observed in other studies, for example mosquito abundance at West Cape, South Africa varied significantly between seasons with more than 10x the number of individuals caught in summer (n=490) opposed to winter (n=34) (Okanga *et al.*, 2013). In a study on *Culex pipiens* in Switzerland, where egg raft density was highly dependent on temperature, a peak in egg laying observed the previous year was not found the next year in July (summer) due to exceptionally low temperatures (Lalubin *et al.*, 2013). The same study did not find significant influence of precipitation or the interaction of precipitation and temperature on egg raft density, although there were no drought conditions as were seen during my study in New Zealand.

The endemic New Zealand mosquito *Opifex fuscus* was collected at two coastal sites, Titahi Bay and the historic settlement area on Cuvier Island. *O. fuscus* is known as the New Zealand rock pool mosquito and larval habitats exist in the coastal spray zone, with the larvae of this species preferring increased salinity (Snell *et al.*, 2010, McGregor, 1965). This species has been displaced by the introduced

Aedes australis in the southeast of the South Island (Cane *et al*., 2010), and it is likely that *O. fuscus* may disappear from more areas as introduced species increase their ranges in the future. In this case, populations on remote islands like Cuvier Island might be essential for the survival of the species.

The sampling methods used also have an influence on the number of different species and individuals caught. Gudex-Cross (2015) emphasized the placement of traps both on the ground and canopy to discern vertical distribution patterns of each species which might be related to feeding patterns and host preference. For example, they found that *A. antipodeus* was mostly caught in ground traps, while *C. astelidae* preferred the canopy and *A. notoscriptus* was caught in equal numbers in both strata. A similar vertical distribution was also found during a study in Auckland (Derraik *et al.*, 2005). Different kinds of traps also influence the outcome of a study. Carlson et al. (2015) even suggest that conclusions made on the role of vectors by examinations using only a single trapping method should be viewed with caution. There is a wide range of mosquito collection techniques available, reaching from sweep nets and insect aspirators to set traps (Chen *et al.*, 2011). While CO_2 baited light traps like the ones used in my study collect host seeking mosquitoes that may feed on a variety of animals, traps baited with readily available birds like chickens or canaries will accomplish a more specific collection (Kimura *et al.*, 2010). Both types of traps mostly collect unfed nulliparus females (Chen *et al.*, 2011, Carlson *et al.*, 2015) which have not come into contact with *Plasmodium* infected birds. To catch older mosquitoes which have taken and digested a blood meal, and therefore had exposure to malaria parasites, gravid traps would be the first choice. Gravid traps have been shown to catch significantly more positive females than CO_2 baited traps (Fryxell *et al.*, 2014), although they also collect a lower number of mosquito species (Chen *et al.*, 2011). On the other hand, certain mosquito species are preferably caught by gravid traps and may be missed by only using other types (Carlson *et al.*, 2015). This considerable trap bias should be addressed by using a multitude of trapping techniques to take into account the specific biology of different mosquito species (Carlson *et al.*, 2015). Future studies in New Zealand should take these points into account and use a variety of traps at different height levels to obtain a wider spectrum of mosquito species and a higher number of individuals preferring different kinds of traps. This approach will hopefully also provide engorged females

on which blood meal identification can be performed. This will reveal valuable information on the so far in New Zealand neglected question of which mosquito bites what kind of bird. The only mosquito in which blood meal identification has been performed in New Zealand so far has been a single engorged female *Culex pervigilans*, in which the blood of an introduced blackbird (*Turdus merula*) was found (Massey *et al.* 2007).

6.5.2 Avian malaria prevalence

My sampling was in most cases scheduled for summer/autumn, which was of advantage for catching infected mosquitoes, as seen by for example by Lalubin et al. (2013) in Switzerland. There, mosquitoes caught in summer were more likely to be infected than females caught in spring. Similarly, in a study by Ferraguit et al. (2013) in Spain, parasite prevalence in vectors was highest in autumn and lowest in spring.

The overall prevalence for avian malaria found in the mosquito pools (counting whole mosquitoes and not discriminating between thorax and abdomen) was 29.4%, which was similar to one study conducted in Cameroon, which identified a prevalence of 30% of 452 screened pools (Njabo *et al.*, 2011). When looking at prevalence in individual mosquito species, my findings are comparable with others worldwide. Two studies in Switzerland looking at *Culex pipiens* for example found an overall prevalence of 6.6% (n=394) in 2006/2007 (Glaizot *et al.*, 2012) and 13.1-20.3%, depending on the season in 2010/2011(Lalubin *et al.*, 2013). In France, looking at the same mosquito species, prevalence ranging from 0% (February) to 15.8% (October) was observed (Zele *et al.*, 2014). A study in the US, looking at different mosquito species, found 10% of 61 pools positive (Fryxell *et al.*, 2014). In studies in Japan, prevalence ranged from 14.3% to 23.9% depending on the area and the examined mosquito species (Kim *et al.*, 2009b, Kim and Tsuda, 2010, Ejiri *et al.*, 2011). When comparing the parasite prevalence between thorax pools, my finding of 12% was comparable to a study in Vanuatu and New Caledonia (Ishtiaq *et al*., 2008) which found a prevalence of 15.5% in tested thorax pools. The same study found a prevalence of 38.5% in individually tested thoraxi and 25% in individual abdomens, indicating that true *Plasmodium* prevalence may be overlooked when examining pooled samples. Ishtiaq et al. (2008) also sampled *A. notoscriptus*, but failed to find *Plasmodium* in both abdomen and thorax owing to the low sample size.

The MIR for different species of mosquitoes is very variable, and can range for example in *C. pipiens* from 3.1% (Kim and Tsuda, 2010) to 0.5% (Ejiri *et al.*, 2009) and 0.03% (Ventim *et al.*, 2012). In other species, like *C. bitaeniorhynchus*, MIR can reach as high as 6.0% (Kim and Tsuda, 2012) or as high as 13% (Fryxell *et al.*, 2014) in the complete mosquito species pool. The MIR I found in the mosquitoes during my study was therefore consistent with findings in other mosquito species worldwide (Ejiri *et al.*, 2011, Kim and Tsuda, 2012, Fryxell *et al.*, 2014),even if they were on the high end of the spectrum. The reason for this may be the low sample size, in particular, for *C. quinquefasciatus*.

6.5.3 Potential avian malaria vectors

As predicted, the prevalence of *Plasmodium* was higher in mosquito abdomen pools than in the corresponding thoraxes, with 26 (59.1%) out of 44 positives in the abdomens and 18 (40.9%) in the thoraxes. Separating abdomens from thoraxes for analysis provides much more information on competent vector status than whole insect DNA extractions and therefore prevents the overestimation of the number of mosquito species involved in malaria transmission (Carlson *et al.*, 2015). There were two potential vectors with *Plasmodium* detected in the thorax, the native *C. pervigilans* and the introduced *A. notoscriptus*. *C. pervigilans* has long been suspected to be a competent vector for avian malaria in NZ (Derraik and Slaney, 2007, Derraik *et al.*, 2008) and mosquitoes of the genus *Culex* are the most common vectors for these parasites worldwide (Kimura *et al.*, 2010, Glaizot *et al.*, 2012). However, I did not find *C. quinquefasciatus* thorax pools that were positive for *Plasmodium*, most likely due to the low sample size of this species. Yet as this mosquito is a proven competent vector, it can safely be assumed that this mosquito also transmits avian *Plasmodium* in New Zealand.

One possible problem with just studying vector thoraxes with molecular methods is the possibility that sporozoites, the parasite life stage before transmission to the vertebrate host, can occur in the haemocoel during their travel from the midgut to the salivary glands (Valkiūnas, 2011). If the vector is not fully competent, they may never reach and fully develop in the salivary glands. Consequently, the amplified parasite DNA from mosquito thoraxes may come from non-infectious parasite stages, and therefore non competent mosquitoes. To be able to fully confirm the competent vector status of different mosquito species in New Zealand, future studies will have

to involve experimental infections and/or microscopic detection of oocytes in the midgut and sporozoites in the salivary glands of the mosquitoes (Glaizot *et al.*, 2012).

In this study, *Plasmodium* parasites were also detected in one thorax pool of Hippoboscid flies. Hippoboscid flies are not considered competent vectors for avian *Plasmodium* and are instead known as vectors for the closely related *Haemoproteus* parasites in pigeons (Valkiūnas, 2005). Haemosporidian parasites infecting dipterans that are not their hosts may prove virulent for these, as seen in the high mortalities for the mosquito *Ochlerotatus cantans* infected with *Haemoproteus* (Valkiūnas *et al.*, 2014a). According to Martinsen *et al.* (2008), the major clades of haemosporidian parasites are associated with vector shifts in the different dipteran families. *Plasmodium* spp. parasitizing birds and reptiles rely on mosquitoes of the family Culicidae, excluding the genus *Anopheles* which is known to transmit parasites infecting mammals. Although this view was widely accepted, recent findings may lead to its revision. Biting midges of the genus *Culicoides*, formerly thought to be only able to transmit haemosporidian parasites of the genus *Haemoproteus*, have recently been found to be infected with *Plasmodium*, but the viability of these vectors to transmit the parasite still needs to be confirmed experimentally (Santiago-Alacron *et al.,* 2012).

Very few studies have been performed on testing the ability of avian *Plasmodium* parasites to develop within bloodsucking vectors other than Culicidae, as seen in some lizard *Plasmodium* species (Santiago-Alarcon *et al.*, 2012b). It is therefore possible that some hippoboscid flies in New Zealand have become competent vectors due to the low number of native mosquito species because of underutilized mosquito habitat (Peacock, 2013, Laird, 1950). There is also the possibility of contamination either during collection of the hippoboscids in the field, or during insect dissection, DNA extraction or PCR preparation although the upmost care was taken during all these analysis steps. However, contrary to what would be expected from contamination, *Plasmodium* sequences from abdomen and thorax pools presented different mixes of lineages (Table 6.4). Although this may indicate a separate infection in thorax and abdomen, it is also possible that standard PCR and sequencing techniques failed to detect all lineages in both pools, as these methods

are notoriously bad at detecting mixed infections. To determine vector status, only experimental infections and microscopic examinations, as suggested for the mosquitoes above, will bring clarification.

6.5.4 *Plasmodium* lineages

Sequence analysis of the *Culex pervigilans* mosquito found by Massey et al (2007) to carry *Plasmodium* showed 99% sequence similarity with *P. relictum*, but the lineage could not be identified because a primer set different from the standard cytochrome b one was used. This emphasizes the importance of adhering to standard analysis methods for producing results that can be compared worldwide between different research groups.

The *Plasmodium* lineages found in mosquitoes during this study are *Plasmodium* (*Huffia*) *elongatum* lineages GRW06 and Linn1 as well as the *P. (Novyella) vaughan*i SYAT05. In one case *Plasmodium relictum* GRW4 was detected. This is concurrent with my prediction that I would find the most common New Zealand lineages found in birds to be represented in the mosquito samples. Both *A. notoscriptus* and *C. pervigilans* carried the malaria lineages Linn1, SYAT05 and GRW6 in both abdomen and thorax and may be competent vectors for these lineages.

The three most common lineages found in my study have also been reported in several studies on mosquito vectors in Europe. SYAT05 was found in *Culex pipiens* in the Czech Republic (Synek *et al.*, 2013) and Switzerland (Glaizot *et al.*, 2012) and in *C. pipiens* and *C. theileri* in Portugal (Ventim *et al.*, 2012). All three were found, among other lineages, in *C. pipiens* in France (Zele *et al.*, 2014) and Switzerland (Lalubin *et al.*, 2013). For a more extensive review, all lineages which have been found so far in vectors have been collected on the MalAvi-database online at http://mbio-serv2.mbioekol.lu.se/Malavi/.

Compared to the number of lineages so far found in New Zealand, diversity was very low during this examination of mosquito vectors. The lineages Linn1, SYAT05 and GRW6 were widespread and were found on all positive sites around the North Island, except for Cape Kidnappers (only SYAT05 and GRW6) and Hen Island (SYAT05). *Plasmodium relictum* GRW4 was only found in Palmerston North. This was most likely due to low sample sizes, especially from the island sites. During my

examination of blood samples from saddlebacks and introduced birds (Chapter 5), a higher diversity of *Plasmodium* lineages was found. Linn1, SYAT05 and GRW6 were still the most common and widespread, but altogether ten different lineages were found. The highest number of different lineages could be reported in the source population of saddlebacks on Hen Island (n=7), followed by Tiritiri Matangi Island (n=5) and Bushy Park (n=4). Cuvier Island and Mokoia Island had the lowest diversity with only 2 different lineages found. The examined mainland sites (Mokoia Island, Auckland, Whangarei, Bushy Park and Cape Kidnappers) had lower *Plasmodium* diversity than Hen and Tiritiri Matangi Island, with four lineages at Auckland, three at Bushy Park, two for both Whangarei and Mokoia Island and one at Cape Kidnappers (Chapter 5). The low diversity of collected lineages may also be connected to the fact that sampling only being performed during late summer and early autumn. Lalubin et al. (2013) reported seasonal changes in lineage composition throughout the year. In their study, SYAT05 decreased from spring to summer in favour of three lineages of the *P. relictum* group (SGS1, GRW11 and PADOM02). Similar seasonal changes have also been observed by Kim and Tsuda (2012). In future studies performed in New Zealand, a wider range of seasons should be taken into account to mediate possible seasonal variations in *Plasmodium* lineage composition.

More than half of all positive mosquito pools examined in this study carried a mix of either two or all three of the three most common lineages. The presence of more than one lineage was found in abdomen and thorax pools. The high incidence of mixes in this study appears to be a by-product of examining pools of mosquitoes instead of looking at individuals, although the presence of several lineages, visible as double peaks on the chromatogram, have been found in other studies examining single mosquitoes (Zele *et al.*, 2014). When detecting mixes, and also *Plasmodium* infections in general, the qPCR/HRM method developed in Chapter 4 was less sensitive than the nested PCR. Real-time PCR techniques developed by other groups were more sensitive in finding infections with haemosporidian parasites, but these methods were also unable to differentiate between different *Plasmodium* species (Njabo *et al.*, 2011, Bell *et al*., 2015).

6.5.5 Implications for wildlife health in New Zealand

In this study, I identified a very likely competent native avian malaria vector, *C. pervigilans,* in New Zealand. With a competent native vector, *Plasmodium* transmission would have been feasible in New Zealand for a very long time and potential endemic lineages have been reported before (Schoener *et al.*, 2014). Therefore, endemic birds may have been exposed to malaria parasites for a long time and may not have been entirely naïve to *Plasmodium* like the endemic birds in Hawaii where these parasites had a devastating impact. During my study, I also found GRW4 in one mosquito pool from Palmerston North, but in the presence of recent findings (Chapter 5; Hellgren, 2015), this does not appear to be a reason for concern. Although deaths in native birds in New Zealand have been caused by the lineages GRW4, Linn1 and GRW6, the latter two seem to have had a bigger impact (Schoener *et al.*, 2014). A recent large-scale study of birds which were found dead in Austria also reported mortalities in 14.6% of 233 cases due to the *Plasmodium* lineages GRW6, SYAT05 and Linn1 (Dinhopl *et al.*, 2015), which were also the most common ones found during this study.

Although the most common *Plasmodium* lineages found in this study appear to be pathogenic and are able to cause mortalities, outbreaks of malaria as a disease are not very common in New Zealand and appear only under certain conditions. Most detected infections in New Zealand present with low level parasitaemia (Gudex-Cross, 2011). Cases of mortality have so far been found only in captive situations for example in dotterel chicks (Reed, 1997), yellowhead/mohua (Alley *et al.,* 2008) or brown kiwi (Banda *et al.*, 2013). Co-infection with avian pox virus also played a role in these mortalities, as seen in the case of the New Zealand dotterel chicks and an outbreak in South Island saddlebacks in the summers of 2002 and 2007 when there were concurrent severe decreases in population numbers (Alley *et al.,* 2010).

This has implications for conservation work in New Zealand which relies heavily on stressful wildlife translocations and captive breeding and rearing, because avian malaria can cause serious disease in immunologically naïve and susceptible birds. Globally, avian malaria is the main haemoparasite affecting birds in zoos and aviaries. For decades, the disease has been known for causing mortalities mainly in captive penguins (Graczyk *et al.*, 1995a, Graczyk *et al.*, 1995b, Graczyk *et al.*, 1993,

Graczyk *et al.*, 1994, Bennett *et al.*, 1993). This is an on-going problem in penguins as recent literature shows (Grim *et al.*, 2004, Bueno *et al.*, 2010, Dinhopl *et al.*, 2011, Silveira *et al.*, 2013) as well as in other bird species (Murata *et al.*, 2008). One reason for the high incidence of infections in zoos and aviaries is that birds imported from areas with different lineages of avian malaria are kept together. These birds have no adaptation to local malaria lineages, or as in the case of penguins from cold climates, are immunologically naïve. This problem is also extended to for example penguins in a rehabilitation setting, as seen reported by (Vanstreels *et al.*, 2015). The latter example has implications for New Zealand, since places like Wildbase, the wildlife clinic at Massey University, Palmerston North, take part in the recovery of injured, diseased or oiled penguins (among other native birds). Recently, mortalities at Wildbase have been caused by *P. elongatum* GRW6 in yellow-eyed penguins (*Megadyptes antipodes*) (E.S. pers. ops.). These birds had been kept in an open air facility as they recovered from injuries and it was suspected that they were infected by local vectors, which are plentiful in that area of Palmerston North and which carry a minimum of four different lineages as reported in this study. In conservation settings like these, mosquito proofing of enclosures is imperative in the future. To minimize the impact of avian malaria outside captive facilities, and to support birds for example in stressful translocation settings, one of the most important counter measures will be the control and reduction of mosquito larval habitat (Atkinson and LaPointe, 2009).

6.6 Conclusion

In conclusion, this study found four *Plasmodium* lineages, Linn1, SYAT05, GRW6 and GRW4 in the mosquitoes tested, with the first three in both abdomens and thoraxes of both *A. notoscriptus* and *C. pervigilans*. These mosquitoes are therefore likely competent vectors for avian malaria in New Zealand and found in high abundance at all sampled sites. One pool of hippoboscid flies was positive for a mixed *Plasmodium* infection in both thorax and abdomen, presenting the possibility that hippoboscids may also be vectors. This is the first detection of avian *Plasmodium* DNA in mosquito thoraxes and hippoboscid flies in New Zealand.

7. General discussion

7.1 General conclusion

The main aims of this thesis were to establish baseline data on the impact of North Island saddleback translocations on their avian malaria (*Plasmodium* spp.) parasites as well as to gain further insight into potential avian malaria vectors New Zealand. The study was also intended to contribute in the development of recommendations for future screening programmes for translocations of native rare passerines. As a result of these studies, a variety of genetic *Plasmodium* lineages previously unrecorded in North Island saddlebacks as well as in New Zealand have been identified. In addition, potential avian malaria vectors, of both native and introduced species, were recorded. Thus, the overall results contained within this thesis contribute to the broad and growing field of avian malaria research in passerines from New Zealand and around the world.

7.1.1 *Plasmodium* lineages in blood

Including the findings made in the course of this thesis, the *Plasmodium* diversity in New Zealand now comprises 22 different lineages found in 39 different bird species (Chapters 2 and 5) (Tompkins *et al*., 2008, McKenna, 2010, Castro *et al*., 2011, Howe *et al.*, 2012, Tompkins *et al*., 2010, Baron *et al.*, 2014, Schoener *et al.*, 2014, Hunter, 2015). It is likely that more parasite lineages will be found when more bird species and individuals are tested in the future, as I found a higher diversity of *Plasmodium* lineages in saddleback in this study than had been found previously, for example by Castro et al. in 2011. I also found a generally higher *Plasmodium* prevalence than had been previously noted in native birds (Baillie *et al*., 2011, Castro *et al*., 2011, Howe *et al.*, 2012, Ewen *et al.*, 2012b), and which was similar to the prevalence in birds overseas (Evans *et al.*, 2009, Beadell *et al.*, 2004, Valkiūnas, 2005). It is likely that at least some of the low prevalence reported previously resulted from the uneven distribution of DNA in samples resulting in some false negatives. In theory it is best practice to always test samples three times, however in Castro et al. (2011), samples were examined only once. During this study, I re-examined 50 of the saddleback blood samples Castro et al. (2011) collected in March 2008 (autumn) and reported in their study. Using the same techniques they

used (Hellgren *et al.*, 2004) I found a higher prevalence of 14.3% in contrast to theirs of 2.8%). It is difficult to predict what this difference would mean in terms of their conclusions without running all the samples again and calculating averages for all sampling periods. However, this observation highlights the importance of taking several samples from the blood rather than a single one, and suggests that in the future PCR's should indeed be carried out in sets of three or more, each representing a different sampling of the blood sample, and use averages instead of single measurements to determine prevalence.

It is very likely that different *Plasmodium* lineages have been transported around the North Island of New Zealand with saddleback translocations. In most cases, it would have been the most common lineages *P. elongatum* GRW6 and Linn1 and *P. vaughani* SYAT05, which had themselves most likely travelled to New Zealand with introduced passerines from Europe. Another possible translocated lineage is KOKAKO01, which is present on both Hen and Tiritiri Matangi Islands. This lineage is most likely native and specific to the endemic wattlebirds family, which includes saddlebacks, kokako and the extinct huia. This lineage was present in blood samples from Tiritiri Matangi Island birds which were translocated to Maungatautari and were examined for this study. In a future study, the newly established saddleback population at Maungatautari should be tested again for the presence of this parasite lineage, to find out if the parasite was able to establish itself at the new site or if it was lost after translocation. If this parasite is indeed native, save guarding its survival along with the translocated host is important for the preservation for New Zealand biodiversity, something which should be encouraged in future translocation projects.

As I showed in Chapter 5 of this study, parasite lineages may have been lost with translocations due to population bottle necks of the host, the NI saddleback. I found that Hen Island had the highest *Plasmodium* diversity in saddlebacks and sites which are not considered "mixing pots" have less than half of the number of lineages. In addition, the number of parasite lineages on small offshore islands was smaller than on the mainland sites, with exception of the source population on Hen Island. This indicates that biodiversity in small islands is smaller than on bigger ones (such as, the mainland); even if parasites manage to be transferred to a new (small) island, the diversity of vectors and small bird population sizes will limit the number of species

that can be supported. To confirm these observations, more samples from a larger range of bird species as well as from a larger number of sites need to be obtained and examined.

By far the most common lineages found in this study were *P. elongatum* GRW6 and Linn1 and *P. vaughani* SYAT05 which have been observed worldwide and are common in birds introduced to New Zealand from Europe. This finding begs the question as to generalist cosmopolitan parasites are indeed dominating the New Zealand diversity, or if the true diversity is not detected by standard methods. For example, are there NZ *Plasmodium* species that are not being detected with the methods at hand? Some support for this possibility stems from the differences in species found when using PCR and smears. It is very likely that during this and other studies previously undertaken in New Zealand, the true *Plasmodium* diversity was underestimated because of the currently inadequate molecular assays and inexperience when performing microscopy.

Another interesting finding in this study (Chapter 5 and 6) is the apparent absence of the avian haemosporidian genus *Haemoproteus*, which is very closely related to avian *Plasmodium*. In other parts of the world, *Haemoproteus* parasites have a much higher diversity than *Plasmodium*, because they are generally considered to be more host specific (Valkiūnas, 2005).These parasites are very common in Europe in the passerine species that were introduced to New Zealand. In the Pacific, these parasites are also common in Australia, New Caledonia and Vanuatu, but are absent from French Polynesia (Clark *et al.*, 2014, Olsson-Pons *et al.*, 2015). The apparent absence in New Zealand is interesting, since the dipteran insect family vectoring these parasites, biting midges (Ceratopogonidae), is present in New Zealand (M. Minor, 2013, pers. com.) and it is likely these parasites have been introduced with European passerines (G. Valkiūnas, 2013, pers. com.). Indeed, Laird (1950) described *Haemoproteus* parasites on blood smears from introduced song thrushes and blackbirds in New Zealand. New Zealand birds appear to be susceptible to *Haemoproteus* and mortalities in captive New Zealand psittacine species have been observed in Europe (Olias *et al.*, 2011). It is possible that New Zealand had *Haemoproteus* species before the arrival of humans, but the extinction and serious population decline of many native birds has also caused the demise of their host

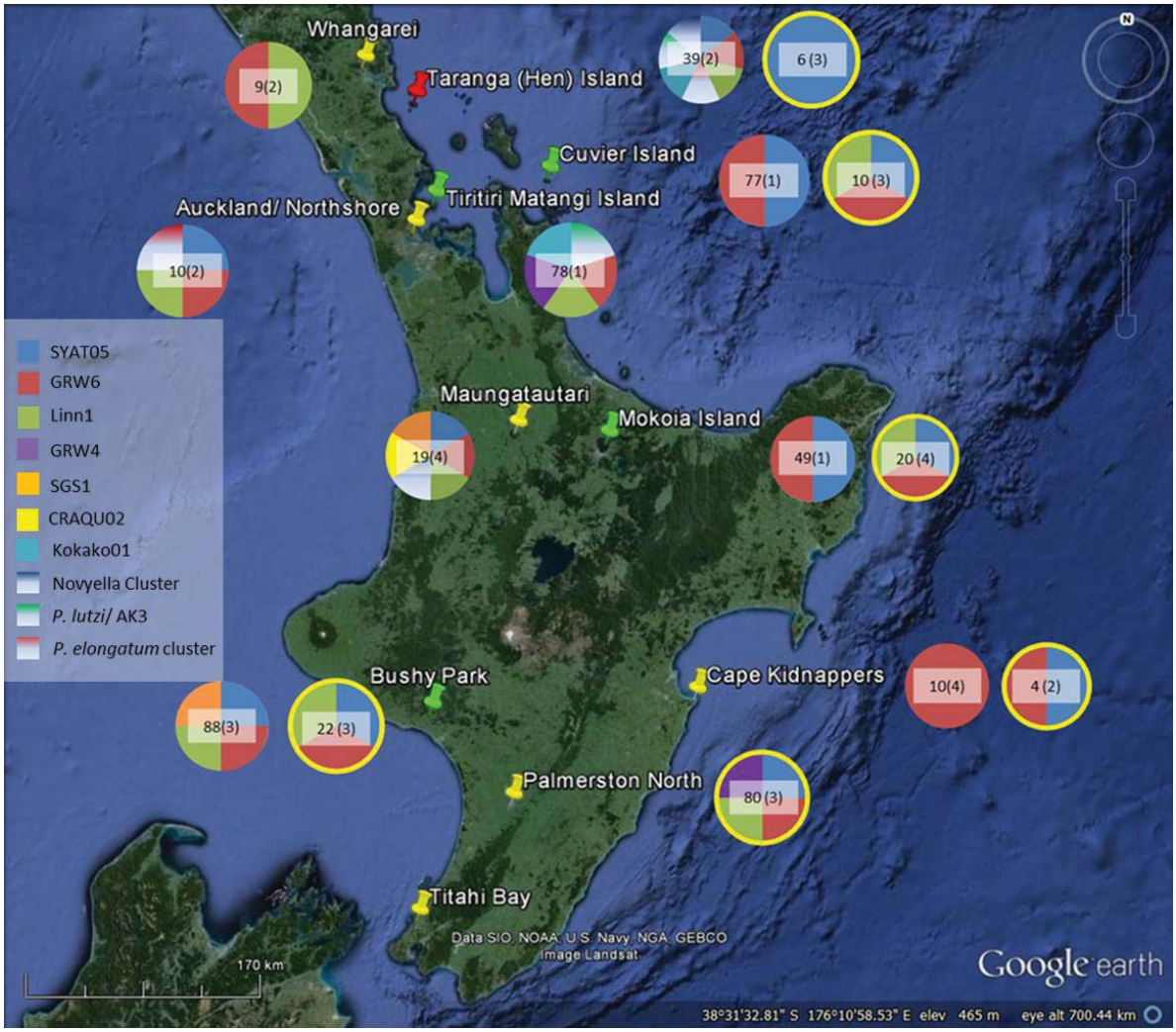
specific parasites. As for the introduced *Haemoproteus* from Europe, the biting midges in New Zealand may have proven unsuitable vectors for these parasites. It is also a possibility that current detection methods are inadequate for finding these parasites.

7.1.2 *Plasmodium* lineages in potential vectors

It is not surprising that the two most common mosquito species in New Zealand, *C. pervigilans* and *A. notoscriptus* (Chapter 6), were also the two most commonly detected species in this study. That both species had avian malaria DNA in their thorax pools lends weight to their role as competent vectors for avian *Plasmodium* in New Zealand. The significance of finding *Plasmodium* DNA in the thorax of hippoboscid flies in this study remains unclear, and their vector status will have to be examined by experimental infections. Very few mosquitoes were collected on the offshore islands in this study and future sampling efforts at these sites are of interest to understand the dynamics between New Zealand's rare endemic birds, both native and introduced vectors and malaria parasites.

When comparing *Plasmodium* lineage diversity in blood versus mosquito samples, I found that blood samples had a higher diversity (Figure 7.1). Still, the most commonly found lineages in blood, *P. elongatum* Linn1 and GRW6 and *P. vaughani* SYAT05, also dominated in the examined mosquitoes. In three cases, on Cuvier and Mokoia Islands and at Cape Kidnappers, the diversity in the examined mosquitoes was higher than in the tested bird blood (Figure 7.1). There are several reasons which may explain these findings. Firstly, the sample size of the collected mosquitoes at most sites was very low due to climatic reasons. Secondly, pooled vector samples were examined in this study. It has been noted by Kimura et al. (2010) that the *Plasmodium* diversity in vectors was greater than in sampled birds, including previously not recovered lineages from birds, when the mosquitoes were examined individually. Finally, it is most likely that the above mentioned problems with standard PCR methods, where the DNA of certain malaria parasites is preferentially amplified, comes into play here as well, since it is more likely for there to be several different parasite species in pooled samples than in individual ones.

