Gastrointestinal Parasites in Endemic, Native, and Introduced New Zealand Passerines with a special focus on Coccidia

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Abstract

There is not much known about the taxonomy, prevalence, epidemiology, and life cycles of gastrointestinal parasites of passerine birds in New Zealand and there is a possibility that many of these parasites might have been introduced by non-native passerines. Approximately half of all New Zealand native passerine species are on the endangered list. Translocations to safe areas are the major management technique used to safeguard them for the future. Under natural conditions, gastrointestinal parasites seldom pose a threat. However, factors such as quarantine for translocation, overcrowding, low genetic diversity, and/or habitat changes may cause an infection outbreak that can severely affect the host species. The effect these parasites might cause under these conditions may therefore endanger translocation and captive breeding programmes. The purpose of this study was to generate baseline data on the gastrointestinal parasites of New Zealand native and introduced passerine birds with an emphasis on the coccidian parasites.

Faeces and tissues were examined from 361 birds from six native and four introduced species. Parasites were identified using flotation and microscopy, in the case of the coccidia also PCR analysis and DNA sequencing were used. Of the samples examined, 90 (24.93 %) were positive for coccidian parasites. Sequencing analysis revealed a close relationship between these parasites and other avian coccidia of the genus *Eimeria*. I found one coccidia species with a unique sequence in North Island robin and one in North Island saddlebacks, at least two different unique sequences in hihi and two in blackbird as well as at least three in tui. In addition, 18 (4.99%) birds were positive for trematodes, 30 (8.31%) for cestodes and three (0.83%) for *Capillaria*. Most of these parasites were reported during this study for the first time.

The results of this study have therefore revealed a whole range of new species of parasite infecting passerines in New Zealand providing a glimpse into the biodiversity of passerine parasites in New Zealand. This knowledge will be useful when taking management decisions particularly for translocations of protected species by alerting managers of possible sources of disease outbreak.

Acknowledgements

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 fantastic supervisors Isabel Castro, Maurice Alley and Laryssa Howe, for their patience and understanding. Without them I might have had much more difficulties and I owe them very much for their help in crossing the difficult 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.

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Preface

This thesis is formatted in a series of distinct research manuscripts ready for publication. Because of this, 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 were studying the epidemiology of avian malaria in New Zealand passerines at the same time as I was doing this research. The laboratory methods were those of the IVABS parasitology laboratory as performed by the technicians Barbara Adlington and Anne Tunnicliffe, suggestions for flotation and parasite indentification came from Dr. Bill Pomroy and Dr. Ian Scott (both IVABS), as well as from Louisa Robertson. The pathology methods and advice were provided by Dr. Maurice Alley (IVABS). The methods of DNA extraction and PCR analysis came from Laryssa Howe (IVABS) and Stephen Trewick (Allan Wilson Centre).

This thesis began as a study on Coccidia in native passerines, but after the discovery of formerly unrecorded helminths it was expanded to include these, as well as gastrointestinal parasites of some introduced birds such as blackbirds (*Turdus merula*). Some parasites have therefore been studied in more detail than others for which only limited material was available. Further study and sample collection of many of these newly identified parasites, is therefore necessary to better understand their classification and importance.

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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.

Chapter 1. Introduction

The relationship between host and parasite is an old one with evidence of intestinal parasites as far back as the early Cretaceous dinosaurs (Poinar & Boucot 2006). By definition, parasites take nutrients from their hosts and usually inflict patho-physiological damage that may cause a negative effect on host fitness by reducing growth, reproduction and survival (Holmstad et al. 2005). Although this may be true in some cases, there is evidence that infections by intestinal parasites in wild birds can be completely asymptomatic and only affect the host under certain conditions (Ritchie et al. 1994). Until now, little is known about the parasites of wild birds and only a limited number of species have been described. Under natural conditions, these parasites seldom pose a threat to the host, and it is only in closed and crowded captive conditions such as a holding facility for translocation or a breeding facility, that parasitic infections can become a problem (Harrison & Lightfoot 2006; Ritchie et al. 1994). In New Zealand, for example, a captive population of endemic hihi/stitchbirds (*Notiomystis cincta*), kept for breeding purposes, has often developed severe infections of *Isospora*-type coccidia which have lead to mortality (Cork et al. 1999; Twentyman 2001).