Figure 7.1: Map of the North Island of New Zealand with sampling sites visited during this study, comparing lineages found in both samples bird blood and mosquito pools; Hen Island, origin of the source population of NI saddleback, has been marked in red, subsequent translocation sites in green and mainland sites (for introduced birds) in yellow. The pie charts present the number of different *Plasmodium* lineages found at a given site each represented by a different colour. Red = GRW6, Blue = SYAT05, Green = LINN1, Turquoise = KOKAKO01; Orange = SGS1, Purple = GRW4, Yellow = CRAQU02, Gradient blue = *Novyella* Cluster, Gradient green = *P. lutzi*/ AK3, Gradient red = *P. elongatum* cluster. The numbers in the centre of the pie charts represent the number of individual birds sampled/mosquito pools examined and the number of bird/mosquito species in brackets. Pie charts with a yellow border represent lineages found in collected mosquito pools, pie charts without border represent the sampled bird blood.



7.1.3 Recommendations for future screening programmes for translocations of rare birds in New Zealand

In Chapter 6, the native mosquito *C. pervigilans* was identified as a very likely avian malaria vector. In using a competent native vector, *Plasmodium* transmission has been feasible in New Zealand for a very long time and potential native lineages have been reported before (Schoener *et al*., 2014). Therefore, endemic birds have been

exposed to malaria parasites probably for a long time and are not entirely naïve to *Plasmodium* unlike the endemic birds in Hawaii where these parasites had a devastating impact. However, it is possible that *Plasmodium* lineages brought over with birds from Europe by Acclimatisation Societies in the 1860s have contributed to the extinction of some endemic bird species like the Huia. In retrospect, it is impossible to judge the impact that the introduction of these parasites had on native avian biodiversity in New Zealand. Yet, the impoverished nature of New Zealand's bird fauna may pose a problem, since there is evidence that suggests that in highly diverse host communities the chance of a parasite being vectored into an appropriate host is diminished (Hellgren et al., 2009). Most infections in New Zealand present with only low level parasitaemia (Gudex-Cross, 2011). Mortality has so far only been observed in captive situations including dotterel chicks (Reed, 1997), yellowhead/mohua (Alley *et al*., 2008) or brown kiwi (Banda *et al*., 2013). A co-infection with avian pox virus also played a role in some of these mortalities, as seen in the case of the New Zealand dotterel chicks and an outbreak in South Island saddlebacks in the summers of 2002 and 2007 with which there were concurrent severe decreases in population numbers (Alley *et al*., 2010). However, the possibility of lethal abortive infections in native birds does remain and can only be resolved by further studies into the pathology and epidemiology of avian malaria infection, preferably by experimental infections of the bird host in question. If avian malaria is expected to cause concern, for example after stressful translocation events or in captive situations, effective vector control should be implemented. This will prevent the infection of susceptible birds as well as transmission of parasites from affected birds which have reverted to an acute infection due to stress related immune suppression.

The most important step for future wildlife health screening for translocations in New Zealand is the development of a standardized protocol concerning a range of possible pathogens, not only avian malaria. It should be agreed upon as to which diseases to screen for and a plan has to be set in place as to how to deal with infections if they are found. In the case of avian malaria in saddlebacks, this study has shown that the most commonly found lineages in saddleback are distributed around the whole of the North Island and are also common in the mosquito populations. Therefore, saddlebacks infected with these lineages prior to

translocation, and which they do not show signs of acute infections, do not pose a risk for introducing new parasites to new areas and translocation should take place. The same appears true for the lineage *P. relictum* GRW4, which caused devastation in Hawaii. Recent research showed that it is likely that this parasite has been in New Zealand longer than it has been in Hawaii (Hellgren *et al*., 2015), and I also found it at two different mainland sites during this study (Chapter 5 and 6; Figure 7.1). It is very likely to be present at other sites as well, although it appears to be not as readily detected by PCR (see above).

The real-time assay described in this study will be helpful in conservation management by providing an initial first overview of avian malaria lineage presence at an examined site, especially if birds are found sick or dead. The assay produced fast and accurate data that can be used in rapid disease screening for *Plasmodium* parasites in native New Zealand birds suffering from acute infection with clinical symptoms as well as birds that have been found dead. The advantage of this method compared to standard PCR and microscopy is the speed, lower cost and the ability to detect some mixed infections. For more detailed prevalence and diversity data, this method did not prove sensitive enough and standard nested PCR as well as microscopy are still necessary. Therefore, this method enables conservation managers and wildlife veterinarians to obtain an overview of *Plasmodium* and, if present, to be able to identify which of the four most common lineages maybe playing a role in morbidity and mortality of monitored bird populations in captivity, at reserves that already have certain native bird species, and at future translocation sites.

Future translocation management decisions also have to take into account the preservation of native parasite species. Birds found positive for potential native *Plasmodium* lineages like Bell01 and Kokako01 (Baillie and Brunton, 2011, Ewen *et al*., 2012b, Howe *et al*., 2012) should be translocated with their parasites provided that the birds show no adverse effects at the time of capture. Until recently, animals in captive breeding programmes as well as translocation settings have been kept as parasite free as possible (Rideout, 2015). Parasites play important roles in ecosystems and the loss of host-specific parasites from endangered species in captive breeding programmes as well as strict indiscriminate parasite treatment of translocated animals poses a substantial threat to the conservation of biodiversity

(Daszak *et al*., 2000). New Zealand has already lost one host specific feather louse, *Rallicola (Aptericola) pilgrimi,* when the host, the little spotted kiwi, became extinct on the mainland (Buckley *et al.*, 2012) and the list is most likely longer due to the lack of baseline data on native parasite diversity. This loss of parasites may be positive for the hosts in the short term, but it may lead to a loss of genetic variation in immunity within the species and therefore make the species more susceptible to disease outbreaks in the long term (Altizer *et al.*, 2003, Smith *et al*., 2009, Sainsbury, 2015). Successful conservation programmes should maintain populations with intact evolutionary processes that include their parasites (Altizer *et al.*, 2003) and there are already management methods available to conserve parasites and at the same time safeguard the health of the hosts (Sainsbury, 2015).

Another possibility to conserve parasites in general would be to introduce a native parasites to a population after it has established and/or the initial stress of the translocation has passed (Isabel Castro, pers. comm.), although this option may not work for all parasites (those which cannot survive outside the host, cannot be cultured, have an unknown biology and life cycle, or are very rare in the source host population). In the case of vector-borne parasites, the introduction of infected vectors would be a possibility.

7.2 Future studies

Future studies are necessary to gain a better picture of avian malaria parasite diversity and their pathology and epidemiology in native birds. In addition, the question about definitive competent vectors remains unresolved. In the light of my results, additional questions need to be addressed. While it might be difficult to establish how much of the original haemosporidian parasite diversity in New Zealand has been lost after the arrival of humans, certain other fields can be examined. Firstly, has the haemosporidian parasite diversity in New Zealand already been well documented or are there more species, especially native species, out there to discover? Secondly, how pathogenic are these parasites to rare native birds in New Zealand and what impact do introduced cosmopolitan lineages have as opposed to native ones? Thirdly, what is the life cycle of these parasites and which vectors are utilised by which parasite? I will discuss these points below.

7.2.1 Identification of *Plasmodium* diversity and mixed infections

More individuals of the lesser sampled bird species in New Zealand need to be examined to document the diversity of *Plasmodium* and to discover previously unknown species and lineages. The study presented here found one lineage previously unrecorded in New Zealand in Australian magpies as well as three new ones in North Island saddleback. Therefore it is likely that with the examination of more birds, both species and individuals, more discoveries will be made. In addition, birds should also be examined for a longer time period in different seasons, similar to the study by Castro et al. (2011), in order to take into account changes in lineage composition throughout the year as well as between years.

Currently, health screening of birds for *Plasmodium* spp. infection relies on either the examination of blood smears or a PCR. Both methods have their advantages and shortcomings, so they are best used side by side in order to achieve the most accurate results (Valkiūnas *et al*., 2006, Valkiūnas *et al.*, 2014b). Still, both methods potentially overlook low level parasitaemia and the true diversity of *Plasmodium* parasites, because different parasite stages can be hard to identify to species level by microscopy and PCR is in many cases unable to detect mixed infections (Valkiūnas *et al.*, 2006a, Palinauskas *et al.*, 2015). Taking into account that mixed infections are more common than single ones (G. Valkiūnas, 2015, pers. com.), current PCR methods are skewing the results towards parasites that are preferentially detected.

To rectify these issues, new molecular markers need to be developed to detect and identify the true diversity of avian *Plasmodium* and for the study of population genetics and phylogenetic relationships (Valkiūnas *et al*., 2014). This is a large task, as molecular markers have only been developed for around 20% of the currently known avian haemosporidian parasite known so far (Valkiūnas *et al.*, 2014b). It will also help provide better understanding of the disease caused by these parasites, especially in the case of lethal abortive infections which cause great damage to tissues before the infection can be seen in the blood (Valkiūnas *et al.,* 2014). The use of genotyping by sequencing techniques may overcome some of the issues with current PCR techniques and this needs to be developed and tested.

For New Zealand, a sensitive detection method is needed to identify the common low parasitaemia in native bird blood samples. This method should also be able to detect the most commonly found *Plasmodium* lineages as well as mixed infections. The best choice of method for future studies would be the use of lineage specific probes for a TaqMan®- assay which would greatly improve the specificity of the real-time PCR. Several different probes can be used in a single multiplex real time PCR assay to detect multiple lineages. So far, the use of this approach is limited by available funding as specific probes are very costly. According to Valkiūnas et al. (2014), the high diversity of avian haemosporidian parasites will make this task difficult in a global setting, but the limited number of lineages found in New Zealand should make it achievable here.

To reach the goal of creating species specific probes, further full gene sequencing of the cytochrome b gene from all known lineages in New Zealand has to be performed, to enable the design of these probes. Yet, the cytochrome b gene may not be the best gene to use for this task, as it has a high AT base content (approximately 73%) in malarial parasites, which makes designing effective primers difficult (Fallon et al., 2003b). Therefore, the possibility of using other genes needs to be explored. For this, more baseline data on the genome of avian malaria parasites in New Zealand is needed, which could be gained by using full genome sequencing.

7.2.2 Origin of avian *Plasmodium* in New Zealand

Learning more about the origin of *Plasmodium* parasites in New Zealand will help to assess current and future risks posed to native birds as well as providing insight into the possible historical impact these parasites have had on New Zealand biodiversity. New Zealand is an old landmass and it's endemic bird fauna is equally ancient and has been isolated for a long time (Ewen *et al.*, 2006). Still, it is very likely that avian *Plasmodium* has been in New Zealand since before the arrival of humans, as the native *Culex* mosquitoes are very likely vectors of these parasites and potential native *Plasmodium* lineages have been identified. While it will be nearly impossible to examine historic specimens for avian malaria and possible extinct lineages, it would be interesting for future work to continue sampling in the Pacific area and compare findings with New Zealand, to establish the taxonomic relationships between parasite lineages around the Pacific and gain insight into their distribution pathways and evolution. For this, it would also be of interest to examine migratory

birds that travel between the Pacific Islands and New Zealand, such as the Shining Cuckoo (*Chrysococcyx lucidas*) and the long tailed cuckoo (*Urodynamis taitensis*) in order to gain insight into the parasite diversity shared between both areas. This may contribute valuable information, as a study examining four passerine families on the Curonian Spit in the Baltic Sea (Krizanaskiene *et al*., 2006) presented evidence that the genetic diversity of haemosporidian parasites may be positively correlated to migratory strategies of the host. The authors found that blackcaps (*Sylvia atricapilla*), a species with three different migration strategies (sedentary population, short distance and long distance migration) harboured the largest proportion of exclusive lineages of haemosporidian parasites observed in any passerine bird thus far (Krizanaskiene *et al*., 2006). In addition, study by Levin et al. (2013) provided evidence that *Plasmodium* parasites were introduced to the Galapagos Islands with migratory birds.

7.2.3 Vector studies

Vector studies in NZ have been neglected and are just starting to become of interest, therefore little is known about the roles of the different mosquito species in New Zealand in the avian malaria life cycle. Future studies on avian malaria vectors in New Zealand should expand on this and other previous studies and should monitor mosquito populations and their *Plasmodium* infections for a longer period of time, in order to negotiate seasonal changes, yearly variation and climatic impact factors. It would be of particular interest to sample more mosquito species and individuals from the remote offshore islands around New Zealand which are used as sanctuaries for rare endemic birds. These studies should use a variety of traps at different height levels in the canopy to obtain a wider spectrum of mosquito species and a higher number of individuals preferring different kinds of traps. This approach will hopefully also provide engorged females on which blood meal identification can be performed, to reveal which mosquito species bites what species of bird.

To be able to fully confirm the competent vector status of different mosquito species in New Zealand, future studies will have to involve experimental infections and/or the microscopic detection of oocytes in the midgut and sporozoites in the salivary glands (Glaizot *et al.*, 2012).

7.3 Outlook

The biological world today is under severe threat by human activities, and we may already have entered a new era of species mass extinction (Wake and Vredenburg, 2008, Barnosky *et al.*, 2011, Pievani, 2014). But all is not lost, habitat restoration, wildlife translocations and captive breeding programmes will remain a tool to mitigate some of the most pressing issues. However, in order to preserve global biodiversity, attention also has to be paid to the vast group of organisms that live with, on, inside and from other, larger organisms, namely their parasites. Parasites are among the most diverse groups of all organisms (Windsor, 1995) and it is likely that they make up the majority of species on earth (Windsor, 1998). Due to this, it can be assumed that parasites make up the unseen majority of species extinctions (Dunn *et al.*, 2009, Stork and Lyal, 1993, Lafferty, 2012) and biodiversity loss may reduce parasite diversity more than previously thought (Lafferty, 2012). Since parasites play an important role in any ecosystem (Lafferty *et al.*, 2008, Tompkins *et al.*, 2011), the potential impact of the loss of such a great part of their diversity is unfathomable. Therefore, because wildlife translocations aim at the conservation of biodiversity, they also have to help preserve ecosystem function in the future which includes the preservation of parasites. As Windsor (1995) writes, “If we truly appreciate biological diversity, we must advocate that all species are precious, even parasites”. He therefore demands an “equal right for parasites” in species conservation. So far, New Zealand has already lost one of its endemic parasites, a feather louse of the little spotted kiwi, during the translocation that saved the vertebrate host (Buckley *et al.*, 2012), but the number is most likely much higher. Some of these parasites may have disappeared without ever being documented. Luckily, conservation management in New Zealand is starting to recognize that parasites are part of the biodiversity in New Zealand’s native fauna, a part that is hardly known and understood and which is also at risk of disappearing before anything is known about them.

Appendix 1: Avian Malaria in New Zealand

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Review

Avian Malaria in New Zealand

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Abstract

Avian malaria parasites of the genus *Plasmodium* have the ability to cause morbidity and mortality in naïve hosts, and their impact on the native biodiversity is potentially serious. Over the last decade, avian malaria has aroused increasing interest as an emerging disease in New Zealand with some endemic avian species, such as the endangered mohua (*Mohua ochrocephala*), thought to be particularly susceptible. To date, avian malaria parasites have been found in 35 different bird species in New Zealand and has been diagnosed as causing death in threatened species such as dotterel (*Charadrius obscurus*), South Island saddleback (*Philesturnus carunculatus carunculatus*), mohua, hihi (*Notiomystis cincta*) and two species of kiwi (*Apteryx* spp.). Introduced blackbirds have been found to be carriers of at least three lineages of *Plasmodium* sp. and because they are very commonly infected they are likely sources of infection for many of New Zealand's endemic birds. The spread and abundance of introduced and endemic mosquitoes as the result of climate change is also likely to be an important factor in the high prevalence of infection in some regions and at certain times of the year.

Although still limited, there is a growing understanding of the ecology and epidemiology of *Plasmodium* sp*.* in New Zealand. Molecular biology has played an important part in this process and has markedly improved our understanding of the taxonomy of the genus *Plasmodium.* This review presents our current state of knowledge, discusses the possible infection and disease outcomes, the implications

for host behaviour and reproduction, methods of diagnosis of infection, and the possible vectors for transmission of the disease in New Zealand

Key words: Diagnosis, Haemosporidia, native birds, pathology, *Plasmodium*, taxonomy.

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1. Introduction

Haemosporidians are single celled, intracellular parasites that have an apical complex (their phylum was formerly known as “Apicomplexa”) which is composed of specialised organelles that are believed to be important in host cell penetration (Atkinson and van Riper III, 1991). Haemosporidians rely on arthropod vectors to complete their life cycle. The order Haemosporidia contains several genera of avian blood parasites, namely *Haemoproteus*, *Parahaemoproteus*, *Leucocytozoon*, *Plasmodium* and the very closely related mammalian parasite *Hepatocystis*.

Opinions about which genera to include vary, so the term “avian malaria” is either used only for the genus *Plasmodium* (Avian Malaria Initiative/MalAvi Database) or for the closely related genera *Plasmodium*, *Haemoproteus*, *Parahaemoproteus* and *Hepatocystis* (Martinsen *et al.,* 2008) which are known to produce malaria pigment (hemozoin) in infected erythrocytes. For the purpose of this review, we will only describe parasites of the genus *Plasmodium*.

Avian malaria parasites need two types of host to complete their life cycle; the fertilisation, formation of zygotes and the asexual sporogony occur in an invertebrate host while the sexual gametogony and asexual merogony take place in a vertebrate host. There are four phases in the life cycle of *Plasmodium* spp. in the vertebrate host. The first phase is the *prepatent* phase in which the parasites undergo the initial cycles of merogony and are not circulating in the blood (Atkinson and van Riper III, 1991; Valkiūnas, 2005). In birds experimentally infected with *Plasmodium*, the *prepatent* phase ranged from two days to several months (Valkiūnas, 2005). The second phase is the *acute* phase in which the parasites begin to appear in the

circulation and rapidly increase in number. This is followed by the *crisis* phase when the parasitemia and pathophysiological stresses caused by the infection reach a peak. Acute infections are generally more severe in hosts which have had no previous contact with the infecting parasite lineage (Atkinson and van Riper III, 1991; Valkiūnas, 2005). If the bird survives the acute and crisis phases, the infection enters a *latent or chronic* phase which might persist for years (Atkinson and van Riper III, 1991; Valkiūnas, 2005).

2. *Plasmodium* species infection in New Zealand birds

Avian malaria parasites have been known to exist in New Zealand for decades, and blood stages in birds were first described by Laird (1950). In 1950, Laird remarked that indigenous birds in New Zealand were remarkably free of haematozoa and he found no evidence of haematozoa in endemic birds, although he suggested that a far wider study would be needed and his sample size was low. Nevertheless, Laird (1950) examined blood smears from 50 different bird species, including six species of passerines, mostly introduced. Another large scale blood-parasite survey of 43 bird species in New Zealand, including native and introduced, performed in the 1970's was unable to detect avian malaria in the examined birds, possibly due to a low sample size of n=1-10 per bird species (Fallis *et al.,* 1976). The findings of Laird and Fallis are in contrast to studies undertaken in the last decade which suggest that avian malaria may be an emerging threat to New Zealand avifauna (Tompkins and Glesson, 2006; Howe *et al*., 2012; Ballie and Brunton, 2011).

2.1 Lineages Identified

To date, avian malaria parasites belonging to 17 lineages have been found in 35 different bird species in New Zealand (Table 1) (Castro *et al.,* 2011; Ewen *et al.,* 2012b; Howe *et al.,* 2012). The most common lineages infecting endemic/native New Zealand bird species but also introduced passerines are *Plasmodium (Huffia) elongatum* lineage GRW06 and *P. (Novyella)* spp. lineage SYAT05; with *P. elongatum* (GRW6) having the widest host range. Other lineages of *Plasmodium* detected in endemic species are *Plasmodium relictum* (lineages GRW4 and SGS1) (Castro *et al*., 2011; Ewen *et al.,* 2012b; Howe *et al.,* 2012) and *Plasmodium sp*. (Linn1), which has not yet been resolved taxonomically, but clusters closely with

species of the subgenus *Huffia* (Howe *et al*. 2012). In addition, although not common, the lineages *Novyella* (AFTRU08) and *P. relictum* (LINOLI01) have been found in bellbirds (*Athornis melanura*) (Baillie and Brunton 2011). All the lineages mentioned above are not known to be endemic to New Zealand and show a high prevalence in European birds introduced to New Zealand (Ewen *et al.* 2012b). In contrast, a lineage, labelled "Kokako01" has to date only been found in kokako (*Callaeas cinerea*) (Howe *et al.,* 2012; Ewen *et al.,* 2012b) and lineage called Bell01 has only been found in native passerines (Baillie and Brunton, 2011), and may be endemic *Plasmodium* lineages (Ewen *et al*., 2012b).

Globally, avian haemosporidia are currently represented by approximately 200 morphospecies, of which 38 are recognised species of *Plasmodium* parasitizing birds worldwide. The richest fauna of *Plasmodium* parasites is found in galliformes (17 species) followed by passeriformes with 16 different species. The genus *Plasmodium* contains several subgenera, namely *Haemamoeba*, *Bennettinia*, *Giovannolaia*, *Huffia* and *Novyella* (Valkiūnas, 2005). Recently, Landau *et al.,* (2010) have proposed a sixth subgenus "*Papernaia*" containing species previously listed as *Novyella* but without a morphological feature; the refringent globule.

Among the species in the subgenus *Haemamoeba* is *Plasmodium relictum*, a pathogenic protozoan which can cause severe infections. *Plasmodium relictum* is to date the most common avian malaria parasite worldwide with an extensive host range covering many bird orders.

The subgenus *Novyella*, contains the species *Plasmodium* (*Novyella*) *vaughani*, which is the second most common *Plasmodium* sp. So far, the pathogenicity of the members of the genus *Novyella* has been insufficiently investigated, but they generally seem to produce mild infections with low mortalities. These parasites can infect a range of bird species, but are most commonly found in passerines.

Plasmodium elongatum belongs to the subgenus *Huffia* and it is another common pathogenic parasite, often causing mortalities (Valkiūnas, 2005). *P. elongatum* has a very wide host range, and passerine birds are thought to act as reservoir hosts.

2.2 Prevalence

The prevalence among avian hosts can vary significantly between sex, region, seasons and years (Table 2). For example, Tompkins and Gleeson (2006) reported a very high prevalence of *Plasmodium* in introduced passerines in the North Island of New Zealand, with up to 100% of the examined blackbirds infected in some regions. However, the prevalence was markedly lower in the south of the South Island with no blackbirds (n=4) caught in the Blue Mountains (Otago) being infected with *Plasmodium*. In addition, Baillie *et al*. (2012) sampled bellbirds from Tiritiri Matangi and Little Barrier (Hauturu) Island and found that female bellbirds had a statistically lower prevalence than males, with prevalence in males up to 2.8 times higher than in females. The overall prevalence of infections ranged from <1% to >45% across the seasons, with prevalence being highest in the winter at Hauturu (Little Barrier Island). The prevalence in males tended to peak during autumn and spring at all sites, with the prevalence in females lower all year round, but with a pronounced peak at the end of the breeding season on Tiritiri Matangi Island. A cluster of infection may also occur in one year but infection is absent in the same location the following year as observed in North Island brown kiwi in an Operation Nest Egg (ONE) facility in Rotorua (Banda *et al.,* 2013). This suggests that a seasonal variation of vector abundance may have been responsible in establishing conditions suitable for the cluster event. The prevalence of each *Plasmodium* species can also vary within host species. For example, Ewen et al (2012b) reported a higher prevalence (~12%) of lineage GRW06 than lineage Bell01 (~4%) in the same population of North Island saddlebacks which may reflect evolutionary relationships between native and exotic lineages of avian malaria.

2.3 Pathogenicity

Recent outbreaks of avian malaria in NZ passerines caused by a variety of *Plasmodium* spp. have been recorded in captive yellowhead/mohua (Alley *et al.,* 2008), European blackbird (Schoener, 2009) and South Island saddleback (*Philesturnus carunculatus carunculatus*) (Alley *et al.,* 2010). In addition mortality events have also be reported in captive dotterel chicks (Reed, 1997) and in brown (*Apteryx mantelli*) and great spotted kiwi (*Apteryx haastii)* (Alley *et al.,* 2012; Howe *et al.,* 2012; Banda *et al.* 2013). This suggests that although most lineages only cause

low level chronic infections (Gudex-Cross, 2011), they are also capable of causing mortality under the right conditions.

These findings are compatible to those from studies in other parts of the world. Generally *Plasmodium* spp. cause little direct mortality in their natural environments and are relatively harmless to wild birds (Bennett *et al.,* 1993; Valkiūnas, 2005), although this depends on the *Plasmodium* species in question. For example, the susceptibility of various bird species to avian malaria is markedly different. When experimentally infected with *Plasmodium relictum* (lineage SGS1), starlings (*Sturnus vulgaris*) were resistant to infection, while house sparrows (*Passer domesticus*) and chaffinches (*Fringilla coelebs*) showed only light parasitemia and siskins (*Carduelis spinus*) and crossbills (*Loxia curvirostra*) were severely affected (Palinauskas *et al.,* 2008).

Avian malaria may play a significant role in concomitant infections with other diseases under certain conditions (Bennett *et al.,* 1993) and can cause serious disease in immunologically naïve and susceptible birds. For example, avian malaria is the main haemoparasite affecting birds in zoos and aviaries. For decades, the disease has been known for causing infections and mortalities mainly in captive penguins (Cranfield *et al*., 1990, Bennett *et al*., 1993, Graczyk *et al*., 1994). This is an on-going problem in penguins as recent literature shows (Pacheco *et al.* 2011) as well as in other species (Bueno *et al*., 2010, Murata *et al.,* 2008). One reason for the high incidence of infections in zoos and aviaries is that birds imported from areas with different lineages of avian malaria are kept together. These birds have no adaptation to local lineages. In addition, often local vectors and other birds which are carriers of parasites are available in the surroundings (Beier and Stoskopf, 1980, Cranfield *et al*., 1990, Pacheco *et al.,* 2011). In New Zealand, this was illustrated in an outbreak of avian malaria in captive mohua (*Mohoua ochrocephala*) at Orana Park in Christchurch (Alley *et al.,* 2008).

2.3.1 Clinical Signs

The clinical signs shown by birds infected with *Plasmodium* spp. depend on the species of bird as well as the lineage of parasite involved. Infected birds can remain

asymptomatic (Ritchie *et al.,* 1995). Studies in wild birds are to a great extent biased towards birds with low parasitemia and chronic infections, because mist netting is used as the main capture method. For example, sick birds in the wild are less likely to move around and are therefore less likely to be caught in the nets. So far, no solution has been found to improve capture rates of heavily infected birds (Valkiūnas 2005).

Acute and crisis phases of infection

In general, birds with the overt disease show the following signs: Vomiting, anorexia, depression, dyspnoea, anaemia of the regenerative haemolytic type, splenomegaly, hepatomegaly and pulmonary oedema. In susceptible Hawaiian amakihi, the birds showed high parasitemias in the acute phase with a decline in food consumption and body weight and then became lethargic making them more susceptible to predators (Atkinson *et al.,* 2000). However, in experimentally infected passerines in Europe, no effect on body mass and temperature was seen, but the birds had a significant decrease in their haematocrit value and hypertrophy of the liver and spleen was seen in the two most severely affected bird species (Palinauskas *et al.,* 2008).

Hepatomegaly with a dark discolouration due to deposition of malaria pigment in tissue macrophages is also known as “black spot disease” and it is visible through the skin (Ritchie *et al.,* 1995). Black spot disease is not always found, as was the case in a North Island brown kiwi where the liver was pale (Banda *et al.,* 2013), but it is common in pet passerines such as canaries, as well as falcons (Ritchie *et al*., 1995, Schmidt *et al.,* 2003) and Hawaiian honeycreepers (LaPointe *et al.,* 2012). Canaries infected with *Plasmodium* spp. have a decreased ability to thermoregulate as well as a lowered oxygen binding capacity in the crisis phase of the disease (Atkinson and van Riper III, 1991). Changes in blood chemistry include decreased pH, elevated plasma protein concentration and decreased oxygen binding capacity of the haemoglobin. The complete blood count (CBC) is decreased (Ritchie *et al*., 1995) and birds that are highly susceptible (and later succumbing) to the infection can present with a haematocrit value as low as 9.4-11.1% (with a mean haematocrit in the control group of 51.4%) (Palinauskas *et al.,* 2011). In general, the white blood cell (WBC) count of infected birds is elevated (Norte *et al.,* 2009), although this is

dependent on the location and time of year and may not correspond to infection status (Norte *et al.,* 2009, Ricklefs and Sheldon, 2007).

Chronic phase of infection

Once a bird has been infected with a malaria parasite and survived the acute stage of the infection, it may maintain the infection for years or even for life. The parasites remain dormant in the hosts' organs (for example liver and spleen), and may be very hard to detect on blood smears (Atkinson and van Riper III 1991; Scheuerlein and Ricklefs, 2004; Valkiūnas, 2005). In chronic infections, parasitemia may be as low as one parasite in one million examined erythrocytes (Zehtindjiev *et al.,* 2009). These chronic asymptomatic infections can resume the haemoparasitic stage if the host becomes immunosuppressed. This may occur at the onset of the breeding season (spring relapse), as a consequence of change of environmental conditions or from other causes of physiological stress (Atkinson and van Riper III, 1991; Scheuerlein and Ricklefs, 2004). Therefore, chronically infected birds could provide a source for the annual initiation of infection if the seasonal relapse of the infection coincides with seasonal peaks in vector abundance (Garvin and Greiner, 2003).

The hosts' immune system is able to develop some degree of resistance to the lineage it is infected with, and therefore parasitemia may be very light as a result of an efficient immune system. According to Atkinson *et al.* (2001b), the persistence of chronic malaria in birds stimulates humoral and cell-mediated immunity to superinfection with closely related isolates of the parasites. Wild caught chronically infected Omao (Hawaiian thrush, *Myadestes obscures*) showed immunity when experimentally re-challenged with *P. relictum* and developed antibodies to a common suite of *Plasmodium* antigens (Atkinson *et al.,* 2001a).

Chronic infections (Atkinson and van Riper III, 1991) may cause some permanent costs to the host (Atkinson and van Riper III, 1991; Garvin and Greiner, 2003; Bensch *et al.,* 2007). For example, Asghar *et al.,* (2011) found that chronic infections can affect both the arrival date at the breeding ground and the number of fledged offspring in migratory birds (Asghar *et al.,* 2011). Kilpatrick *et al.,* (2006) observed that chronically infected adult Hawaiian amakihi had a 17% lower survival rate compared to uninfected birds. Chronically infected females treated with the anti-

malarial drug Malorone, had higher hatching success, higher provisioning rates and a higher fledging success than untreated females (Knowles *et al.,* 2010). In a nine year study, chronic avian malaria caused significant fitness costs to hosts, which varied depending on the infecting lineage; blue tits infected with *P. relictum* had higher survival rate but were less likely to breed successfully than those infected with *P. circumflexum* (Lachish *et al.,* 2011).

2.3.2 Pathology

To date, few studies have examined the pathological effects of particular lineages of *Plasmodium* spp. on different bird species (Valkiūnas, 2005). However, in general, affected birds present with emaciation, an enlargement and a discoloration or paleness of both liver and spleen, and thin watery heart blood; the primary cause of death is believed to be anaemia caused by the malaria parasites (Atkinson *et al.*, 2000, Atkinson *et al.,* 2001a and b; Atkinson and LaPointe, 2009). A blackbird (*Turdus merula*) that was found dead on Mokoia Island, Rotoura, New Zealand had a severe infection with a lineage of *P. elongatum*, and showed very poor body condition. The liver in this bird was very friable and fell apart on touch, the pericardium was filled with a clear yellowish fluid and the spleen was swollen and enlarged. In addition, the kidney was enlarged, pale and granulated with yellow pinpoint lesions over the whole organ (Schoener, 2009; Howe *et al.,* 2012). However, a North Island brown kiwi which succumbed to infection with *P. elongatum* presented with good body condition, mildly extended abdomen, multifocal petechial haemorrhages over epicardial and endocardial surfaces, slightly consolidated deep purple lungs, markedly enlarged spleen and an only mildly enlarged, pale and bronze liver (Banda *et al.,* 2013).

On histopathology, affected birds show interstitial pneumonia of mild to moderate severity (Atkinson *et al.,* 2001b; Howe *et al.,* 2012) with increased numbers of granulocytes in experimentally infected Hawaiian amakihi (Atkinson *et al.,* 2000) and infiltrating interstitial mononuclear cells in naturally infected passerines and kiwi (Howe *et al*., 2012; Alley *et al*., 2012). Moderate multifocal hepatitis is present in the liver of a range of affected New Zealand passerines (Howe et al., 2012). In addition, a diffuse pigment deposition of haemosiderin in the Kupffer cells in the liver and

macrophages in the spleen is common (Atkinson *et al.,* 2000). In a North Island brown kiwi infected with *P. elongatum*, the most severe lesions were found in the lung, liver and spleen. The bird showed severe interstitial pneumonia and the liver presented numerous hypertrophied Kupffer cells which contained intracytoplasmatic merozoites and/or erythrocytic breakdown products including haemosiderin. The splenic parenchyma contained an increased number of macrophages some of which contained intracytoplasmic merozoites, while a larger portion contained erythrocytic breakdown products including haemosiderin (Banda *et al.,* 2013). Extramedullary erythropoiesis and granulopoiesis may be present in liver and spleen (Atkinson *et al.,* 2000). In affected New Zealand birds, namely a North Island brown kiwi and a European blackbird, both infected with *P. elongatum*, the endothelial cells of tissue capillaries often showed an enlarged cytoplasm filled with numerous, small basophilic protozoan merozoites (Howe *et al.,* 2012). In affected Hawaiian amakihi, large numbers of immature erythrocytes were present in the circulation, while the bone marrow showed increased cellularity with depletion of erythrocytes (Atkinson *et al.,* 2000).

2.3.3 Co- Infections

Co-infections with two or more different malaria parasites are common in wild birds (Jarvi *et al.,* 2002) and it is possible to find several haemosporidia lineages in both a population and the individual (Hellgren *et al.,* 2004). Beadell *et al.* (2004) found mixed infections in 29 of 428 individuals (6.78%) in their study on the prevalence of two avian blood parasite genera (*Plasmodium* and *Haemoproteus*) in the Australo-Papuan region. In a recent study in New Zealand, double infections with *P. relictum* and *P. rouxi* were found in introduced blackbirds (Gudex-Cross, 2011).

There is some discrepancy in the literature about the effect of co-infections with different lineages of *Plasmodium* although these are generally considered more virulent than infections with just a single lineage (Arriero and Moeller, 2008; Marzal *et al.,* 2008; Palinauskas *et al.,* 2011). Palinauskas *et al.* (2011) found heavy parasitaemia (over 35% and up to 90% during peaks) in three species of experimentally infected passerines, but did not note any significant effects in body mass. In contrast, Marzal *et al.* (2008) found a negative additive cost in body

condition in individuals from a natural population of house martins experimentally infected by two different *Plasmodium* lineages.

Co-infections of *Plasmodium* parasites with other vector-borne diseases, such as avian pox virus, are also possible. For example, South-Island saddlebacks (*Philesturnus carunculatus*) translocated to two offshore islands in the Marlborough Sounds of New Zealand presented concurrent infections with *Plasmodium elongatum* and avian pox virus in the summers of 2002 and 2007 with concurrent severe decrease in population numbers(Alley *et al.,* 2010). In addition, a New Zealand dotterel (*Charadrius obscurus*) has also been reported as co-infected with both avian pox virus and *Plasmodium* (Reed, 1997).

2.4 Possible vectors in New Zealand

Insect vectors cannot be ignored in the study of avian malaria, because the epidemiology of the disease is strongly related to the vectors' ecology and behaviour (Glaizot *et al.,* 2012). The only confirmed vector of avian malaria in New Zealand is *Culex pervigilans*, a native mosquito (Massey *et al.,* 2007). However, New Zealand possesses 12 species of indigenous mosquitoes as well as four introduced species (Derraik 2004). Of those, other possible vectors are *Culex quinquefasciatus,* an exotic mosquito that has spread rapidly in New Zealand (Tompkins and Gleeson 2006), and *Ochlerotatus (Aedes) australis* (Derraik and Slaney, 2007). In an outbreak of avian malaria in mohua in Orana Park, Christchurch, *Culex pervigilans* was identified as the most likely vector which showed a peak population in midsummer (February) (Derraik and Slaney , 2007).

In Hawaii, where avian malaria is believed to have contributed to the extinction of many native bird species, *Plasmodium* spp. did not reach epizootic proportions until the introduction of the exotic mosquito *Culex quinquefasciatus* (van Riper III *et al.,* 1986). For most species of *Plasmodium*, the insect hosts are known to be mosquitoes of the genera *Culex*, *Aedes* and *Anopheles* (Ritchie *et al.,* 1995; Valkiūnas, 2005). According to Martinsen *et al.* (2008), the major clades of the haemosporidians are associated with vector shifts in the different dipteran families. *Plasmodium* spp. parasitizing birds and reptiles rely on mosquitoes of the family

Culicidae, excluding the genus *Anopheles* which is known to transmit parasites infecting mammals. Although this view was widely accepted, recent findings might lead to its revision. Biting midges of the genus *Culicoides*, formerly thought to be only able to transmit haemosporidians of the genus *Haemoproteus*, have recently been found to be infected with *Plasmodium*, but the viability of these vectors to transmit the parasite still needs to be confirmed experimentally (Santiago-Alacron *et al.,* 2012). The vector competence for transmitting avian malaria varies between mosquito species, and each *Plasmodium* species may use a number of different mosquito species as vectors (Kimura *et al.,* 2010). However, a specific list of vectors for *Plasmodium* sp. has not yet been determined (Valkinuas, 2005; Glaizot *et al.,* 2012). So far, the genus *Culex* seems to provide the most successful vectors worldwide; in different studies it has been found that mosquitoes of this genus contained the biggest diversity of different *Plasmodium* lineages (Kimura *et al.,* 2010; Glaizot *et al.,* 2012). It has also been discovered that the diversity of *Plasmodium* lineages infecting mosquitoes is greater than that infecting birds, therefore including lineages that have not yet been recovered from birds (Kimura *et al.,* 2010). Kimura *et al.* (2010) suspect this is due to most studies in avian malaria being biased towards small common passerines that are easily caught in mist nets. It is therefore possible that more malaria lineages wait to be found in rarer, more elusive bird species.

In the northern hemisphere, most transmission of the parasites between birds occurs in the spring and summer, during the breeding season of the avian hosts. This is also the time when vector populations are increasing with the onset of warm weather, adult birds with chronic, relapsing infections are available as sources of infection, and susceptible juvenile birds are hatching and leaving the nest (Atkinson and van Riper III, 1991). There are differences between years in prevalence of blood parasites (Allander and Bennett, 1994). This may be explained by an annual variation in vector abundance and activity which will influence the parasite transmission in the year before the sample is taken. For example, Tomkins *et al.* (2008) found that a high prevalence of avian malaria in New Zealand birds on Long Island coincided with an unusually high abundance of mosquitoes. The feeding activity of dipteran vectors depends on the weather conditions and can be affected by temperature and wind. Another explanation for the yearly changes might be the winter mortality of the vertebrate hosts. Blood parasites may be stressful for the host,

and during wintertime uninfected birds may survive better than infected ones (Allander and Bennett, 1994).

2.5 Role of Climate Change on Parasite Prevalence

Avian malaria is known from other parts of the world to be an emerging disease. The term “Emerging Disease” describes a disease that has either newly affected a population or species, or is present in the population but is increasing in its geographic range and incidence (Smith *et al.,* 2006). The emergence of vector borne diseases is thought to be likely due to climate change and global warming (Harvell *et al.,* 2002; Valera *et al.,* 2006, Garamszegi, 2011). These changes in the environment may improve the conditions for insect vectors of avian malaria, increasing the distribution range, length of the breeding season and abundance in former vector free (cooler) habitats. In addition, the reproduction of the malaria parasites themselves increases with increasing temperatures (Garamszegi, 2011). It has been noted that the infection rate of *Plasmodium* is strongly associated with temperature anomalies, and has shown an accelerating tendency in the past 20 years. Garamszegi (2011) predicts a two-to threefold increase in the prevalence of malaria in birds for every 1°C rise of global temperatures. Although these changes are associated with warming climate, it remains controversial whether the actual warming is the primary cause or whether other anthropogenic influences like habitat alteration are driving these changes (Harvell *et al.,* 2002). Human activities have been implicated in the introduction of exotic vectors like *Culex quinquefasciatus* (the vector of *Plasmodium* spp.) into New Zealand (Derraik, 2006; Tompkins and Gleeson, 2006).

3. Methods of detection and *Plasmodium* sp. identification

Detection of *Plasmodium* parasites can be undertaken by one of three methods: 1) microscopic examination of blood smears for unique life stages (i.e. trophozoites, merozoites, microgametocytes and macrogametocytes) developing in peripheral blood, 2) histological evidence of meronts in tissues of dead birds, and 3) molecular diagnostic tools. Currently it is recommended that, where possible, both microscopic methods and molecular tools should be used. While molecular tools are of advantage when not all parasite stages are visible under the microscope and a low

parasitemia is present, microscopy is still superior for identifying mixed infections (Perkins *et al.,* 2011) and remains the gold standard for malaria diagnosis worldwide (Ochola *et al*., 2006). Furthermore, some *Plasmodium* species cannot be detected with the currently available molecular tools (Zehtindjiev *et al.,* 2012) and can only be detected in smears.

3.1 Blood smears

Using this method, 175 species of haemosporidian parasites of the genera *Haemoproteus* and *Plasmodium* have been defined up to 2007 (Palinauskas *et al.,* 2007). For blood smears to be useful in the identification of genus and species, it is crucial that the blood smear is of good quality and that the correct staining technique is used (Bruce and Day, 2002). Furthermore, a blood sample should be processed very quickly to prevent the loss of the main diagnostic characters of the parasites from exposure to air (Valkiūnas *et al*., 2008). The commonly recommended stain is Giemsa because rapid stains such as Field's stain, are less stable, bleach out more easily and cannot be used for taxonomic examinations (Valkiūnas, 2005).

Usually, a smear is examined using a light microscope under high magnification (x1000) and immersion oil. Identification is done by sketching or photographing what is observed under the microscope and comparing the images to already universally documented and accepted morphological characteristics (blood stages and morphometric analyses) unique to each parasite (Valkiūnas, 2005). Although, microscopy remains essential in the diagnosis of mixed infections, there are some significant disadvantages when compared to molecular techniques. These include the labour-intensive examination of slides and the potential for missing low level parasitemias (Jarvi *et al.,* 2002; Fallon *et al.,* 2003b). In addition, the identification of parasite morphology is often difficult, and avian malaria parasites are known for their cryptic diversity (where unrelated species may look similar). This can be made worse by distortion of the cells on the smears due to air drying and fixation, environmental conditions at the sampling site or slight differences in the stains used (Perkins *et al.,* 2011). Furthermore, microscopy does not take into account potential differences between the skill and experience of individual technicians (Valkiūnas *et al.,* 2008).

3.1.1 Diagnostic characteristics of Plasmodiidae

The small golden brown or black pigment deposits in infected erythrocytes (haemozoin, malaria pigment), which characterize both *Plasmodium* and *Haemoproteus* parasites, are produced by chemically changing the hosts haemoglobin; it is therefore a product of haemoglobin digestion (Atkinson and van Riper III, 1991; Egan, 2002). Haemozoin contains the ferrous part of the haemoglobin which is not digested by *Plasmodium* and *Haemoproteus* parasites (Valkiūnas, 2005).

Haemozoin is highly refractive when examined by dark- field or polarised light microscopy in both stained and unstained blood smears (Atkinson and van Riper III 1991; Egan, 2002). This pigment is crucial in identifying malaria parasites microscopically, because pigment number, form and location vary between different parasite species (Egan, 2002; Valkiūnas, 2005). Details of the particular characteristics representing the different sub-genera of *Plasmodium* that parasitise avian species have been summarised in (Valkiūnas, 2005).

3.2 Cytology and Histopathology

Histopathological examinations of birds that have died from avian malaria typically display lesions in the lungs, liver, spleen, and occasionally other organs (Schmidt *et al.,* 2003).Thus, rapid diagnosis at necropsy can often be accomplished using routine impression smears of liver, lung or spleen stained with Giemsa or difquik (Harrison and Lightfoot, 2006; Alley *et al.,* 2008).

3.3 Molecular Tools

Various polymerase chain reactions (PCR) based techniques for recognition and genetic classification of haemosporidian parasites have been developed (Waldenström *et al.,* 2004; Valkiūnas *et al.,* 2006). These molecular tools have confirmed the classification of the main subgenera and have also provided information regarding the phylogenetic relationships between genera (Martinsen *et al.,* 2008; Valkiūnas *et al.,* 2008). The most commonly used gene, cytochrome b, has been extremely useful for distinguishing morphospecies (species characterised by

certain morphology). As a result, phylogenetic analysis has revealed that *Plasmodium* spp. of birds form monophyletic groups which in most cases can be aligned to morphological data (Križanauskienė *et al.,* 2006; Hellgren *et al.,* 2007; Palinauskas *et al.,* 2007). However, it is becoming increasingly evident that based on phylogenetic analysis using the cytochrome b gene, the level of *Plasmodium* spp. diversity is greater than that determined using identification based on morphological features alone (Palinauskas *et al.,* 2007). Furthermore, the use of mitochondrial and nuclear DNA is suggesting that the evolution of morphospecies and parasite-vector-host relationships may be significantly more complex than currently acknowledged (Bensch *et al.,* 2000; Bensch *et al.,* 2004; Ricklefs *et al.,* 2004).

Molecular tools have the advantage of detecting low levels of parasites in the blood, which may be missed in peripheral blood smears (Perkins *et al.,* 2011). As a result, PCR techniques are increasingly used to screen blood samples from wild birds for infection with haemosporidian parasites to determine infection prevalence and to scrutinize host specificity and deduce phylogenetic associations (Bensch *et al.,* 2000; Perkins *et al.,* 2011). However, there is a need for evaluation of the sensitivity of these techniques for detecting mixed infections caused by different haemosporidian species belonging to the same and dissimilar genera and subgenera (Valkiūnas *et al.,* 2006). In addition, not all species of *Plasmodium* can be detected with the current molecular tools as was exemplified by a *Plasmodium* species infecting a skylark (*Alauda arvensis*) in Italy that was morphologically distinct on microscopy but failed to be detected by PCR (Zehtindjiev *et al.,* 2012). Nonetheless, once optimised, PCR techniques are faster, cheaper, and more robust than blood smear examination especially for large-scale screening (Freed and Cann, 2003; Valkiūnas *et al.,* 2008). However, until PCR techniques are optimised, smears should be carried out to ensure that all possible parasites are identified.

3.4 Online databases

The MalAvi and GenBank databases contain sequence data of avian blood parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* which are identified by a partial region of their cytochrome b sequences (Bensch *et al.,* 2009; Sayers *et al.,* 2012). MalAvi database (http://mbio-serv4.mbioekol.lu.se/avianmalaria/) currently includes more than 950 parasite lineages. It uses a harmonised classification and all

available information about each parasite is present in a public reference location, which enables easy access by all researchers. This database is updated by researchers and new lineage names remain unique which is done via an automated online system (Bensch *et al.* 2009). Similarly, the GenBank sequence database (http://www.ncbi.nlm.nih.gov/genbank/) is an open access, annotated collection of all publicly available nucleotide sequences and their protein translations (Benson *et al.,* 2011). This database is created and monitored by the National Centre for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database Collaboration (INSDC) (Sayers *et al.,* 2012).

4. Conclusion

There is no question that a variety of avian *Plasmodium* species are now well established throughout New Zealand and some of these are increasing in prevalence. Unfortunately, evidence is mounting that our unique and in some cases critically endangered birds maybe particularly susceptible to this emerging disease. Recent studies in New Zealand and overseas have dramatically increased our understanding of the potential impact that avian malaria could have on our endangered and threatened avifauna. Greater awareness and regular monitoring programs will be necessary to improve our knowledge of these complex host–parasite interactions. A better understanding of *Plasmodium* epidemiology will allow the development of appropriate management strategies for conservation programs that will minimize the impact of avian malaria in New Zealand.

5. Acknowledgements

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Table A1.1: *Plasmodium* spp. lineages identified in New Zealand and their respective avian hosts (from Banda 2012).

Subgenus	*Plasmodium* lineage (GenBank #)	Avian species	Reference(s)
Huffia		NI saddleback	Castro *et al.* 2011
		SI saddleback (Dead)	Alley *et al.* 2010
	***Elongatum* sp. GRW06**	Silvereye	Howe *et al.* 2012
		Brown kiwi	
	(DQ659588, DQ368381)	Blackbird	
		Brown kiwi (dead)	Banda *et al.* 2013
		Sparrow	Marzal *et al.* 2011
		Bellbird	Baillie and Brunton 2011
		Blackbird	
		Song thrush	
		NI saddleback	Ewen *et al.* 2012b
		Sparrow	(MalAvi Database)
		Yellowhammer	
		Whitehead	
		NI robin	
AFTRU5/LINN1 Cluster	**LINN1** (GQ471953)	**Blackbird (Dead)**	Howe *et al.* 2012
		Great Spotted Kiwi (Dead)	Alley et al 2012
	AFTRU5 (MalAvi)	NI Saddleback	Castro *et al.* 2011
	WA39 (EU810610)	Bellbird	Ewen *et al.* 2012b
		Blackbird	(MalAvi Database)
		Song Thrush	
Haemamoeba	**KOKAKO01** (MalAvi)	Kokako	Howe *et al.* 2012
			Ewen *et al.* 2012b
			(MalAvi Database)
	***Relictum* sp. GRW04** (AY099041)	Sparrow	Ewen *et al.* 2012b
			Marzal *et al.* 2011
			(MalAvi Database)
		Red Fronted Parakeet	Ortiz-Catedral 2011
	NZ Hihi (HQ453996)	**Hihi (Dead)**	Howe *et al.* 2012
	***Cathemerium* sp.** (AY377128)	Red-billed gull	Cloutier *et al.* 2011
	***Relictum* sp. SGS1** (AF495571)	Saddleback	Howe *et al.* 2012
		Sparrow	Marzal *et al.* 2011
			(MalAvi Database)
		Myna	Beadel *et al*. 2006

			(MalAvi Database)
		Sparrow Yellowhammer	Ewen *et al.* 2012b (MalAvi Database)
Novyella	***Vaughani* sp. SYATO5** (GenBank DQ847271)	Blackbird Kereru Blackbird Tomtit	Howe *et al.* 2012 Ewen *et al.* 2012b (MalAvi Database)
	LIN3 (GenBank JN415758)	Bellbird	Baillie and Brunton 2011
Unresolved (NZ native *Plasmodium* spp.*?)*	**BELL01** (JQ905572)	Tui Saddleback Bellbird	Ewen *et al.* 2012b (MalAvi Database)
	LIN1 (JN415756)	Bellbird	Baillie and Brunton 2011
	NZRobin30 (JN565686)	North Island Robin	Banda 2012
Unresolved	**PADOM02**	Yellowhammer	MalAvi database (Ruth Brown, unpublished)
Unresolved	**HIHI01**	Hihi	MalAvi database (Ruth Brown, unpublished)
Unresolved	**LIN4** (GenBank JN415759	Bellbird	Baillie and Brunton 2011

[1] Species identified in bold indicate mortality due to *Plasmodium* infection.

Table A1.2: Prevalence of infection in *Plasmodium* positive introduced and endemic species reported during 2006-2013 in New Zealand.

Reference	Host Species	Prevalence	Number sampled	Method of Detection
Tompkins and Gleeson, 2006	Blackbird	0-100%	85	PCR and blood smear
	Starling	0-35%	56	
	House sparrow	0-33%	147	
	Song thrush	4-19%	59	
Ishtiaq et al, 2006	Myna	10%	92	PCR
Sturrock and Tompkins, 2008	Blackbird	7%	60	PCR
	Thrush	11%	27	
Cloutier et al., 2011	Red-billed gulls	7.8%	243	PCR
Baillie and Brunton, 2011	Bellbird	13.4%	693	PCR
Castro et al., 2011	NI saddleback	2.8-10.6%	363	PCR and blood smear
Ortiz-Catedral et al., 2011	Red-fronted parakeets	40.9%	22	PCR
Gudex-Cross, 2011[#]	Bellbird	38.6%	132	Blood smear
	Tui	20%	15	
	Tomtit	13%	8	
	Myna	50%	2	
	Blackbird	100%	4	
	Song thrush	25%	4	
	Silvereye	9.2%	224	
	Fantail	5.3%	19	

Howe et al., 2012	Blackbird	100%	7	PCR and blood smear
	Silvereye	100%	1	
	Hihi (Stichbird)	9%	11	
	Kokako	25%	8	
	Kereru	100%	1	
	NI brown kiwi	10%	10	
Ewen et al., 2012b*	Tui	~5%	30	PCR
	Saddleback	~12%	36	
	Tomtit	~7%	22	
	North Is. Robin	~2%	78	
	Whitehead	~10%	42	
	Bellbird	~7%	78	
	Song Thrush	~25%	19	
	Blackbird	~40%	44	
	House Sparrow	~7%	60	
	Yellowhammer	~17%	23	
Baillie et al., 2012	Bellbird	<1%->45%	457	PCR
Banda et al., 2013	NI brown kiwi	0-78%	32	PCR and blood smear

[#]Master's Thesis, Massey University, 2011.

*Prevalence in host estimated based on data presented in bar graph form. Level of co-infection within host is unclear

Appendix 2: History of pathogen pollution

1. Pathogen pollution- Historic examples

1.1 Human

Pathogen pollution caused by human movements or close contact to other animal species is a very old phenomenon. Molecular work suggests that the human species acquired several infectious diseases from other animal species kept as livestock and pets due to host switching of the pathogen (Clark, 2010). Examples are measles, a morbilivirus closely related to distemper in dogs (*Canis familiaris*), and smallpox, which is an orthopoxvirus closely related to cow (*Bos primigenius*) and camel (*Camelus bactrianus*) pox (Clark, 2010). Today, we see new influenza virus lineages emerge in China where humans have lived close to their livestock (pigs (*Sus scrofa*) and chickens (*Gallus gallus*)) for centuries (Clark, 2010). Pathogens have been transported between human populations with human movements: migration, exploration, trade and invasions. The "Black Death", plague, caused by the bacterium *Yersinia pestis*, and vectored by the rat flea *Xenopsylla cheopis*, originated in China and was brought along the silk-road (trading route) to other parts of the world. In 1347 it reached Europe and caused devastating epidemics during the medieval ages, with a mortality rate of around 50% (Kiple, 1993, Clark, 2010). When smallpox arrived in Japan in the 700s, it killed nearly half of the population, showing a mortality rate of up to 75% (Clark, 2010). One of the most severe examples of pathogen pollution in human history took part during the discovery, exploration and conquest of the Americas by the Europeans. North and South America, together with their human populations, had been isolated from the rest of the world for at least 10,000 years since the last ice age. The arrival of Europeans and African slaves had a catastrophic impact on the indigenous population in terms of disease ecology (Kiple, 1993). The European diseases spread rapidly through the interior of the American continents, devastating the native American populations which were immunologically naïve to the introduced pathogens (Kiple, 1993, Clark, 2010). Examples of introduced European diseases were smallpox, measles and influenza (Kiple, 1993). Together with slaves from Africa, vector-borne diseases, like malaria and yellow fever, were also introduced (Kiple, 1993). The new diseases resulted in high mortality rates of up to 90% with smallpox and up to 74% for others (Kiple,

1993). As a result, the indigenous populations declined by up to 95% in some parts of the Americas (Clark, 2010). Of these diseases, smallpox was the most severe; it reached the island of Hispaniola (today comprising the Dominican Republic and Haiti) in 1518, where it caused the first major epidemic in the new world, before rapidly spreading to Mexico in the mainland (Clark, 2010).

Table A2.1: Historic examples for pathogen pollution and their impact.