Common intestinal parasites in birds belong to different groups of helminths, for example nematodes, cestodes and trematodes, as well as protozoa like coccidia and giardia. In some cases helminth parasites can have significant effects on wild bird populations. For instance, in a study of willow ptarmigans (*Lagopus lagopus)* in the wild it was found that increasing abundance of three different species of helminth parasites were associated with increased chick mortality, reduced annual growth rate of the host population, and reduced host body mass and body condition (Holmstad, et al. 2005). It was also found that a community of two or more different parasite species had an additive effect on host body mass and breeding mortality. In general, most infected birds remained asymptomatic, but in severe cases they showed signs ranging from anorexia and depression to weight loss, melaena, diarrhoea and death. Heavy infection with helminths can also cause

obstruction of the intestines with a mass of worms (Harrison et al. 2006; Ritchie et al. 1994).

Endoparasites may also affect the bird by influencing physiological processes (Holmstad et al. 2005; Poulin 1994; Thompson & Kavaliers 1994) which result in changes in mate choice and reproduction.

As will become clear in the study reported in this thesis, New Zealand passerines like any other birds in the world are hosts to a variety of gastrointestinal parasites. The situation for many endemic passerines in New Zealand is precarious, and several of the most endangered species only survive today on off-shore and mainland islands that are free of introduced mammalian predators where they have been translocated. Many of these environments however are no longer pristine habitats as they were formerly used by Maori and Europeans for forestry and farming. As a result, these environments are highly disturbed and contain young, regenerating forest, which might not be the ideal habitat for the translocated birds. In addition, several conservation islands, such as Tiritiri Matangi, are ecotourism sites and this may also have an effect on the resident and translocated bird populations (Lindsay et al. 2008).

The stress of translocation and release in a less than optimal habitat may predispose immunologically challenged birds to infection from gastrointestinal parasites, such as coccidia. Coccidia are known to cause serious disease in wild non-passerine birds in New Zealand. For example, coccidia have caused mortality in captive adult (Thompson 1978) as well as crèche-reared juvenile kiwi that are about to be released to the wild (Alley 2002). Taken together, all these factors might be enough to reduce translocation and reintroduction success in New Zealand.

This thesis investigates gastrointestinal parasites infecting passerines in New Zealand with an emphasis on coccidia. A more detailed study of coccidia was made because these parasites are known to cause severe disease in captive hihi, an endangered endemic New Zealand passerine (Twentyman

2001). The literature review which follows will give an overview of both coccidian and other gastrointestinal parasites, describe the natural history of the endemic, native and introduced avian species examined in this thesis, discuss disease screening in avian recovery programmes, and finally provide an outline of the aims of this thesis.

1.1 Coccidian Parasite Overview

1.1.1 Coccidian parasites in New Zealand

Coccidian parasites in passerines have been recognized in New Zealand for decades (McKenna 1998). Coccidia have also been found in faeces from kokako (McKenna 2004). However, no descriptions of these coccidia were included in these publications. A coccidial stage was also described in blood samples from silvereyes, identified at the time as "*Atoxoplasma paddae*" (Laird 1959) though the actual identity of this parasite remains to be established. Studies on the excreted oocysts of these parasites were not done by Laird, and molecular methods were not available at that time. Apart from the study of coccidiosis in captive hihi by Twentyman (2001), there have been no other studies of coccidia in wild passerines undertaken in New Zealand. Over the last 10 years since Twentyman's investigation, several useful molecular methods for identifying coccidian species in passerine birds have been developed (Schnitzler et al. 1998; Barta et al. 2001; Barta et al. 2005; Schrenzel et al.2005), and for this reason it was decided to put the main focus of this study on investigating the taxonomy of the coccidia in passerines in New Zealand.