Pathogen	Affected Species	Date	Origin	Destination	Impact	Reference
Variola virus (Smallpox)	Human (*Homo sapiens*)	1518	Europe	Americas	Severe population decline of the indigenous populations	(Kiple, 1993; Clark, 2010)
Morbili virus (Measles)	Human	1635	Europe	Americas	Severe population decline of the indigenous populations	(Kiple, 1993; Clark, 2010)
Influenza virus	Human	1490s	Europe	Americas	Severe population decline of the indigenous populations	(Kiple, 1993; Clark, 2010)
Treponema pallidum (Syphilis)	Human	1490s	Americas	Europe	Sexual transmitted disease STD	(Clark, 2010)
Plasmodium spp. (Human malaria)	Human	1500s	Africa (slave trade)	Americas	Severe population decline of the indigenous populations	(Kiple, 1993; Clark, 2010)
Lyssavirus (Rabies)	North American wildlife Racoons (*Procyon lotor*) from (mammals)	Late 1970s	South Eastern US	Midatlantic US	Epizootic in mammalian wildlife in the US	(Rupprecht *et al.*, 1995)
Rinderpest (Morbilivirus)	Domestic cattle (*Bos taurus*), African buffalo (*Syncerus caffer*), giraffe (*Giraffa camelopardalis*), kudu (*Tragelaphus* spp.), wildebeest	1880s	Europe	Africa	Mortalities of up to 100% in domestic livestock; decimation of buffalo, giraffe, kudu, wildebeest population; local extinction of the Tse-tse fly	

	(*Connochaetes* spp.)					
Canine Distemper (Morbilivirus)	African wild dog (*Lycaon pictus*)	1970s	Domestic dog	African wild dog	Annual population declines in wild dogs of up to 40%	(Ginsberg *et al.*, 1995a, Ginsberg *et al.*, 1995b)
Phocine Distemper (Morbilivirus)	Common seal (*Phoca vitulina*); gray seal (*Halichoerus grypus*)	1988; 2002	Harp seal (*Pagophilus groenlandicus*)	Common seal (*Phoca vitulina*); gray seal (*Halichoerus grypus*)	Severe mortalities in the affected seal populations	(Heidejorgensen *et al.*, 1992, Markussen and Have, 1992, Harding *et al.*, 2002, Harkonen *et al.*, 2006, Jensen *et al.*, 2002)
Myxomatosis	Rabbit (*Oryctolagus cuniculus*)		South America	Europe, Australia		
Squirrel pox (Parapoxvirus)	Red squirrel (*Sciurus vulgaris*)	Early 1900s	American gray squirrels (*Sciurus carolinensis*)	European red squirrels	Population declines in European red squirrels	(Tompkins *et al.*, 2002, Tompkins *et al.*, 2003)
Mycobacterium bovis Bovine tuberculosis	Domestic cattle (*Bos taurus*) and Brushtail possum (*Trichosurus vulpecula*) in NZ	1837	Brushtail possum introduced from Australia→ provided a new very successful host for bovine tuberculosis	NZ	Bovine tuberculosis; Economic losses for farmers	

	Iberian lynx (*Lynx pardinus*) in Spain	1998	Domestic cattle	Iberian lynx	Threat to one of the world's most endangered feline species	(Perez *et al.*, 2001)
BSE (Bovine spongiform encephalitis; caused by a prion)	Domestic cattle, European red deer (*Cervus elaphus elaphus*), domestic cat (*Felis catus*), human	1980s	Sheep cadavers in Britain	Host switching to cattle, deer, cats, humans	BSE epidemic	(Dagleish *et al.*, 2008)
Avian pox	Native Hawaiian Avifauna	Early 1900s	Introduction of the mosquito *Culex quinquefasciatus* as vector		Avian extinction crisis on Hawaii	(Pratt, 2009)
Avian malaria	Native Hawaiian Avifauna	~1920	Introduction of the mosquito *Culex quinquefasciatus* as vector		Avian extinction crisis on Hawaii	(Pratt, 2009)
Pilchard Herpes (PHV)	Pilchards (*Sardinops neopilchardus*)	1995	Probably induced by translocation of bait fish	Australia and New Zealand	Economic losses to the Fisheries sector	(Hyatt *et al.*, 1997, Whittington *et al.*, 1997)

Oyster Herpesvirus (OsHV-1)	Pacific oyster (*Crassostrea gigas*)	2010	?	New Zealand	Economic losses to the Fisheries sector	(Castinel et al., 2015)
Fungus *Aphanomyces astaci* (crayfish plague)	European crayfish	1859	North America (with American crayfish)	Europe	Severe depletion of native European crayfish populations	(Edgerton *et al.*, 2004)
Mite *Varroa destructor*	Honeybee (*Apis mellifera*)	1950s	Indian bee (*Apis cerana*)	worldwide	Economic and ecological impact; Contributing to Colony collapse disorder; Vector for other bee diseases (e.g. Deformed wing virus)	(Rosenkranz *et al.*, 2010)
Fungus *Cryphonectria parasitica* (Chestnut blight)	American chestnut (*Castanea dentata*)	Late 1800s	Japan (from imported Japanese chestnut)	North America	Rapid spread; nearly wiped out American chestnut in its native range	(Loo, 2009)
Fungus *Ophiostoma* spp. (Elm disease); *O. ulmi*	American (*Ulmus americana*) and English elm (*U. procera*)	1910; 1940	Asia	North America and Europe	Severe population declines in American and English elm	(Loo, 2009)

1.2 Domestic cattle and rinderpest

Reviewed in: 2011. The Eradication of Rinderpest from Africa- a great milestone. Page 128. African Union – Interafrican Bureau for Animal Resources (AU-IBAR), Nairobi, Kenya.

Rinderpest is an infectious disease caused by a virus of the genus Morbilivirus. It affects even-toed ungulates of the mammalian order Artiodactyla, namely domestic cattle, domestic buffalo but also wildlife like giraffe, kudu and wildebeest and it is the most lethal and potentially dangerous infectious disease in wild Artiodactyla (Plowright, 1982). Clinical signs in cattle can vary between peracute to acute and mild forms of the disease. The peracute form appears suddenly and the affected animals show in appetence, depression, fever, mucosal congestion, increased heart and breathing rate and death within three days. The typical acute form of the disease has an incubation period of three to nine days, followed by fever, mucosal erosion and diarrhoea and death or recovery. Pregnant cows will abort. Animals that survive rinderpest will have a lifelong immunity to the disease. The subacute of mild form of the disease is common to areas where rinderpest is endemic and presents with only mild clinical signs.

Rinderpest arrived on the African continent, in Egypt, in the mid 1800s by ship from Europe. The disease spread rapidly though Egypt and has since then been endemic in the country with frequent re-occurring outbreaks with severe impact on the countries' domestic cattle. The most severe impact on the African continent, however, was the great rinderpest pandemic which lasted from 1889 to 1897. It is thought that this pandemic had its origin with an invading Italian army in Ethiopia in 1887, which accidently introduced the disease with accompanying cattle. From there it spread rapidly through the entire continent in the following years. The disease had a devastating impact on domestic cattle (with up to 90% mortality) and on the free-ranging populations of the fully susceptible wildlife. Over 90% of buffaloes (*Syncerus caffer*) in Kenya disappeared, and wild ungulate populations in East Africa became so low that local predator numbers declined. In certain areas, the number of ungulates became too low to sustain even micropredators and the tse-tse fly (*Glossina sp*.) became locally extinct (Plowright, 1982; Cunningham, 2003).

Changing grazing patterns due to the missing ungulates may have changed the landscape in Eastern Africa (Cunningham, 2003). Rinderpest remained endemic in Africa during the whole of the 20th century, with reoccurring devastating outbreaks in domestic cattle and wildlife. Since then, a stringent vaccination campaign of livestock and a surveillance programme for both livestock and wildlife, by many cooperating African Nations, has helped to eradicate rinderpest from the continent. The last confirmed outbreak of rinderpest was in buffaloes in the Meru National Park in Kenya in September 2001. After the enzootic, the numbers of wild ungulates in populations across the African continent are slowly recovering, but still have not reached the numbers of before the introduction of the disease (Plowright, 1982).

1.3 Other mammals

Besides rinderpest, there are many other examples of pathogen pollution having a devastating effect on various mammal species and populations (Table 1).

The impact of rabies, caused by a Rhabdovirus of the genus *Lyssavirus*, in the United States has changed from domesticated animals (where it was controlled by vaccination) to wildlife (raccoons, skunks, foxes, bats). A change in human influence in the recreational and economic sector (Rupprecht *et al.*, 1995) brought with it new translocations for recreational (hunting) purposes (Jenkins *et al.*, 1988). This has contributed to the epizootic spread of the disease in wildlife in the US and Canada in the late 1970s. The origin of the rabies enzootic is thought to have been racoons (*Procyon lotor*) from South Eastern US which had been translocated to the Midatlantic states of the US (Rupprecht *et al*., 1995).

Another example is distemper, caused, like rinderpest, by a morbilivirus. Canine distemper, reviewed in (Leisewitz *et al.*, 2001), in Southern Africa poses a threat to wildlife, after many species of the mammalian order Carnivora are susceptible to the virus. Lions, leopards, wild dogs, hyenas and foxes have been found to be infected, with fatal outbreaks in lions and wild dogs. The reservoir for the disease is the large population of unvaccinated domestic dogs in Southern Africa, with the potential to spread fatal disease to native carnivore species (Leisewitz *et al*., 2001). African wild dogs (*Lycaon pictus*) appear to be especially at risk (Alexander *et al.*, 1996) and the disease may have played a role in the population declines in wild dog populations that started in the 1970s (Ginsberg *et al.*, 1995a, Ginsberg *et al.*, 1995b,

Cunningham *et al.*, 2003). Phocine distemper is a common infection in harp seal (*Phoca groenlandica*) with prevalence of up to 98% (Markussen and Have, 1992). In 1988 and 2002, two devastating outbreaks of phocine distemper swept through Europe, causing severe mortalities in Common seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) (Anderson and May, 1986; Markussen and Have, 1992; Harding *et al*., 2002; Jensen *et al*., 2002; Harkonen *et al.*, 2006). The outbreak in 1988 killed about 18000 seals in the North sea (Jensen *et al*., 2002). It is thought that a forced migration of harp seals to the North Sea coast of Norway and Denmark acted as a catalyst, after harp seals are known carriers of the disease (Heidejorgensen *et al*., 1992; Harding *et al.,* 2002; Harkonen *et al*., 2006). This migration might have occurred due to a depletion of fish stocks through overfishing (Heidejorgensen *et al*., 1992).

A parapoxvirus is very likely to be the infectious agent involved in the population decline of red squirrels (*Sciurus vulgaris*) in the UK, where the virus causes disease with high mortalities (Thomas *et al.*, 2003). The virus is thought to have been introduced with American grey squirrels (*Sciurus carolinensis*) in the early 1900s. While American grey squirrels remain asymptomatic carriers and act as a reservoir for the disease, it as a devastating impact on European red squirrels (Tompkins *et al.*, 2002). In addition to the disease transmission, grey squirrels also compete directly with red squirrels for food and territory, and might therefore in future replace the native species (Tompkins *et al*., 2002). The problem of the introduction of grey squirrels ongoing in the UK and on the European continent. In the early 2000's, grey squirrels were introduced into Italy and their eradication is desireable (Signorile *et al.*, 2014).

Additional examples of pathogen pollution affecting mammals include myxomatosis, bovine tuberculosis and bovine spongiform encephalopathy (BSE or "Mad Cow Disease"). Myxomatosis is a disease naturally occurring in American rabbit species, and it is very pathogenic to European rabbits, with up to 99% mortality (Kerr, 2012, Ross, 1982, Trout *et al.*, 1992). The virus was introduced as a biological control agent to Australia (and New Zealand where it did not take) and Europe, what was seen as a "real time experiment of host-pathogen evolution" (Kerr, 2012). In Europe, the virus has a great impact on the native rabbit species, but also on the whole ecosystem with changes in landscape, vegetation and predator-prey ecology. This

endangered for example predators like the Iberian lynx (*Lynx pardinus*) and the Imperial eagle (*Aquila adalberti*) which rely on rabbits as a stable prey source (Ross, 1982, Kerr, 2012). Since the introduction of the disease, the rabbit population in Europe has recovered slowly (Ross, 1982).

While myxomatosis is caused by a virus, bovine tuberculosis was introduced into countries worldwide with domestic cattle. Bovine tuberculosis is caused by *Mycobacterium bovis* and can lead to substantial economic losses. In New Zealand, bovine tuberculosis became a problem after the introduction of the brushtail possum (*Trichosurus vulpecula*) from Australia in 1837 with the purpose of establishing a fur trade. The brushtail possum provided a new and very successful reservoir host for bovine tuberculosis which has been endemic in NZ since then. Bovine tuberculosis also poses a threat the highly endangered Iberian lynx (*Lynx pardinus*) in Spain, where this feline can become infected by domestic cattle (Perez *et al.*, 2001).

BSE is caused by an infectious agent named “prion”, a misfolded protein able to multiply without the need of genetic material like DNA. The practice of feeding meat and bone meal produced from insufficiently heated sheep and cattle cadavers to cattle in the UK in the 1980s is very likely the origin of the BSE epidemic that swept through the UK and in lesser degree Europe. When the misfolded prion protein is consumed by a susceptible species, even over wide species gaps, these animals themselves may become infected. Prion disease with an origin thought in infected cattle cadavers has been found in a wide range of mammalian species, including European red deer (*Cervus elaphus elaphus*) (Dagleish *et al*., 2008), wild bovids and primates (Bons *et al.*, 1999) in British zoos as well as domestic cats (*Felis catus*) and humans.

1.4 Birds

One of the most devastating examples of pathogen pollution caused the extinction of about 50% of the endemic forest birds on the Islands of Hawaii (van Riper III *et al*., 1986). It is hypothesised that avian pox and –malaria were responsible for these extinctions, after the pathogens causing the diseases as well as their vector became introduced to Hawaii by humans (Daszak and Cunningham, 1999). The potential for Hawaiian birds to be infected with both malaria and pox had existed for centuries with the influx of migratory sea and shorebirds carrying such pathogens, but these

diseases were not able to establish until the introduction of the necessary mosquito vectors (van Riper III *et al*., 1986). Therefore, the immunological naïve status of Hawaiian bird populations prior to their exposure following vector introduction is believed to have been a key cause of the subsequent disease impacts (van Riper III *et al*., 1986). In general, highly specialised bird species suffered more than the generalists (Pratt, 2009), after generalists have a wider range of habitats to choose from and therefore escape the vectors. The introduction of the southern house mosquito (*Culex quinquefasciatus*) from mainland USA in 1826 was pivotal for the spread of avian pox and –malaria on Hawaii (van Riper III *et al*., 1986), because insect vectors are compulsory for malaria parasites to complete their life cycle, and for distribution of pox viruses.

It is thought that avian pox arrived before avian malaria in Hawaii, with the earliest identification of avian pox in native Hawaiian birds made in 1902 (Van Riper III *et al.*, 2002). According to van Riper et al. (2002), the extinction of many native Hawaiian bird species in the early 1800s, and the following range reductions of other avian species, corresponds with the introduction of domestic poultry and the arrival of avian pox. Avian pox was therefore one of the main factors for the avian population declines in the 1800s, besides the introduction of mammalian predators and habitat loss due to human activities (Van Riper III *et al*., 2002). The clinical signs in birds infected with poxvirus vary widely depending on the virulence of the virus, susceptibility of the host, type of lesions and other factors (Bolte, 1999). Most commonly, the disease may show as the cutaneous or diphtheric/pharyngeal forms, or both (Bolte *et al.*, 1999, Van Riper III *et al*., 2002). In Hawaii, the diphtheric form is more severe, showing higher mortality rates.

Avian malaria, on Hawaii caused by the introduced *Plasmodium* species *P. relictum*, is thought to have arrived on Hawaii in the 1920s. According to van Riper et al. (1986), the high susceptibility of the novel host was due to lack of a chance to develop appropriate immune responses to the haemoparasites. The clinical signs shown by birds infected with *Plasmodium* spp. depend on the species of bird as well as the lineage of parasite involved. Infected birds can remain asymptomatic (Ritchie *et al*., 1995). In general, birds with the overt disease show the following signs: Vomiting, anorexia, depression, dyspnoea, anaemia of the regenerative haemolytic type, splenomegaly, hepatomegaly and pulmonary oedema. In susceptible Hawaiian

amakihi (*Hemignathus virens*), the birds showed high parasitemias as high as 50% in the acute phase with a decline in food consumption and body weight and then became lethargic making them more susceptible to predators (Atkinson *et al*., 2000). The mortality rate in amakihi can be up to 65% (Atkinson *et al*., 2000). Chronic infections which have no overt clinical signs for years (Atkinson and van Riper III, 1991) may cause some permanent costs to the host. They might affect immune defences, change the activity and behaviour of the host and may diminish the birds' ability to adapt to stressful events in their environment (e.g. lack of food, bad weather conditions, predation, and breeding), causing higher mortality and reduced reproductive success in infected birds (Atkinson and van Riper III, 1991; Garvin *et al*., 2003; Bensch *et al*., 2007).

Both avian pox and malaria play a major role in the ongoing population declines of Hawaiian forest birds. With the advent of climate change, this will become more pronounced, after the high altitude habitat, at the present a mosquito free safe haven for susceptible bird species, will diminish (Pratt, 2009).

1.5 Fish and invertebrates

Economic losses have been caused by pathogen pollution in the fisheries sector. Two examples from New Zealand, which will be discussed in more detail under point 3 in this review, are pilchard herpes and herpes in Pacific oysters.

For commercial reasons (aquaculture), North American crayfish like *Procambarus clarkii* have been introduced to other parts of the world, and *P. clarkii* is now the most cosmopolitan crayfish, found on all continents except Australia and Antarctica (reviewed in (Gherardi, 2006). With these introductions, the devastating crayfish plague, a fungal disease of North American crayfish (caused by several species of the genus *Aphanomyces*), was introduced to Europe (Longshaw, 2011) in the 1850s (Edgerton *et al.*, 2004). North American crayfish are resistant to the disease and appear as asymptomatic carriers of the fungus. Crayfish that are native to Europe, on the other hand, are highly susceptible to the pathogen and may suffer large scale mortalities (Longshaw, 2011). The populations of all species of crayfish native to Europe have suffered since the introduction of the disease 150 years ago and several species have been listed as vulnerable due to the population declines (reviewed in (Edgerton *et al.*, 2004)).

During the first half of the 20th century, the parasitic mite *Varroa destructor*, (reviewed in (Rosenkranz *et al.*, 2010)) a haemophagus ectoparasite of the Indian bee *Apis cerana*, acquired the honeybee *Apis mellifera* as a new host. The details on how this host switch happened are still unknown. The parasite causes varroosis, a disease still seen as one of the greatest threats to apiculture (Rosenkranz *et al*. 2010). The disease has spread worldwide in a relatively short time, helped by human apiculture practices, trade and international movement of bee colonies. The *Varroa*-mite is also a potent vector for several honeybee viruses (Genersch *et al*., 2010; Rosenkranz *et al*., 2010) and can have severe economic and ecological impacts worldwide due to the loss of pollination services (Genersch and Aubert, 2010, Genersch *et al.*, 2010).

1.6 Plants

Pathogen pollution in plants has been reviewed in (Loo, 2009).

Introduced fungal pathogens had a severe impact in North American forests. The fungus *Cryphonectria parasitica*, causative agent of chestnut blight, was introduced in the late 1800s with Japanese chestnut (*Castanea crenata*) from Asia. While the Asian chestnut trees have co-evolved with the fungus and show resistance, American chestnut (*Castanea dentata*) is highly susceptible. The disease spread rapidly through the native range of the American chestnut and wiped out most of the mature trees in just 30 years. Until then, some of the Eastern North American hardwood forests consisted of up to 25% of chestnut trees. The near-extinction of the American chestnut tree also had wider implications. Trees are foundation species for forest ecosystems, and chestnut trees provided a high quality food source for wildlife which has now all but disappeared. Similar devastation and impact on the forest ecosystem was caused by the introduction of fungus species of the genus *Ophiostoma* from Asia to Europe and North America. Two different fungus species, the less aggressive *O. ulmi* and the more aggressive *O. novo-ulmi* cause Dutch elm disease in American (*Ulmus americana*) and English elm (*U. procera*). The less aggressive lineage *O. ulmi* was introduced to Europe and North America in the early 1900s. The more aggressive *O. novo-ulmi*-lineage appeared in North America in the 1940s, and was transferred from there to Europe in the 1960s. These pathogens caused severe population declines in American and English elm trees.

2. Current situation of pathogen pollution

Pathogen pollution is an on-going problem in the modern world (Table 2), and with the increase in trade and travel around the globe might increase.

Table A2.2: Recent examples of pathogen pollution posing a threat to economy and biodiversity.

Pathogen	Affected Species	Date	Origin	Destination	Estimated threat	Reference
West Nile	Birds, mammals, reptiles	1999	Africa	Americas	Rapid spread through Americas; mortality in susceptible wildlife and humans	
Chytridiomycosis	Amphibians world-wide	~1950s; identified 1993	Africa	global	Threat to amphibian species world- wide	(Weldon *et al.*, 2004a)
Babesia canis	Domestic dog (*Canis familiaris*)	Late 1990s	Southern Europe	Germany	Creation of endemic foci and establishment of vectors and disease in Northern Europe	
Mycoplasma gallisepticum (mycoplasmal conjunctivitis)	North American house finches (*Carpodacus mexicanus*)	1990s	Domestic poultry	Native North American finches	Expansion of the *Mycoplasma gallisepticum* epidemic	(Hartup *et al.*, 2001, Dhondt *et al.*, 2006)
Ranavirus	Common frogs (*Rana temporaria*) in England	1980s	North America (pet trade)	England	Population declines in frogs in England	(Teacher *et al.*, 2010)
Colony collapse disorder (caused by several micro and macroparasites)	Honeybee (*Apis mellifera*)			worldwide	Economic and ecological impact	

2.1 Mammals and birds

A new global route for pathogen transmission has opened up with the advent of mass tourism. Today, everyday people have started to take their pets abroad or adopt animals directly from animal shelters or animal welfare organisations abroad. This poses a whole new risk to animal health, animals are imported from other countries, often for animal welfare reasons and travel around the globe with their owners (Englund and Pringle 2003; Menn et al. 2010). One example is a disease that appeared in the late 1990s as a result of the movement of shelter animals from Southern Europe to other European countries like the UK and Germany, canine babesiosis. This disease is caused by *Babesia canis* and its' vector, the tick *Dermacentor reticulatus*. Until the late 1990s, the disease was exotic to Germany, but since then, several endemic foci of the disease have been established (Zahler and Gothe 1997; Zahler et al. 2000; Zahler and Gothe 2001; Beelitz et al. 2008). Not only has the frequency of cases of this arthropod-borne disease in German veterinary practices increased (Menn et al. 2010), but the pathogen and their vector have become established in several regions.

Other examples possibly appeared with global lifestock trade. In 1994, a novel lineage of a common poultry pathogen *Mycoplasma gallisepticum* appeared in native birds in the US (Dhondt *et al.*, 2006). *M. gallisepticum* causes mycoplasmal conjunctivitis which had a severe impact on populations of the eastern house finch (*Carpodacus mexicanus*). The origin of the disease was in the Washington DC area, and spread rapidly through the eastern North American range of the eastern house finch (Fischer *et al*., 1997; Dhondt *et al*., 1998). The pathogen caused a significant decline in its newly acquired host species (Hartup *et a*l., 2001).

West Nile Virus reviewed in "West Nile Virus and Wildlife" (Marra *et al.*, 2004) and "West Nile Virus in the Americas" Pollock (2008)

The arrival of the mosquito borne West Nile virus in North America in 1999 had a devastating impact. The virus, belonging to the family flaviviridae (with the type species, the yellow fever virus) has a very broad host spectrum, not only infecting wildlife like reptiles, birds and mammals, but also killing hundreds of humans. The origin of the virus is believed to be Africa or Asia, and was first isolated in 1937 in the

West Nile district of Uganda (Marra *et al*., 2004). Since then, it has been found to be one of the most wide-spread flaviviruses. A lineage very closely related to the one causing the epidemic in North America was identified in 1998 in Israel (Marra *et al*., 2004). It is unclear how the virus was introduced into the US, but possible explanations include the arrival of an infected mosquito by aircraft, importation of infected birds or transport by migratory birds (Pollock, 2008). After its arrival, the virus spread rapidly through North America, infecting a wide range of species. Birds, of the family Corvidae, such as crows, jays, and magpies, are most commonly affected, and the American crow (*Corvus brachyrhynchos*) makes up the bulk (80%) of positive cases (Pollock, 2008). The virus has also been found in other birds like house sparrows (*Passer domesticus*), common grackles (*Quiscalus quiscula*), house finches (*Carpodacus mexicanus*), American robins (*Turdus migratorius*), Cooper's hawks (*Accipiter cooperii*), and red-tailed hawks (*Buteo jamaicensis*) as well as mammals like squirrels (*Sciurus* species) and rabbits (*Sylvilagus floridanus*) (reviewed in Pollock (2008)). The virus was also found in North American alligators (*Alligator mississippiensis*). West Nile virus is neurotropic and causes encephalitis and meningitis, fever, local bleeding and cell death (reviewed in Marra et al. (2004)). In birds, the initial clinical signs include anorexia, weight loss and apathy. In advanced stages of the disease, birds show head tremors, liver necrosis, central blindness and more severe tremors and seizures. Surviving birds may suffer brain damage. To date, the impact of West Nile virus on North American wildlife cannot yet be estimated, although, should the virus sweep through small populations or species with limited geographic distribution (like the avifauna on Hawaii), the results could be devastating (Pollock, 2008). In the decade since its discovery in New York City there were more than 25000 human cases with more than 1000 deaths (Murray *et al.*, 2010).

2.2 Amphibians

The most striking example and currently largest infectious disease threat to biodiversity (Kilpatrick *et al*., 2010) is the worldwide decline of amphibians. It is due to a pandemic emergence of chytridiomycosis, caused by the parasitic chytrid fungus *Batrachochytrium dendrobatidis* (Kriger and Hero, 2009; Kilpatrick *et al*., 2010; Rodder, Kielgast e*t al*., 2010). (Pathology reviewed in (Campbell *et al.*, 2012)).

This pathogen causes severe skin disease in amphibians. Mainly the ventral abdomen and toes are affected (Berger *et al.*, 2005). The infection is localized in the superficial layers of the epidermis, and causes hyperkeratosis with cytoplasmic degeneration and vacuolation (Berger *et al.*, 1998). Visible lesions of the skin are uncommon and no histologically detectable changes in internal organs have been observed (Berger *et al*., 1998; Voyles *et al*., 2009). The damage to the skin by the pathogen causes abnormal electrolyte homeostasis, with a severe reduction in Na+ absorption across the skin, and loss of Na+, K+ and Cl− from the plasma (Voyles *et al.*, 2009), leading to asystolic cardiac arrest and death (Campbell *et al*., 2012). The clinical signs of the disease include inappetance, lethargy, loss of righting reflex, skin sloughing (Voyles et al., 2009), and slower rehydration (Carver *et al.*, 2010), which become more apparent at the terminal stages of disease.

It is thought that *Batrachochytrium dendrobatidis* has originated in Africa (*Kielgast et al*., 2010). A study by Weldon et al. (2004) examined archived material from *Xenopus* species from Southern Africa and found the first evidence of chytridiomycosis in a specimen from 1938, more than half a century before the identification of the pathogen. In addition, the examined *Xenopus* species rarely show clinical signs of the disease and the prevalence has been stable in the *Xenopus* populations for decades (Weldon *et al.*, 2004b). Another study by Kielgast et al. (2010) also showed a high prevalence of chytridiomycosis and a large number of sub-clinically infected individuals in Kenya. This may suggest that the pathogen comes from the studied areas and host and pathogen have evolved together. The international trade and transport of infected amphibians is thought to be the most important driver for the intercontinental spread of this disease, most likely initialised by the trade with *Xenopus laevis* frogs for human pregnancy testing in the 1930s (Weldon *et al*., 2004) and more recently by the world-wide trade with North American bullfrogs *Rana catesbeiana* (Schloegel *et al.*, 2010). Chytridiomycosis is now globally ubiquitous, spreads rapidly and cannot be eradicated from affected sites (Kriger and Hero, 2009).

Another example for a current decline of an amphibian species due to an introduced pathogen is the appearance of ranavirus in the populations of common frogs (*Rana*

temporaria) in England (reviewed in Teacher *et al*., 2010). Mass mortalities in this frog species have occurred in England since the 1980s, and a disease caused by ranavirus is common in North America. There are two disease syndromes caused by ranavirus in common frogs; ulcerative skin syndrome, characterized by dermal ulceration and haemorrhagic syndrome, characterized by systemic haemorrhaging within the skeletal muscles and visceral organs (Cunningham *et al*., 1996). Other, unspecific signs include lethargy and emaciation and a high mortality rate (Cunningham *et al*., 1996). A study by Teacher *et al*. (2010) has shown that common frogs in the UK are experiencing reoccurring outbreaks of ranaviral disease with significant localized population declines. It is thought that this pathogen was introduced to England from North America, potentially due to translocations of the bullfrog (*Rana catesbiana*) or goldfish (*Carassius auratus*) for the pet trade (Cunningham, Daszak & Rodriguez, 2003).

3. Situation of pathogen pollution in New Zealand

New Zealand is not exempt from the occurrence of pathogen pollution. The impact of the introduction of the Australian brushtail possum, acting as a new reservoir for bovine tuberculosis, has already been described above. Since the year 2000, the *Varroa*-mite has been in New Zealand, bringing with it a threat not only to bee keepers, but also whole economic sectors relying on the pollination services provided by honey bees (Goodwin, 2004).

In 1995, a large-scale epizootic in pilchards (*Sardinops neopilchardus*), caused by the pilchard herpes virus, appeared in Australia and New Zealand (Bons *et al*., 1999). It is thought that the mortality of pilchards during the epizootic ranged as high as high 75% (Gaughan *et al.*, 2000). The virus has had not only an economic impact on pilchard fisheries in Australia and New Zealand (Whittington *et al.*, 1997), but also had a significant impact on wildlife, especially piscivorous bird species (Dann *et al.*, 2000, Bunce and Norman, 2000). Little blue penguins (*Eudyptula minor*) showed increased mortality rates and breeding failures following the food shortage (Dann *et al.*, 2000) and Australasian gannets (*Morus serrator*) switched to other prey items with a lower calorific value (Bunce and Norman, 2000). It was suggested that the pathogen might have been induced by translocation of bait fish or the importation of

frozen pilchard as bait into Australia, but this has not been proven (Hine, 1995; Whittington *et al*., 1997).

The oyster herpes virus (OsHV-1), causing an outbreak in Pacific oysters (*Crassostrea gigas*) in New Zealand in 2010, is believed to have been in the country since 1992 (Castinel *et al*., 2015). The outbreak had a significant impact on the New Zealand shellfish industry, but the origin of the pathogen remains unknown.

In 1999, chytridiomycosis has been found in New Zealand in southern bell frogs (*Litoria raniformis*), a frog species introduced from Australia (Waldman *et al.*, 2001). This is of concern, after New Zealand has three unique endemic frog species of the genus *Leiopelma*, of which especially the Archey's frog (*L. archeyi*) is in rapid decline (Bell et al., 2004). There is evidence that disease plays a major role in the decline of this frog species, supported, according to Bell (2004), by (1) the rapidity and severity of decline, (2) the progressive (south to north) nature of decline, and (3) finding frogs with chytriodiomycosis at the time of decline.

Due to human activities, the exotic mosquito *Culex quinquefasciatus*, a vector of avian malaria (*Plasmodium* spp.), has been introduced to New Zealand. This may be of special interest, after the introduction of the same mosquito species to Hawaii is believed to have been the catalyst for the extinction of about 50% of the native bird fauna (van Riper, 1986). There is a possibility that an expansion in vector range of both native and introduced vectors (like *C. quinquefasciatus*) expose susceptible native New Zealand bird species to vector borne diseases like avian malaria and –pox for the first time or increase the parasite burden of species that are already infected (Tompkins and Gleeson, 2006).

Appendix 3: Serial dilutions and melt curves of qPCR for most common NZ Plasmodium lineages using primer pair HRMF and HaemR2

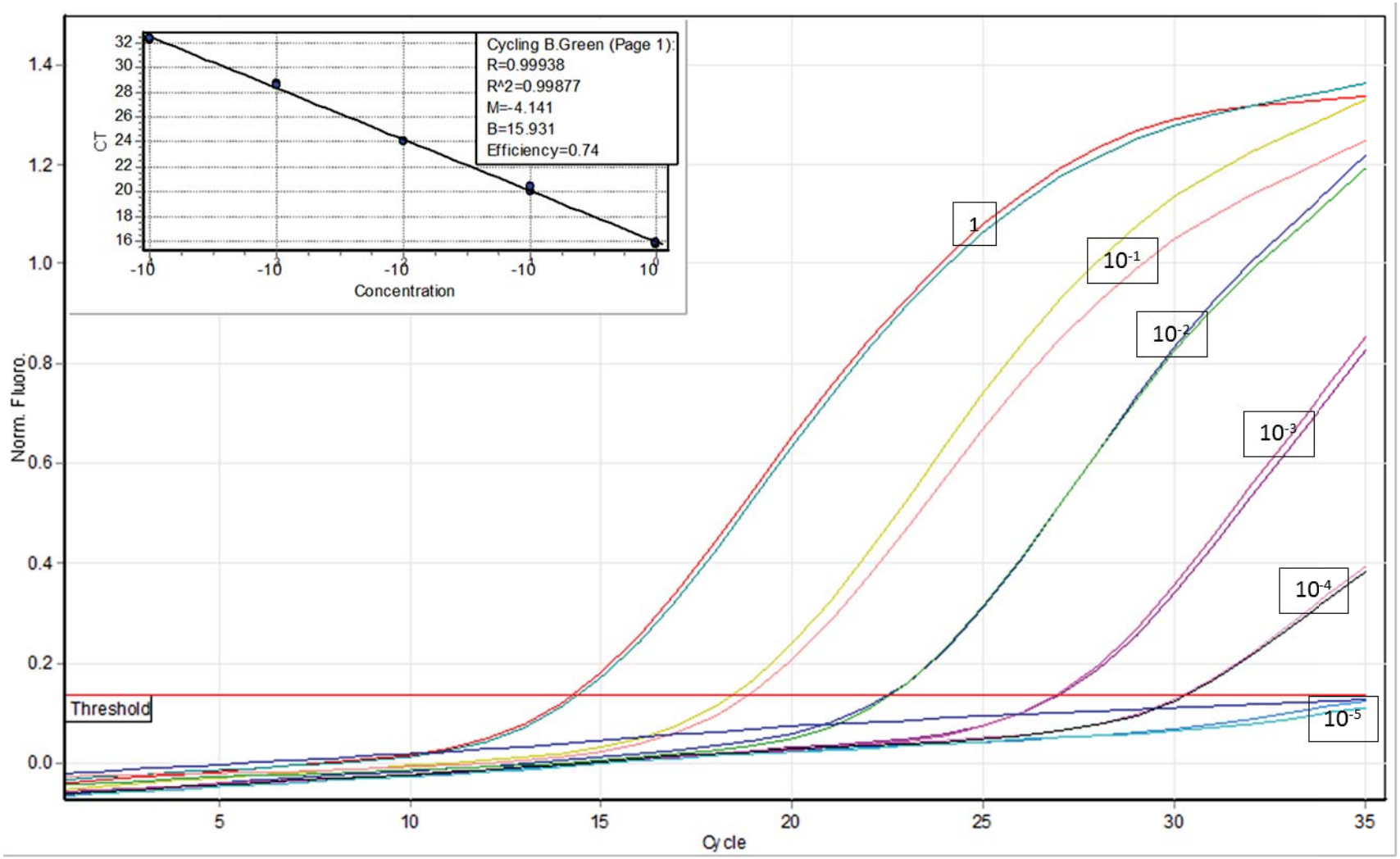

Figure A3.1: Five 10-fold serial dilutions of a 1ng/µl standard starting at 0.1ng/µl and standard curve for *P. elongatum* GRW6 using primers HRMF and HaemR2; detection limit is 10^{-4} ng/µl of DNA

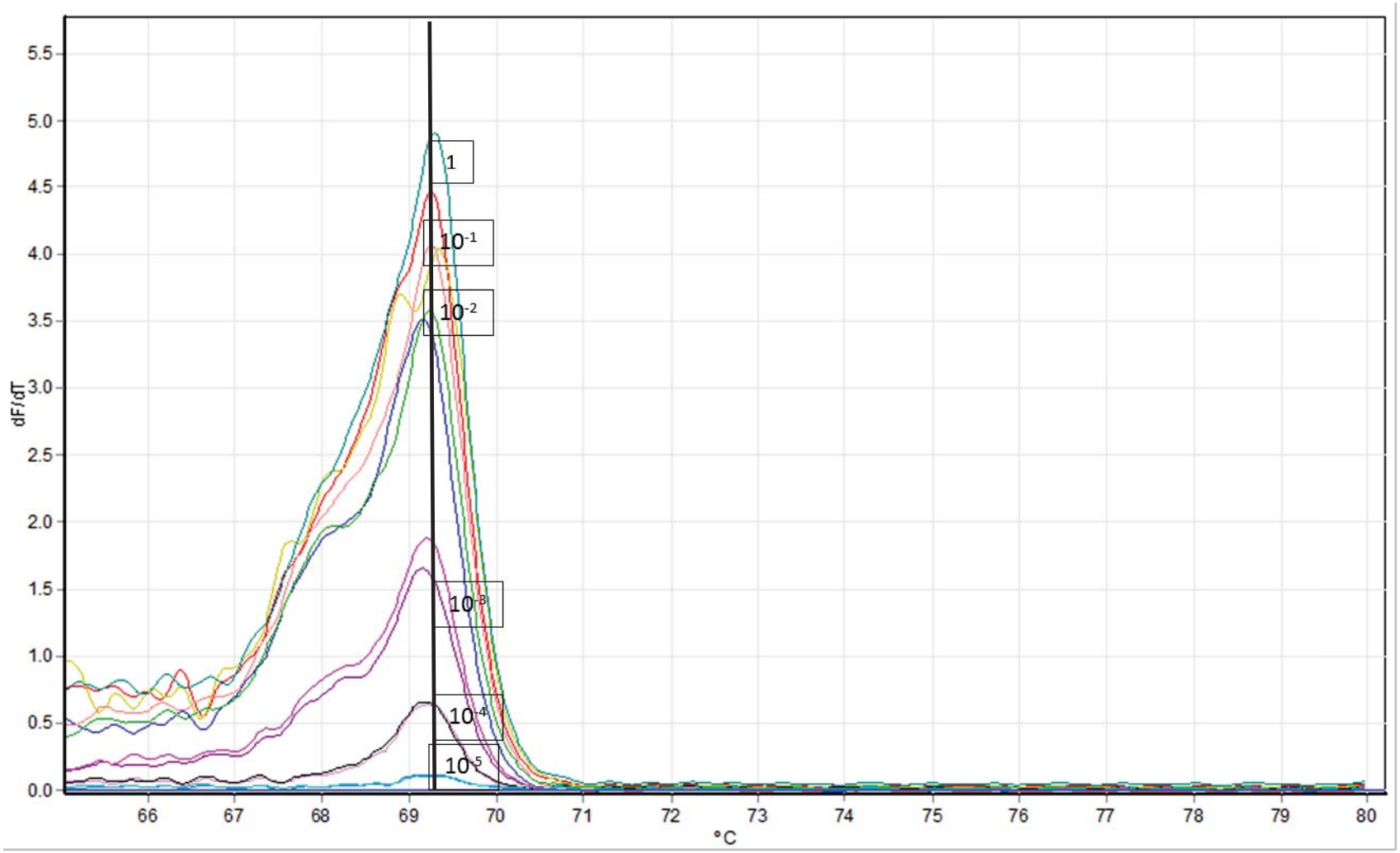

Figure A3.2: Melt curve of five 10-fold serial dilutions of a 1ng/µl standard starting at 0.1ng/µl and standard curve for *P. elongatum* GRW6 using primers HRMF and HaemR2; detection limit is 10^{-4} ng/µl of DNA

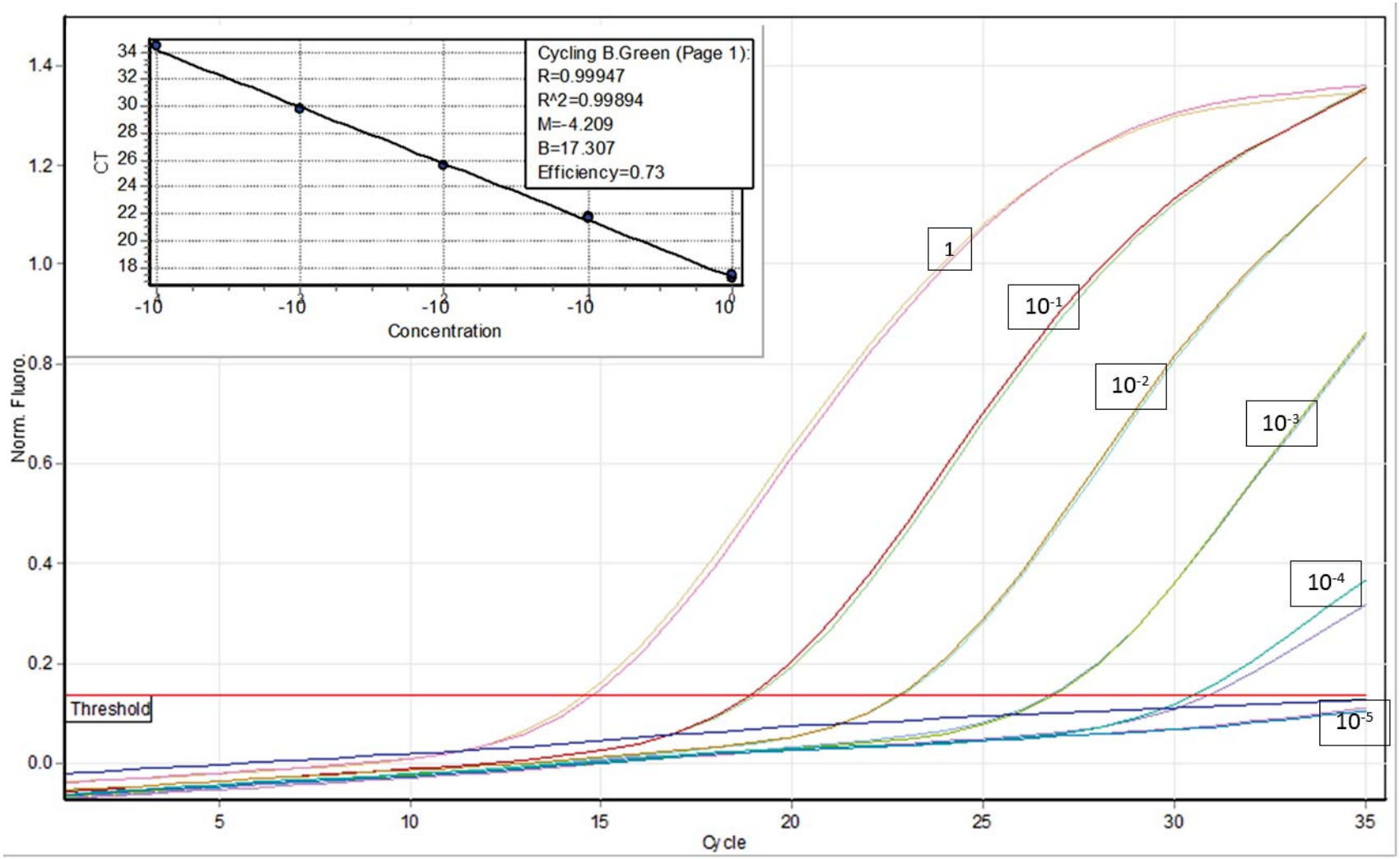

Figure A3.3: Five 10-fold serial dilutions of a 1ng/µl standard starting at 0.1ng/µl and standard curve for *P. relictum* GRW4 using primers HRMF and HaemR2; detection limit is 10^{-4} ng/µl of DNA

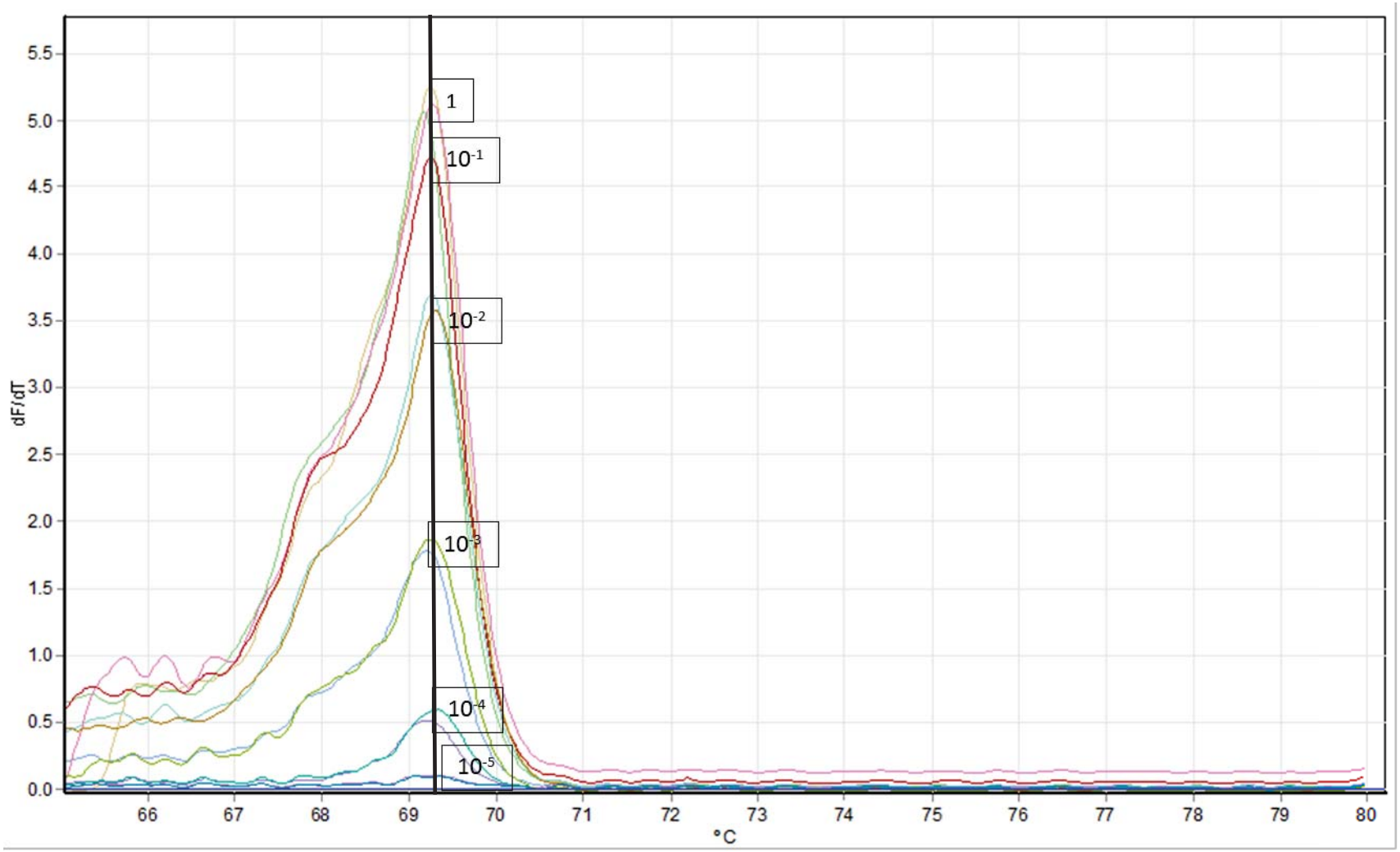

Figure A3.4: Melt curve of five 10-fold serial dilutions of a 1ng/µl standard starting at 0.1ng/µl and standard curve for *P. relictum* GRW4 using primers HRMF and HaemR2; detection limit is 10^{-4} ng/µl of DNA

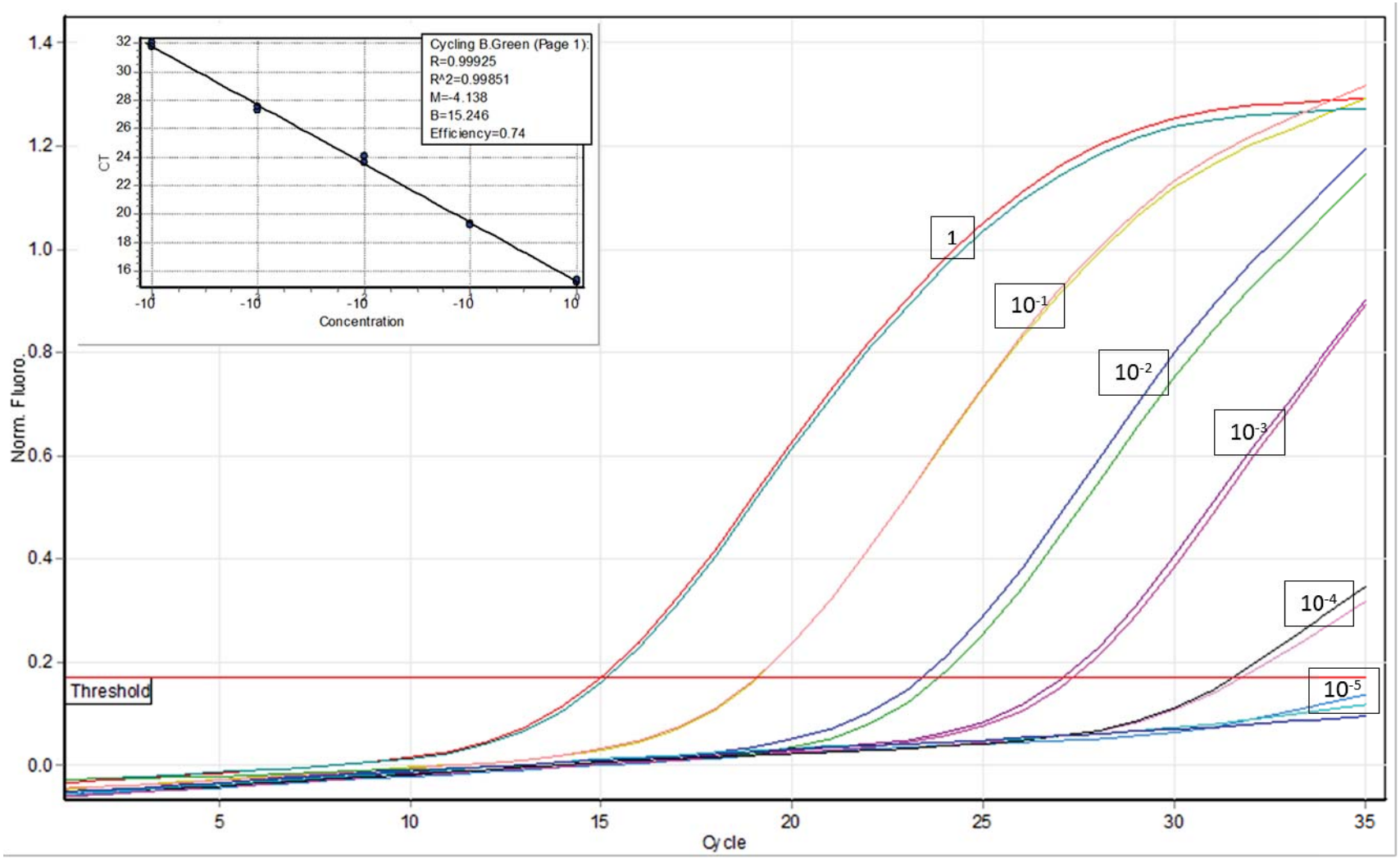

Figure A3.5: Five 10-fold serial dilutions of a 1ng/µl standard starting at 0.1ng/µl and standard curve for *P.* spp. LINN1 using primers HRMF and HaemR2; detection limit is 10^{-4} ng/µl of DNA

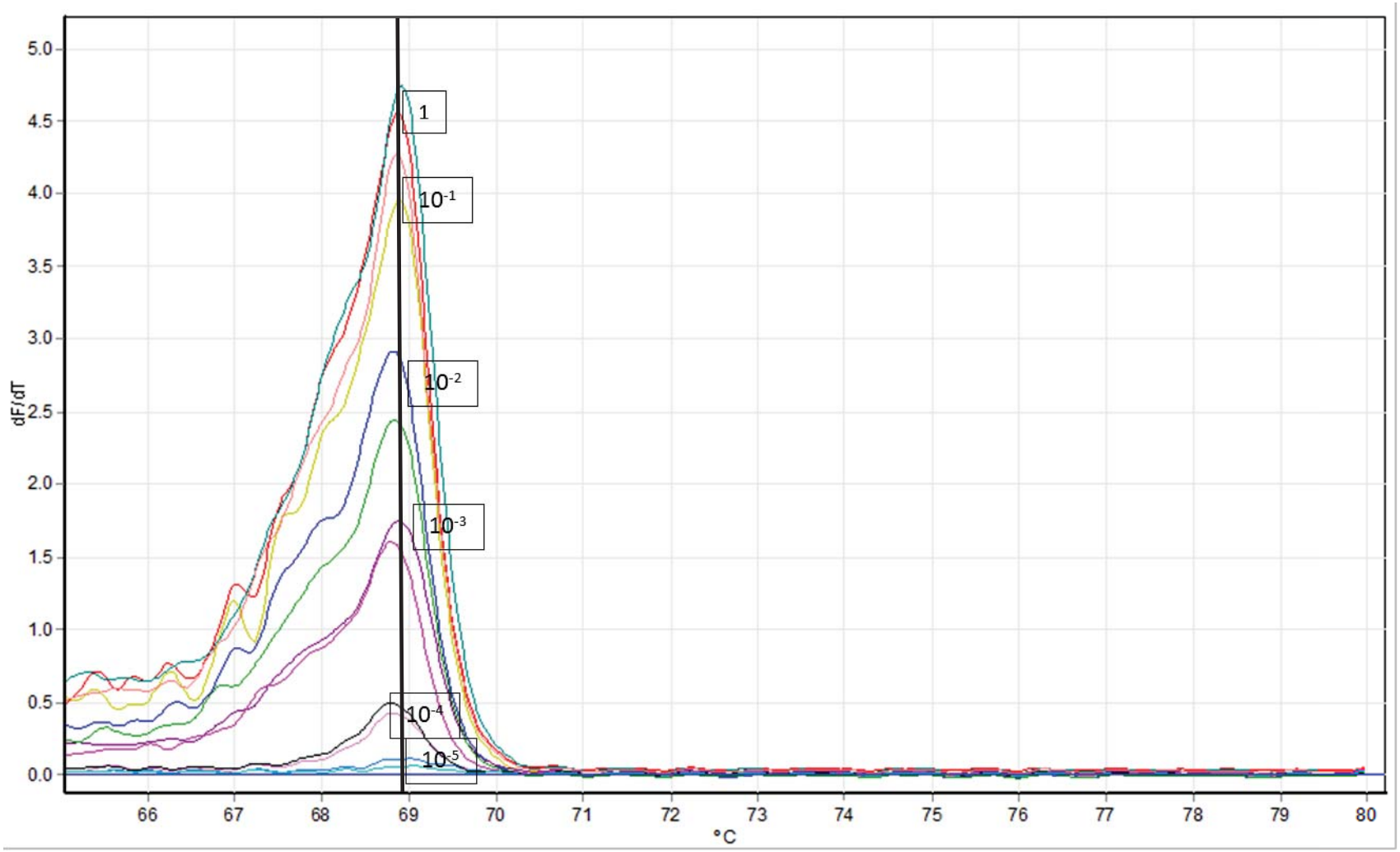

Figure A3.6: Melt curve of five 10-fold serial dilutions of a 1ng/µl standard starting at 0.1ng/µl and standard curve for *P.* spp. LINN1 using primers HRMF and HaemR2; detection limit is 10^{-4} ng/µl of DNA

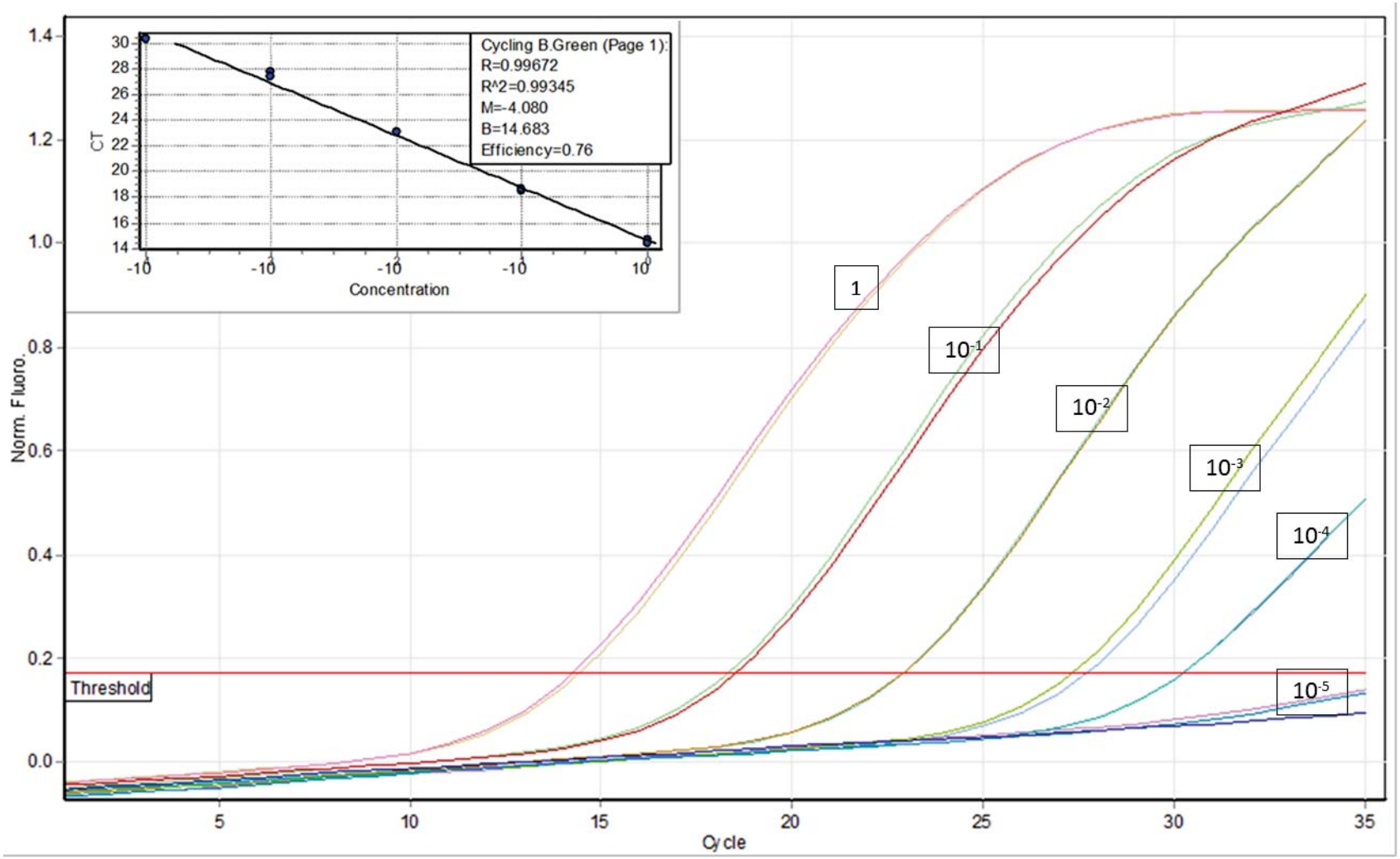

Figure A3.7: Five 10-fold serial dilutions of a 1ng/µl standard starting at 0.1ng/µl and standard curve for *P. vaughani* SYAT05 using primers HRMF and HaemR2; detection limit is 10^{-4} ng/µl of DNA

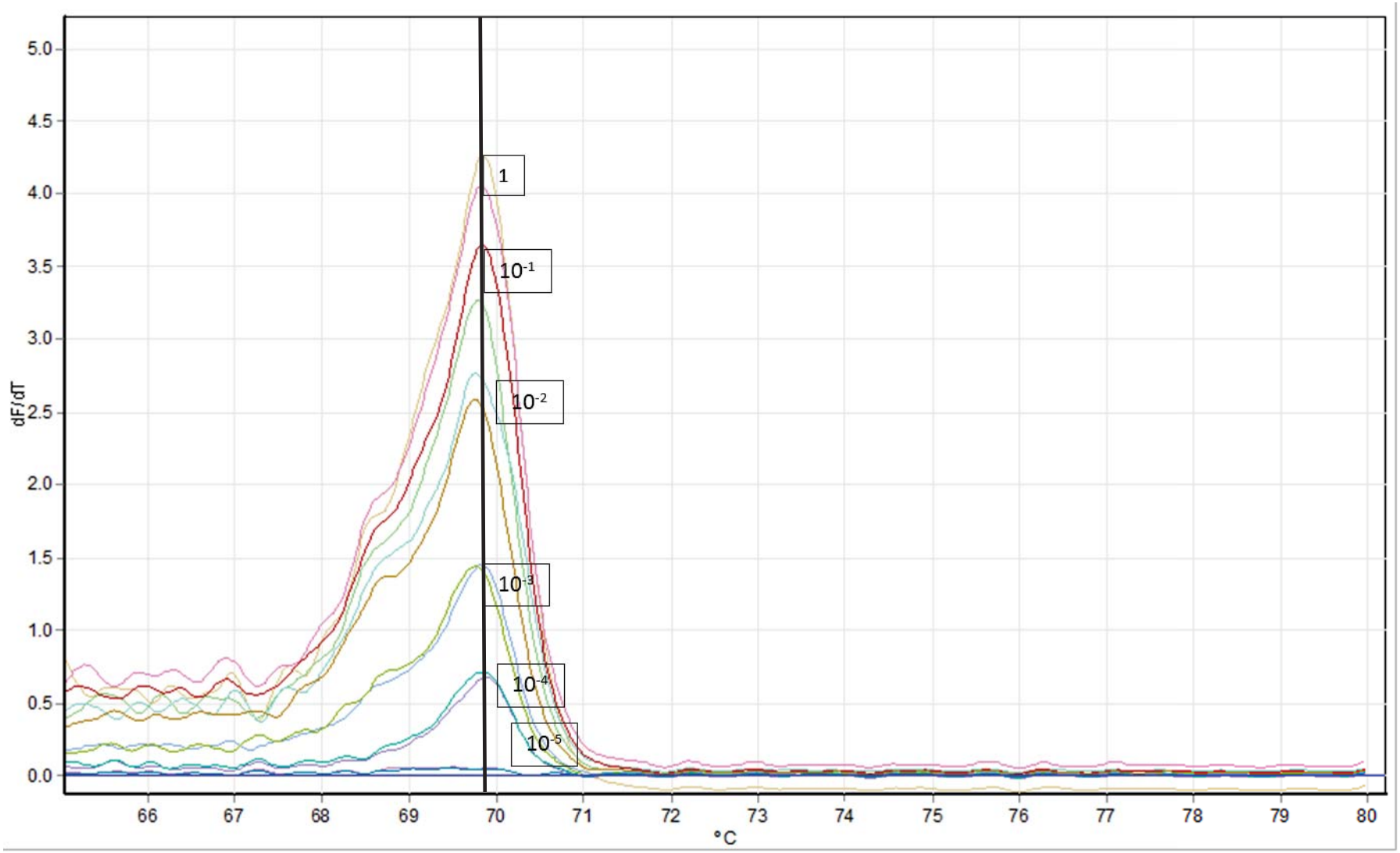

Figure A3.8: Melt curve of five 10-fold serial dilutions of a 1ng/µl standard starting at 0.1ng/µl and standard curve for *P. vaughani* SYAT05 using primers HRMF and HaemR2; detection limit is 10^{-4} ng/µl of DNA

Appendix 4: Identifying *Plasmodium* species mixed infections in mosquito vectors by interpreting peak variation in electropherograms using Geneious version 6.1.7 software

Figure A4: Electropherogram showing simultaneus different coloured peaks corresponding with base differences in three different Plasmodium lineages, Linn1, GRW6 and SYAT05

Appendix 5: Capture and handling of the birds, including measurements taken

(Please refer to Colour Plates 2 and 3)

The birds were caught in mist nets using recordings of saddleback song and blackbird alarm calls as lure. The bags for restraining the birds were used only once on each side and washed, disinfected and dried at the end of each day. The birds were carefully placed into these bags by hand. Hands were disinfected after handling each bird, to reduce the spread of transmissible diseases.

After capture, each of the following measurements and information were taken: weight, tarsus length, wing length, body condition, presence of a brood patch in females, and wattle size in saddlebacks only. Length measurements were made with callipers to an accuracy of 0.1mm. For assessment of body condition, the amount of muscle and fat tissue covering the sternum and the keel (Pectoral muscle scores (0-3) as well as the amount of fat in the thoracic inlet of the bird were estimated with modified scale of 0-4 according to the methods described by (Melville, 2011). Each bird was given a metal band for individual identification and to prevent resampling.

The health status of every bird was assessed by evaluating the body condition, looking for wounds and/ or lesions as well as colour of mucous membranes (eyes and mouth) and behaviour and responsiveness of the bird in the hand. Animals of ill health or showing unusual stress were released without further manipulation. The birds were restrained using a blood-sampling cone or the hand. Blood samples were taken by venepuncture of the brachial vein directly into capillary tubes. The area of puncture was cleaned with 70% ethanol prior to pricking and pressure was applied to the puncture following blood extraction to stop the flow of blood. One drop of blood was used to prepare blood smears, and the remaining blood was placed into sampling tubes containing heparin. On Hen Island, where no cooling facilities were available, blood was collected in plastic microcentrifuge tubes containing 98% ethanol.

Saddlebacks, which are known to consume nectar, were given freshly prepared sugar water after handling and prior to release. Birds that were recaptured were examined for health status as described above and released without further manipulation.

Appendix 6: Raw data

Table A6.1: Measurements of sampled birds

Location	Sampling Date	Bird Species	Bird number	weight	Sex	Body Condition	Thoracic Inlet	Tarsus LONG	Head Bill	Wing Length	left length	left width	right length	right width
Auckland/ Northshore	8/03/2012	Blackbird	D 203920	105	J	5 out of 9	5 out of 5	3.76	4.96	120				
Auckland/ Northshore	6/03/2013	Blackbird	D 203917	89	J	3 out of 9	5 out of 5	3.78	5.39	125				
Auckland/ Northshore	6/03/2013	Blackbird	D 203918	102	J	3 out of 9	4 out of 5	3.88	5.24	129				
Auckland/ Northshore	7/03/2013	Songthrush	C 86208	71	J	2 out of 9	1 out of 5	3.21	4.33	118				
Auckland/ Northshore	7/03/2013	Songthrush	C 89167	75	J	3 out of 9	3 out of 5	3.54	4.22	111				
Auckland/ Northshore	7/03/2013	Blackbird	D 203919	102	J	5 out of 9	3 out of 5	3.83	5.22	128				
Auckland/ Northshore	7/03/2013	Blackbird	DP 3404	90	J	3 out of 9	1 out of 5	3.89	5.34	128				
Auckland/ Northshore	7/03/2013	Blackbird	DP 3405	110	J	5 out of 9	5 out of 5	3.5	5.4	132				
Auckland/ Northshore	7/03/2013	Blackbird	DP 3406	109	J	6 out of 9	5 out of 5	3.92	5.12	128				
Auckland/ Northshore	8/03/2013	Blackbird	D 203921	103	J	4 out of 9	5 out of 5	3.6	5.08	127				
Bushy Park	1/03/2012	Saddleback	D 188682	73	M	5 out of 9		3.96	5.93	9.2	1	0.52	1.14	0.52
Bushy Park	1/03/2012	Saddleback	D 19180	95	M	6 out of 9		3.8	5.8	9.3	1.1	0.7	1.05	0.65
Bushy Park	1/03/2012	Saddleback	D 19181	80	M	4 out of 9		3.8	5.8	9	1.2	0.65	1.05	0.7
Bushy Park	1/03/2012	Saddleback	D 191815	87	M	4 out of 9		4.7	6	9	0.9	0.7	1	0.65
Bushy Park	1/03/2012	Saddleback	D 204201	75	M	5 out of 9		3.75	5.93	8.43	1	0.9	1.1	0.7
Bushy Park	1/03/2012	Saddleback	D 204202	66	J	4 out of 9		4	5.8	8.9	0.62	0.42	0.6	0.21
Bushy Park	1/03/2012	Saddleback	D 204203	65	J	6 out of 9		3.85	5.93	8.5	0.52	0.31	0.42	0.2
Bushy Park	1/03/2012	Saddleback	D 204204	65	J (?)	5 out of 9		4	5.72	9	0.32	0.15	0.2	0.1
Bushy Park	1/03/2012	Saddleback	D 204205	63	J(?)	4 out of 9		4.1	5.8	9	0.6	0.35	0.4	0.35
Bushy Park	1/03/2012	Saddleback	D 204206	77	M	6 out of 9		3.75	6.35	9.3	0.93	0.62	0.94	0.02
Bushy Park	1/03/2012	Saddleback	D 204207	65	F	4 out of 9		3.2	5.8	8.7	0.71	0.4	0.69	0.45
Bushy Park	1/03/2012	Saddleback	D 204208	69	F (?)	5 out of 9		4	5.83	8.5	0.5	0.45	0.65	0.4
Bushy Park	1/03/2012	Saddleback	D 204209	80	M	5 out of 9		3.5	6.2	9	1.1	0.5	0.8	0.4
Bushy Park	1/03/2012	Saddleback	D 204210	80	M	5 out of 9		3.7	5.9	9	0.8	0.5	0.9	0.6
Bushy Park	1/03/2012	Saddleback	D 204211	80	M	5 out of 9		3.7	6.1	9	1	0.5	1	0.55
Bushy Park	1/03/2012	Saddleback	D 204213	80	M (?)	6 out of 9		3.5	5.3	9.5	0.8	0.2	0.8	0.2
Bushy Park	1/03/2012	Saddleback	D 204214	70	F	5 out of 9		4.2	5.9	8	0.65	0.3	0.6	0.35

Bushy Park	1/03/2012	Saddleback	D 204215	70	J (?)	6 out of 9		4.2	5.6	9	0.45	0.4	0.55	0.35
Bushy Park	1/03/2012	Saddleback	D 204216	72	J (?)	5 out of 9		4.3	5.85	9	0.6	0.25	0.45	0.3
Bushy Park	1/03/2012	Saddleback	D 204217	83	M	6 out of 9		4.5	6	8	1	0.6	0.9	0.5
Bushy Park	1/03/2012	Saddleback	D 204218	72	J	5 out of 9		4.4	5.7	9.2	0.6	0.4	0.5	0.3
Bushy Park	1/03/2012	Saddleback	D 204219	NA	M	5 out of 9		4.5	5.9	9.2	0.9	0.45	0.8	0.45
Bushy Park	1/03/2012	Saddleback	D 204220	69	F	4 out of 9		4.3	5.8	8.5	0.8	0.45	0.8	0.4
Bushy Park	1/03/2012	Saddleback	D 204221	80	M	5 out of 9		4.65	5.75	9.5	1	0.6	1	0.6
Bushy Park	1/03/2012	Saddleback	D 204222	56	J	4 out of 9		4.8	5.6	9	0.3	1.5	3.5	0.2
Bushy Park	1/03/2012	Saddleback	D 204223	66	F	5 out of 9		4.35	5.8	8.7	0.7	0.4	0.7	0.5
Bushy Park	1/03/2012	Saddleback	D 204224	83	M	6 out of 9		4.6	6.1	9.5	0.9	0.6	1	0.6
Bushy Park	1/03/2012	Saddleback	D 204225	80	M	4 out of 9		4.7	5.9	9.5	0.9	0.6	1	0.55
Bushy Park	1/03/2012	Saddleback	D 204226	80	M	5 out of 9		4.8	6.3	9.5	0.9	0.6	1	0.6
Bushy Park	1/03/2012	Saddleback	D 204227	75	F (?)	5 out of 9		4.35	6	8.7	0.8	0.5	0.7	0.5
Bushy Park	1/03/2012	Saddleback	D 204228	77	F	6 out of 9		4.3	5.5	8	0.7	0.4	0.8	0.4
Bushy Park	1/03/2012	Saddleback	D 204229	70	F	5 out of 9		4.4	5.75	8	0.7	0.4	0.7	0.4
Bushy Park	1/03/2012	Saddleback	D 204230	68	F	6 out of 9		4.4	5.8	9.2	0.7	0.35	0.65	0.35
Bushy Park	1/03/2012	Saddleback	D 204231	69	J	4 out of 9		4.6	5.7	8.7				
Bushy Park	1/03/2012	Saddleback	D 204299	75	M	6 out of 9		4.75	6	9	1	0.5	1	0.5
Bushy Park	1/03/2012	Saddleback	D 204300	75	M	4 out of 9		3.54	5.93	9	1.14	0.73	1	0.72
Bushy Park	1/03/2012	Saddleback	D204212	63	F (?)	4 out of 9		3.4	5.8	8.4	0.8	0.35	0.8	0.4
Bushy Park	14/01/2013	Saddleback	204215	66	F	5 out of 9	2 out of 5	4.38	5.9	8.1	0.63	0.46	0.46	0.4
Bushy Park	14/01/2013	Saddleback	204241	76	?	4 out of 9	4 out of 5	4.62	6.16	9.3	0.84	0.49	0.88	0.45
Bushy Park	14/01/2013	Saddleback	204242	79	?	4 out of 9	1.5 out of 5	4.64	5.07	9.5	0.69	0.51	0.75	0.45
Bushy Park	14/01/2013	Saddleback	204259	77	F	5 out of 9	2 out of 5	4.8	6	9.4	0.97	0.59	1.1	0.56
Bushy Park	14/01/2013	Saddleback	204278	76	M	4 out of 9	1.5 out of 5	4.63	6	9.5	1.12	0.65	1.1	0.58
Bushy Park	14/01/2013	Saddleback	204279	76	M	5 out of 9	2 out of 5	escape	escape	escape	escape	escape	escape	escape
Bushy Park	15/01/2013	Saddleback	191812	79	M	3.5 out of 9	2 out of 5	4.66	5.98	9.7	1.29	0.7	1.05	0.64
Bushy Park	15/01/2013	Saddleback	204273	80	M	4.5 out of 9	1.5 out of 5	4.79	6.11	8.9	1.12	0.64	1.11	0.7
Bushy Park	15/01/2013	Saddleback	204274	73	F	4 out of 9	2 out of 5	4.65	6.06	9.8	0.75	0.42	0.83	0.39
Bushy Park	15/01/2013	Saddleback	204275	81	M	4 out of 9	2 out of 5	4.54	6.14	9.6	1.04	0.64	1.26	0.65
Bushy Park	15/01/2013	Saddleback	204276	75	F	4 out of 9	1.5 out of 5	4.36	5.81	9.5	0.86	0.41	0.86	0.38
Bushy Park	15/01/2013	Saddleback	204277	76	M	4 out of 9	1 out of 5	4.59	5.93	9.6	1.08	0.59	1.05	0.57
Bushy Park	16/01/2013	Saddleback	204206	94.5	M?	5 out of 9	1 out of 5	4.67	6.21	9.3	1	0.62	1.3	0.6

Bushy Park	16/01/2013	Saddleback	204210	74	?	4 out of 9	4 out of 5	4.45	6.06	9.7	1	0.54	0.98	0.56
Bushy Park	16/01/2013	Saddleback	204264	80	J	6 out of 9	5 out of 5	4.82	5.77	9.8	0.53	0.33	0.54	0.29
Bushy Park	16/01/2013	Saddleback	204265	57	J	6 out of 9	4 out of 5	4.81	5.83	9.5	0.41	0.21	0.55	0.21
Bushy Park	16/01/2013	Saddleback	204266	80	M	4 out of 9	4 out of 5	4.67	6.1	9.6	1.09	0.61	1.1	0.57
Bushy Park	16/01/2013	Saddleback	204267	76	F	5 out of 9	3 out of 5	4.44	6.12	9.5	0.8	0.43	0.85	0.46
Bushy Park	16/01/2013	Saddleback	204268	84	M	4 out of 9	3 out of 5	4.57	6.11	9.5	0.96	0.53	0.98	0.54
Bushy Park	16/01/2013	Saddleback	204269	70	J	4 out of 9	3 out of 5	4.77	5.79	9.7	0.51	0.27	0.45	0.24
Bushy Park	16/01/2013	Saddleback	204270	98	F	5 out of 9	2 out of 5	4.66	5.87	9.4	0.86	0.48	0.96	0.54
Bushy Park	16/01/2013	Saddleback	204271	63	J	5 out of 9	3 out of 5	4.88	5.7	8.5	0.45	0.31	0.43	0.3
Bushy Park	16/01/2013	Blackbird	204272	88	M	escape	escape	4	5.2	12.5				
Bushy Park	17/01/2013	Saddleback	191873	77	F	5 out of 9	3 out of 5	4.3	5.84	9.5	0.85	0.45	0.8	0.55
Bushy Park	18/01/2013	Saddleback	204216	78	M	4 out of 9	2 out of 5	4.55	5.99	9.4	0.88	0.47	0.88	0.53
Bushy Park	18/01/2013	Saddleback	204219	81	M	4 out of 9	4 out of 5	4.76	6.06	9.8	1	0.56	1.07	0.6
Bushy Park	18/01/2013	Saddleback	204260	72	F	5 out of 9	4 out of 5	4.37	5.86	9.8	0.58	0.34	0.61	0.38
Bushy Park	18/01/2013	Saddleback	204261	85	F	4 out of 9	1 out of 5	4.7	5.84	escape	0.9	0.52	0.9	0.54
Bushy Park	18/01/2013	Saddleback	204262	77	M	escape	escape	4.9	6.15	9.5	escape	escape	escape	escape
Bushy Park	18/01/2013	Saddleback	204263	75	F	4 out of 9	0 out of 5	4.7	6.2	9.6	0.7	0.38	0.77	0.4
Bushy Park	19/02/2013	Saddleback	204225	74	M	5 out of 9	2 out of 5	4.68	5.99	98	0.9	0.55	0.92	0.56
Bushy Park	19/02/2013	Saddleback	204226	76	M	6 out of 9	3 out of 5	4.72	62.5	103	1.14	0.69	1.15	1.13
Bushy Park	19/02/2013	Saddleback	204243	67	F ?	4 out of 9	1 out of 5	4.36	5.89	89	0.68	0.41	0.72	0.37
Bushy Park	19/02/2013	Saddleback	204244	78	F	5 out of 9	2 out of 5	4.6	6.05	moult	0.73	0.4	0.8	0.38
Bushy Park	19/02/2013	Saddleback	204245	83	M	5 out of 9	1 out of 5	4.71	6.12	98	0.92	0.51	0.94	0.47
Bushy Park	19/02/2013	Saddleback	204246	87	M	5 out of 9	2 out of 5	4.69	6.02	96	1.01	0.48	0.92	0.52
Bushy Park	19/02/2013	Blackbird	204247	95	J (M?)	5 out of 9	2 out of 5							
Bushy Park	19/02/2013	Saddleback	204256	73	J	5 out of 9	2 out of 5	4.53	6.04	96	0.56	0.32	0.56	0.28
Bushy Park	20/02/2013	Blackbird	204248	95	J	3 out of 9	4 out of 5	3.94	5.25	121				
Bushy Park	20/02/2013	Blackbird	204249	97	J	3 out of 9	5 out of 5	3.89	5.25	130				
Bushy Park	20/02/2013	Songthrush	204250	66	J	3 out of 9	3 out of 5	3.75	4.66	117				
Bushy Park	20/02/2013	Saddleback	204251	68	F	5 out of 9	3 out of 5	4.37	5.9	91	0.84	0.45	0.6	0.43
Bushy Park	20/02/2013	Blackbird	204252	89	J (M?)	4 out of 9	2 out of 5	3.77	5.04	117				
Bushy Park	20/02/2013	Blackbird	204253	99	J (M?)	4 out of 9	2 out of 5	3.93	5.19	129				
Bushy Park	20/02/2013	Saddleback	204254	72	F	4 out of 9	3 out of 5	4.47	6.05	97	0.76	0.43	0.61	0.4
Bushy Park	20/02/2013	Blackbird	204255	88	J	3 out of 9	3 out of 5	3.87	5.07	122				

Bushy Park	20/02/2013	Saddleback	204266	79	M	5 out of 9	1 out of 5	4.71	6.15	97	1.07	0.55	1.03	0.51
Bushy Park	20/02/2013	Blackbird	DP 3402	97	J	3 out of 9	4 out of 5	3.74	5.04	130				
Bushy Park	20/02/2013	Blackbird	DP 3403	96	J (M?)	3 out of 9	5 out of 5	3.69	5.19	130				
Bushy Park	20/02/2013	Blackbird	DP 3499	98	J (M)	4 out of 9	4 out of 5	4	5.13	128				
Bushy Park	20/02/2013	Blackbird	DP 3500	92	J	4 out of 9	3 out of 5	3.82	5.21	127				
Cape Kidnappers	24/01/2013	Dunnock	A 167776	21	M	4 out of 9	5 out of 5	20	32.9	69				
Cape Kidnappers	1/02/2013	Chaffinch	A 167778	35	F	5 out of 9	3 out of 5	27	32	79				
Cape Kidnappers	1/02/2013	Dunnock	A 167779	21	J	4 out of 9	3 out of 5	25.7	32.2	65				
Cape Kidnappers	1/02/2013	Yellowammer	A 167780	22	J	5 out of 9	3 out of 5	23.7	30	75				
Cape Kidnappers	1/02/2013	Yellowammer	A 167781	22	?	4 out of 9	2 out of 5	24.1	31.4	76				
Cape Kidnappers	1/02/2013	Greenfinch	A 167782	27	M	3 out of 9	4 out of 5	21.9	32.4	84				
Cape Kidnappers	1/02/2013	Greenfinch	A 167783	28	F	5 out of 9	4 out of 5	21.5	32.4	81				
Cape Kidnappers	1/02/2013	Dunnock	A 167784	19	F	4 out of 9	4 out of 5							
Cape Kidnappers	1/02/2013	Sparrow	B 124501	31	J	4 out of 9	3 out of 5							
Cape Kidnappers	1/02/2013	Sparrow	B 124502	27	F	5 out of 9	3 out of 5	23.1	30.9	75				
Cuvier	25/04/2013	Saddleback	D 200124	83	M	6 out of 9	1 out of 5	42.5			0.9	0.49	0.83	0.41
Cuvier	25/04/2013	Saddleback	D 200135											
Cuvier	25/04/2013	Saddleback	D 200136	71	F	4 out of 9	3 out of 5	38.7			0.6	0.34	0.55	0.35
Cuvier	25/04/2013	Saddleback	D 200139	82	M	7 out of 9	1 out of 5	41.8					0.97	0.47
Cuvier	25/04/2013	Saddleback	D 200142	70	J	3 out of 9	5 out of 5	41						
Cuvier	25/04/2013	Saddleback	D 200143	72	F	4 out of 9	4 out of 5	39.1						
Cuvier	25/04/2013	Saddleback	D 200144	79	M	5 out of 9	4 out of 5	38.5					0.97	0.57
Cuvier	25/04/2013	Saddleback	D 200145	80	M	5 out of 9	4 out of 5	41.2					1.09	0.63
Cuvier	25/04/2013	Saddleback	D 200153	66	J/F			38.4					0.81	
Cuvier	25/04/2013	Saddleback	D 200154	75	J/M	6 out of 9	4 out of 5	39.6					0.65	0.4
Cuvier	25/04/2013	Saddleback	D 200156	65	J/F	5 out of 9	3 out of 5	36.9					0.46	0.36
Cuvier	25/04/2013	Saddleback	D 200170	69	F	4 out of 9	3 out of 5	39.1					0.9	0.45
Cuvier	25/04/2013	Saddleback	D 200172	69	F	5 out of 9	4 out of 5	39.6					0.71	0.48
Cuvier	25/04/2013	Saddleback	D 200173	62	F	4 out of 9	3 out of 5	38					0.78	0.41
Cuvier	25/04/2013	Saddleback	D 200174	65	F	6 out of 9	4 out of 5	38.9					0.73	0.5
Cuvier	25/04/2013	Saddleback	D 200175	70	F	5 out of 9	5 out of 5	40					0.9	0.51
Cuvier	25/04/2013	Saddleback	D 200177	85	M	5 out of 9	3 out of 5	41.8					0.89	0.3
Cuvier	25/04/2013	Saddleback	D 200178	67	F	3 out of 9	1 out of 5	39.3					0.82	0.48

Cuvier	25/04/2013	Saddleback	D 200179	68	F	6 out of 9	5 out of 5	39.1					0.9	0.53
Cuvier	25/04/2013	Saddleback	D 200180	65	J/M	5 out of 9	1 out of 5	39.6					0.6	0.31
Cuvier	25/04/2013	Saddleback	D 200187	74	J/M	4 out of 9	4 out of 5	42.2					0.44	0.25
Cuvier	25/04/2013	Saddleback	D 200188	79	M			40.8					0.8	0.46
Cuvier	25/04/2013	Saddleback	D 200189	68	F			40					0.79	0.44
Cuvier	25/04/2013	Saddleback	D 200190	84	M	5 out of 9	2 out of 5	41.8					0.82	0.56
Cuvier	26/04/2013	Saddleback	D 200191	69	M	6 out of 9	2 out of 5	40.4					0.98	0.57
Cuvier	26/04/2013	Saddleback	D 200193	73	M			40.3					1.01	0.59
Cuvier	26/04/2013	Saddleback	D 200194	65	F	6 out of 9	5 out of 5	37.7					0.77	0.42
Cuvier	26/04/2013	Saddleback	D 200195	81	M	4 out of 9	2 out of 5	41.1					1.28	0.56
Cuvier	26/04/2013	Saddleback	D 200196	77	F	6 out of 9	5 out of 5	39.7					0.77	0.47
Cuvier	26/04/2013	Saddleback	D 200197	64	J	6 out of 9	5 out of 5	36.5					0.75	0.47
Cuvier	26/04/2013	Saddleback	D 200198	65	J	6 out of 9	5 out of 5	38.1					0.32	0.2
Cuvier	26/04/2013	Saddleback	D 200281	71	F			37.2					0.84	0.47
Cuvier	26/04/2013	Saddleback	D 200282	67	M	6 out of 9	3 out of 5	38.2					0.95	0.49
Cuvier	26/04/2013	Saddleback	D 200283	67	F	6 out of 9	4 out of 5	38.2					0.65	0.34
Cuvier	26/04/2013	Saddleback	D 200286	78	J	5 out of 9	5 out of 5	41.6					0.49	0.2
Cuvier	26/04/2013	Saddleback	D 200287	70	F	4 out of 9	2 out of 5	38.6					0.86	0.45
Cuvier	26/04/2013	Saddleback	D 200290	80	F	5 out of 9	4 out of 5	39.8					0.87	0.53
Cuvier	26/04/2013	Saddleback	D 200292	78	F (?)	5 out of 9	4 out of 5	40.2					0.98	0.42
Cuvier	26/04/2013	Saddleback	D 200294	64	F	6 out of 9	2 out of 5	38					0.7	0.42
Cuvier	26/04/2013	Saddleback	D 200295	69	F	5 out of 9	1 out of 5	41					0.53	0.24
Cuvier	26/04/2013	Saddleback	D 200296	62	F			38.7						
Cuvier	26/04/2013	Saddleback	D 200297	86	F	6 out of 9	5 out of 5	40.3						
Cuvier	26/04/2013	Saddleback	DP 0318	66	F	7 out of 9	4 out of 5						0.72	0.36
Cuvier	26/04/2013	Saddleback	DP0316		F	5 out of 9	2 out of 5							
Hen Island	14/11/2012	Saddleback	204298	92	J	4 out of 9	4 out of 5	3.9	5.7	8.8	0.4	0.3	0.5	0.2
Hen Island	15/11/2012	Saddleback	204296	82	M	3 out of 9	2 out of 5	4.5	6.5	9.3	1.35	0.65	1.4	0.8
Hen Island	15/11/2012	Saddleback	204297	68	F	5 out of 9	4 out of 5	4.1	5.8	9.8	0.6	0.4	0.6	0.35
Hen Island	16/11/2012	Saddleback	204292	92	M	5 out of 9	3 out of 5	4.7	6.45	10	1.05	0.7	1.2	0.6
Hen Island	16/11/2012	Saddleback	204293	84	M	4 out of 9	1 out of 5	4.3	6.3	9.5	1	0.6	1.2	0.2
Hen Island	16/11/2012	Saddleback	204294	70	F	3 out of 9	2 out of 5	4.2	5.9	8.9	0.7	0.4	0.7	0.4
Hen Island	16/11/2012	Saddleback	204295	76	M	5 out of 9	2 out of 5	4.6	5.6	9.7	0.85	0.45	0.8	0.45

Hen Island	17/11/2012	Saddleback	204232	78	M	4 out of 9	1 out of 5	4.1	6.2	9.5	1	0.6	1.1	0.5
Hen Island	18/11/2012	Saddleback	204233	80	M	4 out of 9	3 out of 5	4.7	5.9	9	1.1	0.5	1.05	0.6
Hen Island	18/11/2012	Blackbird	204234	91	M	3 out of 9	3 out of 5	3.75	5.4	12.6				
Hen Island	21/11/2012	Saddleback	204291	70	F	4 out of 9	4 out of 5	4.5	5.8	9.2	0.85	0.4	0.8	0.4
Hen Island	22/11/2012	Saddleback	204290	64	F	4 out of 9	3 out of 5	4.3	5.5	8.7	0.65	0.35	0.65	0.35
Hen Island	24/11/2012	Saddleback	204235	82	M (?)	5 out of 9	2 out of 5	4.1	6.0	9.6	1.05	0.7	1.1	0.65
Hen Island	24/11/2012	Saddleback	204236	67	F	3 out of 9	4 out of 5	4.2	5.9	9.9	0.65	0.4	0.7	0.4
Hen Island	24/11/2012	Saddleback	204237	65	F	3 out of 9	3 out of 5	4.3	6.0	9	0.55	0.25	0.6	0.25
Hen Island	24/11/2012	Saddleback	204285	72	F	4 out of 9	4 out of 5	4.2	6.1	9	0.7	0.45	0.6	0.45
Hen Island	24/11/2012	Saddleback	204286	86	M	4 out of 9	4 out of 5	4.4	6.17	9	0.95	0.6	0.9	0.55
Hen Island	24/11/2012	Saddleback	204287	84	M	4 out of 9	5 out of 5	4.83	6.51	9.5	1	0.71	10.5	0.68
Hen Island	24/11/2012	Saddleback	204288	80	M	flew away	flew away	flew away	flew away	flew away	flew away	flew away	flew away	flew away
Hen Island	24/11/2012	Saddleback	204289	77	M	3 out of 9	3 out of 5	4.9	6.2	10	1	0.7	10.4	0.7
Hen Island	25/11/2012	Saddleback	204238	85	M	3 out of 9	5 out of 5	46.5	5.9	9.8	1.15	0.75	1.2	0.6
Hen Island	25/11/2012	Saddleback	204239	84	F	3 out of 9	3 out of 5	4.85	6.2	9.5	0.95	0.5	0.9	0.5
Hen Island	25/11/2012	Saddleback	204282	87	M	3 out of 9	3 out of 5	4.6	6	9.4	1.05	0.55	0.95	0.6
Hen Island	25/11/2012	Saddleback	204283	68	J	4 out of 9	4 out of 5	43.5	5.5	9	0.6	0.3	0.7	0.25
Hen Island	25/11/2012	Saddleback	204284	66	F	3 out of 9	4 out of 5	3.8	57.5	9.2	0.75	0.34	0.71	0.32
Hen Island	26/11/2012	Saddleback	204240	88	M	5 out of 9	2 out of 5	4	6.3	9.5	1.2	0.7	1.3	0.6
Hen Island	26/11/2012	Saddleback	204280	85	M	4 out of 9	2 out of 5	4.2	6.1	9.7	1.05	0.65	10.5	0.7
Hen Island	26/11/2012	Saddleback	204281	73	F	4 out of 9	4 out of 5	40.5	6	9	0.7	0.4	0.7	0.4
Whangarei	9/03/2013	Blackbird	DP 3407	97	J	4 out of 9	3 out of 5	4.05	5.26	129				
Whangarei	9/03/2013	Blackbird	DP 3408	100	J	4 out of 9	2 out of 5	4	5.3	132				
Whangarei	9/03/2013	Blackbird	DP 3409	98	J	4 out of 9	3 out of 5	3.78	5.09	122				
Whangarei	10/03/2013	Songthrush	C 86209	72	J	4 out of 9	2 out of 5	3.12	4.67	114				
Whangarei	10/03/2013	Blackbird	DP 3410	90	J	2 out of 9	1 out of 5	3.85	5.13	127				
Whangarei	10/03/2013	Blackbird	DP 3411	92	J	2 out of 9	3 out of 5	3.56	4.93	117				
Whangarei	10/03/2013	Blackbird	DP 3412	83	J	2 out of 9	4 out of 5	3.79	5.14	126				
Whangarei	11/03/2013	Blackbird	DP 3413	93	J	4 out of 9	4 out of 5	3.69	5	122				
Whangarei	11/03/2013	Blackbird	DP 3414	96	J	3 out of 9	2 out of 5	4.45	5.26	125				

Table A6.2: PCR and sequencing results of sampled birds

Location	Sampling Date	Bird Species	Bird number	PCR (fb= false band; += positive; -= negative; w+=weak positive	Sequencing mixed infections	Sequencing
			D 140902			
Tiritiri	Aug-05	Saddleback		pos		LINN 1
Tiritiri	Aug-05	Saddleback	D 143083	neg (fb)		
Tiritiri	Aug-05	Saddleback	D 143943	pos (same number as below. But one sample neg, the other strong pos!)		P. elongatum
Tiritiri	Aug-05	Saddleback	D 143943	neg (same number as above. But one sample neg, the other strong pos!)		
Tiritiri	Aug-05	Saddleback	D 163887	neg		
Tiritiri	Aug-05	Saddleback	D 184818	neg		
Tiritiri	Aug-05	Saddleback	D 184819	neg		
Tiritiri	Aug-05	Saddleback	D 184821	neg		
Tiritiri	Aug-05	Saddleback	D 186986	neg (fb)		
Tiritiri	Aug-05	Saddleback	D 186989	neg (fb)		
Tiritiri	Aug-05	Saddleback	D 186990	neg		
Tiritiri	Aug-05	Saddleback	D 186990	neg		
Tiritiri	Aug-05	Saddleback	D 186991	w+		
Tiritiri	Aug-05	Saddleback	D 186992	neg		
Tiritiri	Aug-05	Saddleback	D 186995	neg		
Tiritiri	Aug-05	Saddleback	D 186996	w+	Mixed infection?	Mixed infection?
Tiritiri	Aug-05	Saddleback	D 186997	neg (most Tiri samples with fb)		
Tiritiri	Aug-05	Saddleback	D 186998	neg (fb)		
Tiritiri	Aug-05	Saddleback	D 187000	neg		
Hen	Jun-06	Saddleback	D 174719	pos (fb)		P. elongatum
Hen	Jun-06	Saddleback	D 179172	pos (fb)		Kokako 01
Hen	Jun-06	Saddleback	D 184854	neg (fb)		
Hen	Jun-06	Saddleback	D 184857	neg (fb)		
Hen	Jun-06	Saddleback	D 184858	neg (fb)		
Hen	Jun-06	Saddleback	D 184859	neg (fb)		
Hen	Jun-06	Saddleback	D 184860	w+ (weak positive band) (fb)		
Hen	Jun-06	Saddleback	D 184875	pos (all Hen samples with fb)		P. elongatum
Hen	Jun-06	Saddleback	D 184876	pos (fb)	mixed	LINN 1

Hen	Jun-06	Saddleback	D 187709	pos (fb)	P. elongatum
Hen	Jun-06	Saddleback	D 187710	neg (fb)	
Mokoia	Sep-09	Saddleback	D 136312	pos	Novyella strain
Mokoia	Sep-09	Saddleback	D 136313	neg	
Mokoia	Sep-09	Saddleback	D 136316	neg (fb)	
Mokoia	Sep-09	Saddleback	D 136317	neg	
Mokoia	Sep-09	Saddleback	D 136319	neg	
Mokoia	Sep-09	Saddleback	D 136320	neg	
Mokoia	Sep-09	Saddleback	D 136323	neg	
Mokoia	Sep-09	Saddleback	D 136393	neg	
Mokoia	Sep-09	Saddleback	D 136395	neg	
Mokoia	Sep-09	Saddleback	D 136395	neg	
Mokoia	Sep-09	Saddleback	D 136398	neg	
Mokoia	Sep-09	Saddleback	D 136400	neg	
Mokoia	Sep-09	Saddleback	D 136401	pos	P. elongatum
Mokoia	Sep-09	Saddleback	D 136409	pos (?) (came up once positive, but negative was pos too. On second run neg)	
Mokoia	Sep-09	Saddleback	D 136499	neg	
Mokoia	Sep-09	Saddleback	D 136500	pos (?) (came up once positive, but negative was pos too. On second run neg)	
Mokoia	Sep-09	Saddleback	D 138399	neg (fb)	
Mokoia	Sep-09	Saddleback	D 183428	neg (fb)	
Mokoia	Sep-09	Saddleback	D 183465	neg	
Mokoia	Sep-09	Saddleback	D 190016	neg	
Mokoia	Sep-09	Saddleback	D 190020	neg	
Mokoia	Sep-09	Saddleback	D 68012	neg (fb)	
Mokoia	Sep-09	Saddleback	D 68015	neg (fb)	
Mokoia	Sep-09	Saddleback	D 68021	neg	
Mokoia	Sep-09	Saddleback	D 68022	neg (fb)	
Mokoia	Sep-09	Saddleback	D 68025	neg	
Mokoia	Sep-09	Saddleback	D 68027	neg	
Mokoia	Sep-09	Saddleback	D 68029	neg	
Mokoia	Sep-09	Saddleback	D 68030	neg	
Mokoia	Sep-09	Saddleback	D 68032	neg	
Mokoia	Sep-09	Saddleback	D 68033	w+	

Mokoia	Sep-09	Saddleback	D 68034	neg (fb)		
Mokoia	Sep-09	Saddleback	D 68035	neg		
Mokoia	Sep-09	Saddleback	D 68036	neg		
Mokoia	Sep-09	Saddleback	D 68039	neg		
Mokoia	Sep-09	Saddleback	D 68043	w+		
Mokoia	Sep-09	Saddleback	D 68044	neg		
Mokoia	Sep-09	Saddleback	D 68045	pos		P. elongatum
Mokoia	Sep-09	Saddleback	D 68048	neg		
Mokoia	Sep-09	Saddleback	D 68049	neg		
Mokoia	Sep-09	Saddleback	D 68051	neg		
Mokoia	Sep-09	Saddleback	D 68099	neg		
Mokoia	Sep-09	Saddleback	D 89940	neg		
Mokoia	Sep-09	Saddleback	D 89943	neg		
Mokoia	Sep-09	Saddleback	D 89944	neg		
Mokoia	Sep-09	Saddleback	D 89960	w+		
Mokoia	Sep-09	Saddleback	D 89967	neg		
Mokoia	Sep-09	Saddleback	D 89971	neg		
Mokoia	Sep-09	Saddleback	D 89972	pos	Mixed infection?	Mixed infection?
Bushy Park	Mar-12	Saddleback	D 188682	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 19180	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 19181	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 191815	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204201	pos (redone) w+		
Bushy Park	Mar-12	Saddleback	D 204202	pos (redone) neg (fb)		P.elongatum
Bushy Park	Mar-12	Saddleback	D 204203	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204204	pos (redone) neg (fb)		LINN 1
Bushy Park	Mar-12	Saddleback	D 204205	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204206	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204207	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204208	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204209	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204210	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204211	neg (fb)		

Bushy Park	Mar-12	Saddleback	D 204213	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204214	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204215	pos (redone) w+	mixed	Possibly 2 species
Bushy Park	Mar-12	Saddleback	D 204216	w+		
Bushy Park	Mar-12	Saddleback	D 204217	pos	mixed	LINN 1 (other sequence 17(2) possibly mixed infection?
Bushy Park	Mar-12	Saddleback	D 204218	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204219	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204220	pos (redone) neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204221	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204222	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204223	pos		P. elongatum
Bushy Park	Mar-12	Saddleback	D 204224	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204225	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204226	pos (redone) w+		
Bushy Park	Mar-12	Saddleback	D 204227	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204228	pos (redone) neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204229	pos (redone) neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204230	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204231	neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204299	pos (redone) neg (fb)		
Bushy Park	Mar-12	Saddleback	D 204300	neg (fb)		
Bushy Park	Mar-12	Saddleback	D204212	neg (fb)		
Auckland/ Northshore	8/03/2012	Blackbird	D 203920	pos +++	mixed (F)	Plas. Spp.
Hen Island	14/11/2012	Saddleback	204298	NA, no blood in Alcohol		
Hen Island	15/11/2012	Saddleback	204296	pos		P. elongatum
Hen Island	15/11/2012	Saddleback	204297	NA, no blood in Alcohol		
Hen Island	16/11/2012	Saddleback	204292	pos	Mixed?	Novyella
Hen Island	16/11/2012	Saddleback	204293	pos	Mixed?	P. elongatum
Hen Island	16/11/2012	Saddleback	204294	pos		
Hen Island	16/11/2012	Saddleback	204295	pos		P. elongatum
Hen Island	17/11/2012	Saddleback	204232	pos		Plas. Spp.

Hen Island	18/11/2012	Saddleback	204233	pos		P. elongatum
Hen Island	18/11/2012	Blackbird	204234	pos		P. elongatum
Hen Island	21/11/2012	Saddleback	204291	pos		P. elongatum
Hen Island	22/11/2012	Saddleback	204290	pos		Novyella strain
Hen Island	24/11/2012	Saddleback	204235	pos		Novyella strain
Hen Island	24/11/2012	Saddleback	204236	pos	Mixed?	elongatum?
Hen Island	24/11/2012	Saddleback	204237	pos	Mixed ?	Kokako 01
Hen Island	24/11/2012	Saddleback	204285	pos		Novyella strain
Hen Island	24/11/2012	Saddleback	204286	pos	Mixed Infection	Novyella OR P. elongatum
Hen Island	24/11/2012	Saddleback	204287	pos		Kokako 01
Hen Island	24/11/2012	Saddleback	204288	pos		Novyella OR P. elongatum
Hen Island	24/11/2012	Saddleback	204289	pos		clusters with Novyella
Hen Island	25/11/2012	Saddleback	204238	pos		P. elongatum
Hen Island	25/11/2012	Saddleback	204239	pos	Mixed?	elongatum?
Hen Island	25/11/2012	Saddleback	204282	pos		Novyella strain
Hen Island	25/11/2012	Saddleback	204283	neg? Negative control had weak band, same brightness		
Hen Island	25/11/2012	Saddleback	204284	pos		P. elongatum
Hen Island	26/11/2012	Saddleback	204240	pos		Novyella strain
Hen Island	26/11/2012	Saddleback	204280	pos		P. elongatum
Hen Island	26/11/2012	Saddleback	204281	neg? Negative control had weak band, same brightness		
Bushy Park	14/01/2013	Saddleback	204215	pos +++		Novyella strain
Bushy Park	14/01/2013	Saddleback	204241	unlab 1? Was neg.		
Bushy Park	14/01/2013	Saddleback	204242	neg (fb)		
Bushy Park	14/01/2013	Saddleback	204259	pos + (fb)	mixed (F)	P.elongatum
Bushy Park	14/01/2013	Saddleback	204278	neg (fb)		
Bushy Park	14/01/2013	Saddleback	204279	unlab 1? Was neg.		
Bushy Park	15/01/2013	Saddleback	191812	neg (fb)		
Bushy Park	15/01/2013	Saddleback	204273	neg		
Bushy Park	15/01/2013	Saddleback	204274	pos +++		P.elongatum
Bushy Park	15/01/2013	Saddleback	204275	neg		
Bushy Park	15/01/2013	Saddleback	204276	neg (fb)		
Bushy Park	15/01/2013	Saddleback	204277	pos +++		SGS 1

Bushy Park	16/01/2013	Saddleback	204206	pos +++		P.elongatum
Bushy Park	16/01/2013	Saddleback	204210	neg		
Bushy Park	16/01/2013	Saddleback	204264	pos +++	mixed (F)	Novyella strain
Bushy Park	16/01/2013	Saddleback	204265	neg		
Bushy Park	16/01/2013	Saddleback	204266	w+		P.elongatum
Bushy Park	16/01/2013	Saddleback	204267	neg		
Bushy Park	16/01/2013	Saddleback	204268	unlab 2 or 3? Both pos +++		Novyella OR P. elongatum
Bushy Park	16/01/2013	Saddleback	204269	w+		
Bushy Park	16/01/2013	Saddleback	204270	unlab 1? Was neg.		
Bushy Park	16/01/2013	Saddleback	204271	w+		
Bushy Park	16/01/2013	Blackbird	204272	unlab 2 or 3? Both pos +++		Novyella OR P. elongatum
Bushy Park	17/01/2013	Saddleback	191873	neg		
Bushy Park	18/01/2013	Saddleback	204216	pos ++	mixed (F)	Novyella strain
Bushy Park	18/01/2013	Saddleback	204219	unlab 1? Was neg.		
Bushy Park	18/01/2013	Saddleback	204260	pos +++		
Bushy Park	18/01/2013	Saddleback	204261	w+		
Bushy Park	18/01/2013	Saddleback	204262	neg (fb)		
Bushy Park	18/01/2013	Saddleback	204263	w+		
Cape Kidnappers	24/01/2013	Dunnock	A 167776			
Cape Kidnappers	1/02/2013	Chaffinch	A 167778			
Cape Kidnappers	1/02/2013	Dunnock	A 167779	pos ++		P. elongatum
Cape Kidnappers	1/02/2013	Yellowammer	A 167780			
Cape Kidnappers	1/02/2013	Yellowammer	A 167781			
Cape Kidnappers	1/02/2013	Greenfinch	A 167782			
Cape Kidnappers	1/02/2013	Greenfinch	A 167783			
Cape Kidnappers	1/02/2013	Dunnock	A 167784			
Cape Kidnappers	1/02/2013	Sparrow	B 124501			
Cape Kidnappers	1/02/2013	Sparrow	B 124502			
Bushy Park	19/02/2013	Saddleback	204225	neg (fb)		
Bushy Park	19/02/2013	Saddleback	204226	neg		
Bushy Park	19/02/2013	Saddleback	204243	neg (fb)		
Bushy Park	19/02/2013	Saddleback	204244	neg (fb)		
Bushy Park	19/02/2013	Saddleback	204245	pos +++		Novyella strain

Bushy Park	19/02/2013	Saddleback	204246	pos+		Novyella strain
Bushy Park	19/02/2013	Blackbird	204247	pos +++		Plas. Spp.
Bushy Park	19/02/2013	Saddleback	204256	neg		
Bushy Park	20/02/2013	Blackbird	204248	neg		
Bushy Park	20/02/2013	Blackbird	204249	pos +++		
Bushy Park	20/02/2013	Songthrush	204250	pos +++		Plas. Spp.
Bushy Park	20/02/2013	Saddleback	204251	neg		
Bushy Park	20/02/2013	Blackbird	204252	unlab 1? Was neg.		
Bushy Park	20/02/2013	Blackbird	204253	pos +++		Novyella strain
Bushy Park	20/02/2013	Saddleback	204254	w+		
Bushy Park	20/02/2013	Blackbird	204255	pos +++		P.elongatum
Bushy Park	20/02/2013	Saddleback	204266	w+		
Bushy Park	20/02/2013	Blackbird	DP 3402	pos +++		P. elongatum
Bushy Park	20/02/2013	Blackbird	DP 3403	pos +++		Plas. Spp.
Bushy Park	20/02/2013	Blackbird	DP 3499	pos +		Plas. Spp.
Bushy Park	20/02/2013	Blackbird	DP 3500	pos +	mixed (F)	Novyella strain
Auckland/ Northshore	6/03/2013	Blackbird	D 203917	w+		
Auckland/ Northshore	6/03/2013	Blackbird	D 203918	pos+	mixed (F)	Plas. Spp.
Auckland/ Northshore	7/03/2013	Songthrush	C 86208	pos +++		Novyella strain
Auckland/ Northshore	7/03/2013	Songthrush	C 89167	pos +++	mixed (F)	P.elongatum
Auckland/ Northshore	7/03/2013	Blackbird	D 203919	pos +++	mixed (F)	Novyella strain
Auckland/ Northshore	7/03/2013	Blackbird	DP 3404	neg		
Auckland/ Northshore	7/03/2013	Blackbird	DP 3405	pos +++		Novyella strain
Auckland/ Northshore	7/03/2013	Blackbird	DP 3406	pos +++	mixed (F)	Relictum cluster
Auckland/ Northshore	8/03/2013	Blackbird	D 203921	pos +++		Novyella strain

Whangarei	9/03/2013	Blackbird	DP 3407	pos +++		Novyella strain
Whangarei	9/03/2013	Blackbird	DP 3408	pos +++		Novyella strain
Whangarei	9/03/2013	Blackbird	DP 3409	pos +++	mixed (F)	Novyella strain
Whangarei	10/03/2013	Songthrush	C 86209	pos +++	mixed (F)	Plas. Spp.
Whangarei	10/03/2013	Blackbird	DP 3410	pos +++		Novyella strain
Whangarei	10/03/2013	Blackbird	DP 3411	pos +++		Novyella strain
Whangarei	10/03/2013	Blackbird	DP 3412	pos +++	mixed (F)	Novyella strain
Whangarei	11/03/2013	Blackbird	DP 3413	pos +++		Novyella strain
Whangarei	11/03/2013	Blackbird	DP 3414	pos +++	mixed (F)	Novyella strain
Cuvier	25/04/2013	Saddleback	D 200124	neg		
Cuvier	25/04/2013	Saddleback	D 200135	pos+	mixed (F +R)	
Cuvier	25/04/2013	Saddleback	D 200136	neg		
Cuvier	25/04/2013	Saddleback	D 200139	neg		
Cuvier	25/04/2013	Saddleback	D 200142	neg		
Cuvier	25/04/2013	Saddleback	D 200143	neg		
Cuvier	25/04/2013	Saddleback	D 200144	neg		
Cuvier	25/04/2013	Saddleback	D 200145	neg		
Cuvier	25/04/2013	Saddleback	D 200153	neg		
Cuvier	25/04/2013	Saddleback	D 200154	neg		
Cuvier	25/04/2013	Saddleback	D 200156	neg		
Cuvier	25/04/2013	Saddleback	D 200170	neg		
Cuvier	25/04/2013	Saddleback	D 200172	pos +		P. elongatum
Cuvier	25/04/2013	Saddleback	D 200173	pos+		P. elongatum
Cuvier	25/04/2013	Saddleback	D 200174	neg		
Cuvier	25/04/2013	Saddleback	D 200175	neg		
Cuvier	25/04/2013	Saddleback	D 200177	neg		
Cuvier	25/04/2013	Saddleback	D 200178	neg		
Cuvier	25/04/2013	Saddleback	D 200179	neg		
Cuvier	25/04/2013	Saddleback	D 200180	neg		
Cuvier	25/04/2013	Saddleback	D 200187	pos +	mixed (F +R)	P. elongatum
Cuvier	25/04/2013	Saddleback	D 200188	neg		
Cuvier	25/04/2013	Saddleback	D 200189	neg		
Cuvier	25/04/2013	Saddleback	D 200190	neg		

Cuvier	26/04/2013	Saddleback	D 200191	neg	
Cuvier	26/04/2013	Saddleback	D 200193	neg	
Cuvier	26/04/2013	Saddleback	D 200194	neg	
Cuvier	26/04/2013	Saddleback	D 200195	pos+	P. elongatum
Cuvier	26/04/2013	Saddleback	D 200196	neg	
Cuvier	26/04/2013	Saddleback	D 200197	neg	
Cuvier	26/04/2013	Saddleback	D 200198	neg	
Cuvier	26/04/2013	Saddleback	D 200281	neg	
Cuvier	26/04/2013	Saddleback	D 200282	pos +++	Novyella strain
Cuvier	26/04/2013	Saddleback	D 200283	neg	
Cuvier	26/04/2013	Saddleback	D 200286	neg	
Cuvier	26/04/2013	Saddleback	D 200287	neg	
Cuvier	26/04/2013	Saddleback	D 200290	neg	
Cuvier	26/04/2013	Saddleback	D 200292	neg	
Cuvier	26/04/2013	Saddleback	D 200294	neg	
Cuvier	26/04/2013	Saddleback	D 200295	neg	
Cuvier	26/04/2013	Saddleback	D 200296	neg	
Cuvier	26/04/2013	Saddleback	D 200297	neg	
Cuvier	26/04/2013	Saddleback	DP 0318	neg	
Cuvier	26/04/2013	Saddleback	DP0316	neg	
Tiritiri	Jun-13	Saddleback	3290- 1	neg	
Tiritiri	Jun-13	Saddleback	3290- 10	neg	
Tiritiri	Jun-13	Saddleback	3290- 11	neg	
Tiritiri	Jun-13	Saddleback	3290- 12	neg	
Tiritiri	Jun-13	Saddleback	3290- 13	neg	
Tiritiri	Jun-13	Saddleback	3290- 14	neg	
Tiritiri	Jun-13	Saddleback	3290- 15	neg	
Tiritiri	Jun-13	Saddleback	3290- 16	pos	P. elongatum
Tiritiri	Jun-13	Saddleback	3290- 17	pos	P. elongatum
Tiritiri	Jun-13	Saddleback	3290- 18	neg	
Tiritiri	Jun-13	Saddleback	3290- 19	neg	
Tiritiri	Jun-13	Saddleback	3290- 2	neg	
Tiritiri	Jun-13	Saddleback	3290- 20	neg	

Tiritiri		Jun-13	Saddleback	3290- 21	pos		Relictum cluster
Tiritiri		Jun-13	Saddleback	3290- 22	pos		KOKAKO 01
Tiritiri		Jun-13	Saddleback	3290- 23	pos	mixed ?	? P. elongatum ?
Tiritiri		Jun-13	Saddleback	3290- 24	neg		
Tiritiri		Jun-13	Saddleback	3290- 25	pos		P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 26	neg		
Tiritiri		Jun-13	Saddleback	3290- 27	pos	mixed ?	P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 28	neg		
Tiritiri		Jun-13	Saddleback	3290- 29	neg		
Tiritiri		Jun-13	Saddleback	3290- 3	neg		
Tiritiri		Jun-13	Saddleback	3290- 30	pos		P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 31	pos		P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 32	neg		
Tiritiri		Jun-13	Saddleback	3290- 33	pos		P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 34	pos		P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 35	neg		
Tiritiri		Jun-13	Saddleback	3290- 36	neg		
Tiritiri		Jun-13	Saddleback	3290- 37	pos		P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 38	pos		P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 39	pos		P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 4	w+ ?		
Tiritiri		Jun-13	Saddleback	3290- 40	neg		
Tiritiri		Jun-13	Saddleback	3290- 41	pos; fb		P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 42	neg		
Tiritiri		Jun-13	Saddleback	3290- 5	neg		
Tiritiri		Jun-13	Saddleback	3290- 6	pos		P. elongatum
Tiritiri		Jun-13	Saddleback	3290- 7	w+ ?		
Tiritiri		Jun-13	Saddleback	3290- 8	w+ ?		
Tiritiri		Jun-13	Saddleback	3290- 9	neg		
				D 17/04			
Cuvier	April 2006 (?)		Saddleback	Escape	neg (fb)		
Cuvier	April 2006 (?)		Saddleback	D 184852	re-done w+ neg (fb)		
Cuvier	April 2006 (?)		Saddleback	D 184871	neg (fb)		

Cuvier	April 2006 (?)	Saddleback	D 184872	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184873	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184874	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184877	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184878	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184879	re-done w+ neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184880	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184881	re-done w+ neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184882	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184883	re-done pos +++ neg (fb)		P. elongatum
Cuvier	April 2006 (?)	Saddleback	D 184889	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184890	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184891	re-done pos +++ neg (fb)	mixed (F)	P. elongatum
Cuvier	April 2006 (?)	Saddleback	D 184892	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184893	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184894	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184895	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184896	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184897	re-done w+ neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 184898	neg (fb)		
Cuvier	April 2006 (?)	Saddleback	D 185143	neg (fb)		
Tiritiri	Aug-Dec 2006	Saddleback	D 164548	neg (fb)		
Tiritiri	Aug-Dec 2006	Saddleback	D 179758	neg		
Tiritiri	Aug-Dec 2006	Saddleback	D 184347	neg (fb)		
Tiritiri	Aug-Dec 2006	Saddleback	D 184850	neg		
Tiritiri	Aug-Dec 2006	Saddleback	D 184862	w+	Mixed infection?	Mixed infection?
Tiritiri	Aug-Dec 2006	Saddleback	D 187701	neg		
Tiritiri	Aug-Dec 2006	Saddleback	D 187704	w+	mixed	Mixed infection?
Tiritiri	Aug-Dec 2006	Saddleback	D 187705	pos		Fulvetta strain ?
Tiritiri	Aug-Dec 2006	Saddleback	D 187706	neg		
Tiritiri	Aug-Dec 2006	Saddleback	D 187716	pos		P. relictum
Tiritiri	Aug-Dec 2006	Saddleback	D 187717	pos		P. elongatum
Tiritiri	Aug-Dec 2006	Saddleback	D 187718	pos		P. elongatum

Tiritiri	Aug-Dec 2006	Saddleback	D 187719	neg			
Tiritiri	Aug-Dec 2006	Saddleback	D 187721	w+		Mixed infection?	P. elongatum (LIN 2)
Tiritiri	Aug-Dec 2006	Saddleback	D 187738	neg (fb)			
Tiritiri	Aug-Dec 2006	Saddleback	D 189720	neg			
Tiritiri	Aug-Dec 2006	Saddleback	No ID "Rose"	pos			P. elongatum
Cuvier		Saddleback	D 200185				
Cuvier		Saddleback	D 200284				
Cuvier		Saddleback	D 200285				
Cuvier		Saddleback	D 200289				
Cuvier		Saddleback	D 200291				
Cuvier		Saddleback	D 200293				
Cuvier		Saddleback	D 200299				
Cuvier		Saddleback	D 200919				
Cuvier		Saddleback	DP 0317				
Cuvier		Saddleback	DP 0319				

Table A6.3: Blood smears results

Location	Species	Bird number	PCR	Mixed Infection	Sequence	Blood smear	Blood stages	Blood smear Diagnosis
Bushy Park	Saddleback	D 188682	neg (fb)			neg		
Bushy Park	Saddleback	D 19180	neg (fb)			pos	trophozoites on blood smear	
Bushy Park	Saddleback	D 19181	neg (fb)			pos		
Bushy Park	Saddleback	D 191815	neg (fb)			bad slide		
Bushy Park	Saddleback	D 204201	**pos (redone) w+**			neg		
Bushy Park	Saddleback	D 204202	**pos** (redone) neg (fb)		**GRW6**	pos	immature gamont, trophozoites	
Bushy Park	Saddleback	D 204203	neg (fb)			neg		
Bushy Park	Saddleback	D 204204	**pos** (redone) neg (fb)		**LINN 1**	pos (many immature	trophozoites	

						erythrocytes)		
Bushy Park	Saddleback	D 204205	neg (fb)			neg		
Bushy Park	Saddleback	D 204206	neg (fb)			neg		
Bushy Park	Saddleback	D 204207	neg (fb)			neg		
Bushy Park	Saddleback	D 204208	neg (fb)			neg		
Bushy Park	Saddleback	D 204209	neg (fb)			neg		
Bushy Park	Saddleback	D 204210	neg (fb)			neg		
Bushy Park	Saddleback	D 204211	neg (fb)			neg		
Bushy Park	Saddleback	D 204213	neg (fb)			bad slide		
Bushy Park	Saddleback	D 204214	neg (fb)			bad slide		
Bushy Park	Saddleback	D 204215	**pos (redone) w+**	**mixed**	**Possibly 2 species**	pos	meronts, trophs + young gametozyte	**P. vaughani**
Bushy Park	Saddleback	D 204216	**w+**			pos (many immature erythrocytes)	meronts+ trophs	
Bushy Park	Saddleback	D 204217	**pos**	**mixed**	**LINN 1 (other sequence 17(2) possibly mixed infection?**	pos	meronts+ trophs	**P. elongatum?**
Bushy Park	Saddleback	D 204218	neg (fb)			neg		
Bushy Park	Saddleback	D 204219	neg (fb)			pos	trophozoites	
Bushy Park	Saddleback	D 204220	**pos** (redone) neg (fb)			pos	trophozoites	
Bushy Park	Saddleback	D 204221	neg (fb)			pos	trophozoites	
Bushy Park	Saddleback	D 204222	neg (fb)			neg		
Bushy Park	Saddleback	D 204223	**pos**		**P. elongatum**	bad slide	trophozoites	
Bushy Park	Saddleback	D 204224	neg (fb)			pos	Trophozoites and Meronts	**Novyella**
Bushy Park	Saddleback	D 204225	neg (fb)			neg		
Bushy Park	Saddleback	D 204226	**pos (redone) w+**			pos	trophozoites	
Bushy Park	Saddleback	D 204227	neg (fb)			pos	trophozoites	
Bushy Park	Saddleback	D 204228	**pos** (redone) neg (fb)			pos	trophozoites	
Bushy Park	Saddleback	D 204229	**pos** (redone) neg (fb)			neg	**Not sure if trophozoite or not**	
Bushy Park	Saddleback	D 204230	neg (fb)			neg		
Bushy Park	Saddleback	D 204231	neg (fb)			neg		
Bushy Park	Saddleback	D 204299	**pos** (redone) neg (fb)			neg		

Bushy Park	Saddleback	D 204300	neg (fb)			neg		
Bushy Park	Saddleback	D204212	neg (fb)			neg		
Hen Island	Saddleback	D 184858	NA, no blood in Alcohol			**pos; had been sampled in 2006; was negative on PCR then**	**Trophozoites**	
Hen Island	Saddleback	204298	NA, no blood in Alcohol			pos	positive, few, trophozoites and meronts	**P. elongatum?**
Hen Island	Saddleback	204296	**pos**		**GRW6**	pos	positive, few	
Hen Island	Saddleback	204297	NA, no blood in Alcohol			pos	positive, immature gamont	
Hen Island	Saddleback	204292	**pos**	Mixed?	**SYAT05**	pos (many immature erythrocytes)	positive, few, trophozoites and meronts	**P. vaughani + P. elongatum**
Hen Island	Saddleback	204293	**pos**	Mixed?	**GRW6**	pos	positive, meronts	**P. elongatum**
Hen Island	Saddleback	204294	**pos**			pos		
Hen Island	Saddleback	204295	**pos**		**GRW6**	pos		
Hen Island	Saddleback	204232	**pos**		**LINN1**	pos	positive, few; trophozoites, meronts	**P. elongatum**
Hen Island	Saddleback	204233	**pos**		**GRW6**	pos (many immature erythrocytes)	positive, few	**P. elongatum**
Hen Island	Blackbird	204234	**pos**		**GRW6**	**pos; elongatum-parasites in immat. erys(many immature erythrocytes)**	positive, immature erys, trophozoites, young gamont, meronts	**P. elongatum**
Hen Island	Saddleback	204291	**pos**		**GRW6**	pos (many immature erythrocytes)	positive, few, trophozoites	**P. elongatum**
Hen Island	Saddleback	204290	**pos**		**SYAT05**	pos		
Hen Island	Saddleback	204235	**pos**		**SYAT05**	pos	positive, few, young gamont	**P. elongatum?**
Hen Island	Saddleback	204236	**pos**	Mixed?	**GRW6**	pos		
Hen Island	Saddleback	204237	**pos**	Mixed ?	**Kokako 01**	pos		
Hen Island	Saddleback	204285	**pos**		**SYAT05**	pos (many immature erythrocytes)	positive, few, trophozoites and meronts	
Hen Island	Saddleback	204286	**pos**	Mixed Infection	**SYAT05 and GRW6**	pos		
Hen Island	Saddleback	204287	**pos**		**Kokako 01**	pos	trophozoites	
Hen Island	Saddleback	204288	**pos**		**SYAT05 and GRW6**	pos	Trophozoites and	

								Meronts	
Hen Island	Saddleback	204289		**pos**		**clusters with Novyella**	pos; Novyella	Trophozoites and Meronts	**Novyella**
Hen Island	Saddleback	204238		**pos**		**GRW6**	pos		
Hen Island	Saddleback	204239		**pos**	Mixed?	**elongatum?**	pos	positive, few	**P. elongatum**
Hen Island	Saddleback	204282		**pos**		**SYAT05**	pos; bowtie-Rouxi	positive, few, meronts, young gamont, 1 meront-bow tie--> rouxi?	**P. vaughani (P. rouxi)**
Hen Island	Saddleback	204283		neg? Negative control had weak band, same brightness			pos (many immature erythrocytes)	positive, trophozoites, small gamonts	
Hen Island	Saddleback	204284		**pos**		**GRW6**	pos (many immature erythrocytes)	trophozoites, meronts	**P. rouxi and elongatum**
Hen Island	Saddleback	204240		**pos**		**SYAT05**	pos		
Hen Island	Saddleback	204280		**pos**		**GRW6**	pos		
Hen Island	Saddleback	204281		neg? Negative control had weak band, same brightness			pos; bowtie-Rouxi		**P. rouxi**
Bushy Park	Saddleback		204215	**pos +++**		**SYAT05**	pos		
Bushy Park	Saddleback	204241		unlab 1? Was neg.			bad slide		
Bushy Park	Saddleback	204242		neg (fb)			neg		
Bushy Park	Saddleback	204259		**pos + (fb)**	mixed (F)	**GRW6**	bad slide		
Bushy Park	Saddleback	204278		neg (fb)			neg		
Bushy Park	Saddleback	204279		unlab 1? Was neg.			pos		
Bushy Park	Saddleback		191812	neg (fb)			neg		
Bushy Park	Saddleback	204273		neg			bad slide		
Bushy Park	Saddleback	204274		**pos +++**		**GRW6**	neg		
Bushy Park	Saddleback	204275		neg			neg		
Bushy Park	Saddleback	204276		neg (fb)			neg		
Bushy Park	Saddleback	204277		**pos +++**		**SGS 1**	neg		
Bushy Park	Saddleback		204206	**pos +++**		**GRW6**	pos		
Bushy Park	Saddleback		204210	neg			pos		
Bushy Park	Saddleback	204264		**pos +++**	mixed (F)	**SYAT05**	pos		
Bushy Park	Saddleback	204265		neg			neg		
Bushy Park	Saddleback	204266		**w+**		**GRW6**	pos		

Bushy Park	Saddleback	204267		neg			neg		
Bushy Park	Saddleback	204268		**pos +++**		**SYAT05 and GRW6**	pos	positive, meronts, trophs, young gamonts	
Bushy Park	Saddleback	204269		**w+**			pos		
Bushy Park	Saddleback	204270		unlab 1? Was neg.			neg		
Bushy Park	Saddleback	204271		**w+**			bad slide		
Bushy Park	Blackbird	204272		**pos +++**		**SYAT05 and GRW6**	pos; vaughani	positive, meronts, Novyella?	**P. vaughani**
Bushy Park	Saddleback		191873	neg			bad slide		
Bushy Park	Saddleback		204216	**pos ++**	mixed (F)	**SYAT05**	pos		
Bushy Park	Saddleback		204219	unlab 1? Was neg.			neg		
Bushy Park	Saddleback	204260		**pos +++**			pos		
Bushy Park	Saddleback	204261		**w+**			pos		
Bushy Park	Saddleback	204262		neg (fb)			neg		
Bushy Park	Saddleback	204263		**w+**			neg		
Cape Kidnappers	Dunnock	A 167776					neg		
Cape Kidnappers	Chaffinch	A 167778					neg		
Cape Kidnappers	Dunnock	A 167779		**pos ++**		**GRW6**	pos; most likely mixed infection of elongatum + ?	gametocytes	**P. elongatum + ?**
Cape Kidnappers	Yellowammer	A 167780					neg		
Cape Kidnappers	Yellowammer	A 167781					neg		
Cape Kidnappers	Greenfinch	A 167782					neg		
Cape Kidnappers	Greenfinch	A 167783					bad slide		
Cape Kidnappers	Dunnock	A 167784					bad slide		
Cape Kidnappers	Sparrow	B 124501					pos	trophozoites	
Cape Kidnappers	Sparrow	B 124502					neg		
Bushy Park	Saddleback		204225	neg (fb)			neg		
Bushy Park	Saddleback		204226	neg			neg		
Bushy Park	Saddleback		204243	neg (fb)			neg		
Bushy Park	Saddleback		204244	neg (fb)			neg		
Bushy Park	Saddleback		204245	**pos +++**		**SYAT05**	neg		
Bushy Park	Saddleback		204246	**pos+**		**SYAT05**	neg		

Bushy Park	Blackbird		204247	**pos +++**		**LINN1**	neg		
Bushy Park	Saddleback		204256	neg			neg		
Bushy Park	Blackbird		204248	neg			bad slide		
Bushy Park	Blackbird		204249	**pos +++**			pos		
Bushy Park	Songthrush		204250	**pos +++**		**LINN1**	pos; relictum	Trophozoites, meronts, gametocytes	**P. relictum**
Bushy Park	Saddleback		204251	neg			neg	positive, meronts, gamonts	
Bushy Park	Blackbird		204252	unlab 1? Was neg.			neg		
Bushy Park	Blackbird		204253	**pos +++**		**SYAT05**	pos; vaughani; many immat erys.	positive, meronts, gamonts, immature erys	**P. vaughani**
Bushy Park	Saddleback		204254	**w+**			pos; vaughani		**P. vaughani**
Bushy Park	Blackbird		204255	**pos +++**		**GRW6**	pos		
Bushy Park	Saddleback		204266	**w+**			pos		
Bushy Park	Blackbird	DP 3402		**pos +++**		**GRW6**	bad slide		
Bushy Park	Blackbird	DP 3403		**pos +++**		**LINN1**	neg		
Bushy Park	Blackbird	DP 3499		**pos +**		**LINN1**	pos	positive, meronts, gametocytes	**P. elongatum???**
Bushy Park	Blackbird	DP 3500		**pos +**	mixed (F)	**SYAT05**	pos; elongatum???	Trophozoites, meronts and gametocytes	**P. elongatum + Novyella**
Auckland/ Northshore	Blackbird	D 203920		**pos +++**	mixed (F)	**LINN1**	pos; relictum	Trophozoites, meronts and gametocytes	**P. relictum**
Auckland/ Northshore	Blackbird	D 203917		**w+**			pos		
Auckland/ Northshore	Blackbird	D 203918		**pos+**	mixed (F)	**LINN1**	pos; relictum; elongatum-parasites in immat. erys(many immature erythrocytes)		**P. elongatum + relictum**
Auckland/ Northshore	Songthrush	C 86208		**pos +++**		**SYAT05**	pos; vaughani, immat. erys		**P. vaughani**
Auckland/ Northshore	Songthrush	C 89167		**pos +++**	mixed (F)	**GRW6**	pos, elongatum. Immat. erys		**P.elongatum**
Auckland/ Northshore	Blackbird	D 203919		**pos +++**	mixed (F)	**SYAT05**	pos		
Auckland/ Northshore	Blackbird	DP 3404		neg			neg		

Auckland/ Northshore	Blackbird	DP 3405	**pos +++**		**SYAT05**	pos; vaughani; many immat erys.	**P. vaughani**
Auckland/ Northshore	Blackbird	DP 3406	**pos +++**	mixed (F)	**Relictum cluster**	pos	
Auckland/ Northshore	Blackbird	D 203921	**pos +++**		**SYAT05**	pos; rouxi, vaughani, relictum	**P. rouxi, vaughani + relictum**
Whangarei	Blackbird	DP 3407	**pos +++**		**SYAT05**	pos; vaughani; many immat erys.	**P. vaughani**
Whangarei	Blackbird	DP 3408	**pos +++**		**SYAT05**	pos; vaugani, elongatum, many immat. erys + parasites in immat. Erys	**P. vaughani + elongatum**
Whangarei	Blackbird	DP 3409	**pos +++**	mixed (F)	**SYAT05**	pos, many immat. erys	
Whangarei	Songthrush	C 86209	**pos +++**	mixed (F)	**LINN1**	pos; elongatum- parasites in immat. erys(many immature erythrocytes)	**P. elongatum**
Whangarei	Blackbird	DP 3410	**pos +++**		**SYAT05**	pos, many immat. erys	
Whangarei	Blackbird	DP 3411	**pos +++**		**SYAT05**	pos; vaughani; many immat erys.	**P. vaughani**
Whangarei	Blackbird	DP 3412	**pos +++**	mixed (F)	**SYAT05**	pos, relictum, elongatum, many immat. Erys	**P. relictum**
Whangarei	Blackbird	DP 3413	**pos +++**		**SYAT05**	pos, vaughani, many immat. erys	**P. vaughani**
Whangarei	Blackbird	DP 3414	**pos +++**	mixed (F)	**SYAT05**	pos, relictum, vaughani, many immat. Erys	**P. relictum + vaughani**
Cuvier	Saddleback	D 200124	neg			bad slide	
Cuvier	Saddleback	D 200135	**pos+**	mixed (F +R)		NO SLIDE	
Cuvier	Saddleback	D 200136	neg			pos	
Cuvier	Saddleback	D 200139	neg			bad slide	
Cuvier	Saddleback	D 200142	neg			bad slide	
Cuvier	Saddleback	D 200143	neg			bad slide	
Cuvier	Saddleback	D 200144	neg			bad slide	
Cuvier	Saddleback	D 200145	neg			neg	
Cuvier	Saddleback	D 200153	neg			pos	
Cuvier	Saddleback	D 200154	neg			pos	
Cuvier	Saddleback	D 200156	neg			pos	
Cuvier	Saddleback	D 200170	neg			bad slide	
Cuvier	Saddleback	D 200172	**pos +**		**GRW6**	pos	
Cuvier	Saddleback	D 200173	**pos+**		**GRW6**	bad slide	
Cuvier	Saddleback	D 200174	neg			pos	
Cuvier	Saddleback	D 200175	neg			neg	

Cuvier	Saddleback	D 200177	neg			neg	
Cuvier	Saddleback	D 200178	neg			pos	
Cuvier	Saddleback	D 200179	neg			pos	
Cuvier	Saddleback	D 200180	neg			neg	
Cuvier	Saddleback	D 200187	**pos +**	mixed (F +R)	**GRW6**	pos	
Cuvier	Saddleback	D 200188	neg			bad slide	
Cuvier	Saddleback	D 200189	neg			pos	
Cuvier	Saddleback	D 200190	neg			bad slide	
Cuvier	Saddleback	D 200191	neg			pos	
Cuvier	Saddleback	D 200193	neg			bad slide	
Cuvier	Saddleback	D 200194	neg			pos	
Cuvier	Saddleback	D 200195	**pos+**		**GRW6**	bad slide	
Cuvier	Saddleback	D 200196	neg			bad slide	
Cuvier	Saddleback	D 200197	neg			pos	
Cuvier	Saddleback	D 200198	neg			bad slide	
Cuvier	Saddleback	D 200281	neg			pos	
Cuvier	Saddleback	D 200282	**pos +++**		**SYAT05**	pos	
Cuvier	Saddleback	D 200283	neg			neg	
Cuvier	Saddleback	D 200286	neg			neg	
Cuvier	Saddleback	D 200287	neg			neg	
Cuvier	Saddleback	D 200290	neg			bad slide	
Cuvier	Saddleback	D 200292	neg			bad slide	
Cuvier	Saddleback	D 200294	neg			bad slide	
Cuvier	Saddleback	D 200295	neg			bad slide	
Cuvier	Saddleback	D 200296	neg			bad slide	
Cuvier	Saddleback	D 200297	neg			bad slide	
Cuvier	Saddleback	DP 0318	neg			bad slide	
Cuvier	Saddleback	DP0316	neg			NO SLIDE	
Tiritiri	Saddleback	3290- 1	neg			bad slide	
Tiritiri	Saddleback	3290- 10	neg			bad slide	
Tiritiri	Saddleback	3290- 11	neg			bad slide	
Tiritiri	Saddleback	3290- 12	neg			bad slide	
Tiritiri	Saddleback	3290- 13	neg			pos	Trophozoites

Tiritiri	Saddleback	3290- 14	neg			neg		
Tiritiri	Saddleback	3290- 15	neg			neg		
Tiritiri	Saddleback	3290- 16	**pos**		**GRW6**	bad slide		
Tiritiri	Saddleback	3290- 17	**pos**		**GRW6**	bad slide		
Tiritiri	Saddleback	3290- 18	neg			neg		
Tiritiri	Saddleback	3290- 19	neg			bad slide		
Tiritiri	Saddleback	3290- 2	neg			bad slide		
Tiritiri	Saddleback	3290- 20	neg			neg		
Tiritiri	Saddleback	3290- 21	**pos**		**Relictum cluster**	bad slide		
Tiritiri	Saddleback	3290- 22	**pos**		**KOKAKO 01**	bad slide		
Tiritiri	Saddleback	3290- 23	**pos**	mixed ?	**? P. elongatum ?**	pos	Trophozoites and Meronts	**P. elongatum?**
Tiritiri	Saddleback	3290- 24	neg			bad slide		
Tiritiri	Saddleback	3290- 25	**pos**		**GRW6**	pos		
Tiritiri	Saddleback	3290- 26	neg			bad slide		
Tiritiri	Saddleback	3290- 27	**pos**	mixed ?	**GRW6**	pos		
Tiritiri	Saddleback	3290- 28	neg			bad slide		
Tiritiri	Saddleback	3290- 29	neg			neg		
Tiritiri	Saddleback	3290- 3	neg			bad slide		
Tiritiri	Saddleback	3290- 30	**pos**		**P. elongatum**	pos		
Tiritiri	Saddleback	3290- 31	**pos**		**P. elongatum**	pos		
Tiritiri	Saddleback	3290- 32	neg			neg		
Tiritiri	Saddleback	3290- 33	**pos**		**GRW6**	pos		
Tiritiri	Saddleback	3290- 34	**pos**		**GRW6**	pos		
Tiritiri	Saddleback	3290- 35	neg			pos		
Tiritiri	Saddleback	3290- 36	neg			pos		
Tiritiri	Saddleback	3290- 37	**pos**		**GRW6**	pos, parasites in immat. erys	Trophozoites, meronts and gametocytes	**P. elongatum**
Tiritiri	Saddleback	3290- 38	**pos**		**GRW6**	pos		
Tiritiri	Saddleback	3290- 39	**pos**		**GRW6**	pos		
Tiritiri	Saddleback	3290- 4	w+ ?			bad slide		
Tiritiri	Saddleback	3290- 40	neg			pos		
Tiritiri	Saddleback	3290- 41	**pos; fb**		**GRW6**	pos		

Tiritiri	Saddleback	3290- 42	neg		pos
Tiritiri	Saddleback	3290- 5	neg		bad slide
Tiritiri	Saddleback	3290- 6	**pos**	**GRW6**	bad slide
Tiritiri	Saddleback	3290- 7	w+ ?		bad slide
Tiritiri	Saddleback	3290- 8	w+ ?		bad slide
Tiritiri	Saddleback	3290- 9	neg		bad slide

Table A6.4: PCR and Sequencing in sampled mosquito pools

Tube Label	**Location**	**Sampling Date**	**Mozzie Species**	**Number of mosquitoes in pool**	**classic PCR** (fb= false band; += positive; -= negative; w+=weak positive		**Sequencing**	**Sequencing**	**New Real Time PCR**	
					Thorax	**Abdomen**	**Thorax**	**Abdomen**	**Thorax**	**Abdomen**
Auck Noto	Auckland Northshore	1/02/2013	Aedes notoscriptus	21						
Bushy Park	Bushy Park	1/02/2014	Larvae- all Larvae collected in February 2014 were of Cx. Pervigilans;							
Bushy perv	Bushy Park	1/02/2014	Culex pervigilans	1	**pos** (weak band)					
Bushy antipod	Bushy Park	1/02/2014	Ochlerotatus antipodeus	9						
Bushy Noto	Bushy Park	1/02/2014	Aedes notoscriptus	8						
Bushy perv 2	Bushy Park	1/02/2013	Culex pervigilans	20	**pos** (weak band)	**pos**		**MIX** Linn1 + GRW6		linn 1
Bushy Noto 2	Bushy Park	1/02/2013	Aedes notoscriptus	12	**pos** (weak band)	**pos**	Linn1	Linn1		
Bushy Antip 2	Bushy Park	1/02/2013	Ochlerotatus antipodeus	4						
Bushy unknown 2	Bushy Park	1/02/2013	unidentified	16		**pos**		Linn1		elongatum?
Bushy perv 3	Bushy Park	1/02/2013	Culex pervigilans	20		**pos**		MIX GRW6+???		
Bushy perv 4	Bushy Park	1/02/2013	Culex pervigilans	21		**pos**		Linn1		weak; mix of all?
Bushy unknown 3	Bushy Park	1/02/2013	unidentified	6		**pos**		SYAT05		weak; Novyella/ Mixed?
Cape Kid Noto	Cape Kidnappers	1/02/2013	Aedes notoscriptus	1	**pos**		MIX GRW6+ ???		e+ linn 1?	
Cape Kid perv	Cape Kidnappers	1/02/2013	Culex pervigilans	5		**pos**		SYAT05 (99% ident with SYAT05; G vs. A)		

Hippo Cuv	Cuvier Island	1/04/2013	Saddleback Hippoboscid	4	**pos**	**pos**	MIX Linn1+ GRW6	MIX Linn1+ SYAT05	weak pos; broad
Hippo Cuv Kakariki	Cuvier Island	1/04/2013	Kakariki Hippoboscid	1					
Cuv Opifex 1	Cuvier Island	1/04/2013	Opifex fuscus	19					
Cuv Noto 1	Cuvier Island	1/04/2013	***Aedes notoscriptus***	20					
Cuv Noto 2	Cuvier Island	1/04/2013	Aedes notoscriptus	7					
Noto Hen	Hen Island	1/11/2012	Aedes notoscriptus	3		**pos**		SYAT05 (99% ident with SYAT05; G vs. A)	
Cx astelidae Hen	Hen Island	1/11/2012	Culex astelidae	1					
Ochlerotatus antipodeus Hen	Hen Island	1/11/2012	Ochlerotatus antipodeus	8					
Mok Noto 1	Mokoia Island	1/02/2014	Aedes notoscriptus	20					
Mok Unbek	Mokoia Island	1/02/2014	Unidentified	2					
Mok quinque	Mokoia Island	1/02/2014	Culex quinquefasciatus	3					
Mok Culex unbek	Mokoia Island	1/02/2014	unidentified Culex; Cx. Rotoruae??	2	**pos**		SYAT05 (99% ident with SYAT05; G vs. A)		
Mok Anti 1	Mokoia Island	1/02/2014	***Ochlerotatus antipodeus***	4					
Mok Noto 2	Mokoia Island	1/03/2012	Aedes notoscriptus	20					
Mok Anti 2	Mokoia Island	1/03/2012	Ochlerotatus antipodeus	9					
Mok perv 2	Mokoia Island	1/03/2012	Culex pervigilans	8	**pos** (weak band)	**pos** (weak band)			
Mok perv 3	Mokoia Island	1/03/2012	Culex pervigilans	21	**pos** (weak band)	**pos**	**MIX Linn1+SYAT05+ GRW6**	**MIX Linn1+SYAT05+GRW6**	
Mok quinque 2	Mokoia Island	1/03/2012	Culex quinquefasciatus	1					
1 Turitea Noto	Palmerston North	1/01/2014	Aedes notoscriptus	20	neg	neg			
2 Turitea Noto	Palmerston North	1/01/2014	Aedes notoscriptus	22	neg	neg			
Turitea quinque	Palmerston North	1/01/2014	Culex quinquefasciatus	7	neg	**pos**		MIX LINN 1 + GRW6	
3 Palmerston pervigilans	Palmerston North	1/01/2014	Culex pervigilans	4	neg	**pos**		LINN 1	
Home Noto 1	Palmerston North	1/01/2014	Aedes notoscriptus	20	neg	neg			
Home Noto 2	Palmerston North	1/01/2014	Aedes notoscriptus	33	neg				
Noto engorged (human blood)	Palmerston North	1/01/2014	Aedes notoscriptus	4	neg	neg (pos on Danielles PCR)			

Isabel	Palmerston North	5/07/2013	Aedes notoscriptus	2	neg	**pos**		MIX SYAT05+ ???? (99% ident with SYAT05; G vs. A)			left shift of peak; shift to left of linn 1
Tur 1	Palmerston North	1/01/2014	Aedes notoscriptus	21		**pos**		Linn1			weak; mix?
Tur 2	Palmerston North	1/01/2014	Aedes notoscriptus	20	neg	neg					
Tur 3	Palmerston North	1/01/2014	Aedes notoscriptus	21							
Home 1	Palmerston North	1/01/2014	Aedes notoscriptus	20		**pos**		MIX Linn1+???			Linn 1
Home 2	Palmerston North	1/01/2014	Aedes notoscriptus	20	**pos**		MIX SYAT05+???			Linn1 + elongatum?	
Home 3	Palmerston North	1/01/2014	Aedes notoscriptus	21	**pos**		MIX SYAT05+???			peak with leftshift (left of linn 1)	
Home 4	Palmerston North	1/01/2014	Aedes notoscriptus	20	**pos**		SYAT05			mix; linn 1 +elongatum?	
Home 5	Palmerston North	1/01/2014	Aedes notoscriptus	20							
Home 6	Palmerston North	1/01/2014	Aedes notoscriptus	20							
Noto Home 7	Palmerston North	1/01/2014	Aedes notoscriptus	20		**pos** (weak band)					
Noto Home 8	Palmerston North	1/01/2014	Aedes notoscriptus	20	**pos**	**pos**	MIX Linn1+ SYAT05	MIX Linn1+ SYAT05		mix?	elongatum?
Noto Home 9	Palmerston North	1/01/2014	Aedes notoscriptus	20							
Noto Home 10	Palmerston North	1/01/2014	Aedes notoscriptus	20	**pos**		SYAT05				
Noto Home 11	Palmerston North	1/01/2014	Aedes notoscriptus	20		**pos**		**GRW4**			elongatum?
Home Perv 1	Palmerston North	1/01/2014	Culex pervigilans	2	**pos**	**pos**	MIX Linn1 +GRW6	MIX Linn1+ GRW6		linn1? Left shift	left shift left of linn 1
Home Perv 2	Palmerston North	1/01/2014	Culex pervigilans	5							
HP1 (2)	Palmerston North	1/01/2014	Culex pervigilans	4	neg	neg					
Home unknown	Palmerston North	1/01/2014	unidentified	17	**pos**	**pos** (weak band)					
Noto Home 12	Palmerston North	1/01/2014	Aedes notoscriptus	20							
Noto Home 13	Palmerston North	1/01/2014	Aedes notoscriptus	20							
Tur Noto 1	Palmerston North	1/01/2014	Aedes notoscriptus	20		**pos**		Linn1			weak; mix of all?
Tur Noto 2	Palmerston North	1/01/2014	Aedes notoscriptus	20							
Home Noto 14	Palmerston North	1/01/2014	Aedes notoscriptus	20	**pos** (weak band)	**pos** (weak band)					
HN2	Palmerston North	1/01/2014	Aedes notoscriptus	20							

HN17	Palmerston North	1/01/2014	Aedes notoscriptus	20					
TN4	Palmerston North	1/01/2014	Aedes notoscriptus	20					
TN5	Palmerston North	1/01/2014	Aedes notoscriptus	20		**pos**		Linn1	
HN12	Palmerston North	1/01/2014	Aedes notoscriptus	20					
TN14	Palmerston North	1/01/2014	Aedes notoscriptus	20					
TN15	Palmerston North	1/01/2014	Aedes notoscriptus	20	**pos**		MIX SYAT05+ Linn1+ ???		mix; weak
TN11	Palmerston North	1/01/2014	Aedes notoscriptus	20	**pos** (weak band)	**pos** (weak band)			
HN16	Palmerston North	1/01/2014	Aedes notoscriptus	20					
Opifex 1	Titali Bay	1/03/2013	Opifex fuscus	15		**pos** (weak band)			
Opifex 2	Titali Bay	1/03/2013	Opifex fuscus	9					
Whang Noto	Whangarei Mair Park	1/02/2013	Aedes notoscriptus	9					

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