1.1.2 Taxonomy

Coccidian parasites are obligate intracellular protozoan parasites. As members of the phylum *Apicomplexa,* they possess an apical complex at one end of the cell that allows these parasites to enter their host cells (Rommel et al. 2000). They are often host specific; for example, experiments

have shown that isosporan oocysts from sparrows (*Passer domesticus*) cannot infect canaries (*Serinus canaria*) (Box, 1970) and oocysts from hihi are not able to infect starlings (*Sturnus vulgaris*) (Twentyman, 2001).

Conventionally, the taxonomic groups within the Apicomplexa were separated based entirely on the morphology of their oocysts after sporulation, with the genus *Eimeria* possessing four sporocysts with two sporozoites each in the oocyst, the genus *Isospora* possessing two sporocysts with four sporozoites each and the genus *Caryospora* possessing one sporocyst with four to eight sporozoites. Recently, it has been suggested that the tetrasporozoic and diplosporocystic *Isospora* species from mammals, that were missing a Stieda body, should be transferred to the genus "*Cystoisospora*" while the tetrasporozoic and diplosporocystic species of birds, possessing Stieda bodies, should remain in the genus *Isospora* under the family *Eimeriidae* (see Figure 2 A+B) (Barta, et al. 2005). However, the taxonomic classification of the coccidia remains controversial, particularly because of the existence of a separate genus "*Atoxoplasma*"; the name that was given to "*Isospora*-type coccidia with extraintestinal stages (Ball et al.1998; Martinez & Munoz 1998; Twentyman 2001; Upton et al. 2001; Remple 2004; Adkesson et al. 2005; Barta, et al., 2005; Schrenzel, et al. 2005; Sanchez-Cordon et al. 2007; Gill & Paperna 2008). In addition, with the development of molecular techniques and studies examining the genetic diversity of the small subunit rRNA of coccidian parasites, it has been suggested that the genus *Isospora* is a polyphyletic group and should be divided into 2 separate genera belonging to two different families (Carreno et al. 1998; Morrison et al. 2004). Thus it is now recognized that *Isospora* spp. in mammals are more closely related to *Neospora, Toxoplasma* and *Sarcocystis* than to *Eimeria*, while *Caryospora* is closely related to *Eimeria* spp. (Carreno et al. 1998; Morrison et al. 2004). Genetic evidence from studies of avian coccidia of the genus *Isospora* now shows that they are closely related to *Eimeria* and should, therefore, belong to the family *Eimeriidae* (Carreno et al. 1998; Morrison et al. 2004).

1.1.3 The coccidian life cycle in birds

A host becomes infected with coccidia by ingesting the sporulated oocysts that have been shed in the faeces from another host. The shedding of the oocysts by the host bird follows a diurnal periodicity and mainly occurs in the late afternoon and evening (Box 1977; Hudman et al. 2000; Brown et al. 2001; Martinaud et al. 2009). Consequently, even a highly infected bird can show negative oocyst output when sampled in the morning (Dolnik2006). Studies have demonstrated that the peak of oocyst shedding in greenfinches was between 2pm and 5pm (Brown et al. 2001). It has been suggested that the release of oocysts in the late afternoon is an adaptive trait to avoid desiccation and UV radiation, thus reducing the mortality of the oocysts in the external environment (Martinaud et al. 2009). To test this hypothesis, experiments were performed with blackbirds infected with *Isospora turdi.* The results revealed that even after the short exposure of faeces containing oocysts to natural sunlight, oocyst survival dramatically decreased due to the effect of warmth and UV light, particularly UVB (Martinaud et al. 2009).

Sporulation of the oocysts occurs in warm, moist environments and is necessary before infection can occur, because unsporulated oocysts will pass through the gut of the “would- be” host unchanged (Rommel et al. 2000 Horak et al. 2004; Belli et al. 2006). The time needed for sporulation varies in different *Isospora* species. About 70% of oocysts contained in blackbird faeces, incubated at room temperature in 2% potassium dichromate solution sporulate within 24 hours (Martinaud et al. 2009), while sporulation times in hihi remain unclear and might range from two to six days (Twentyman 2001).

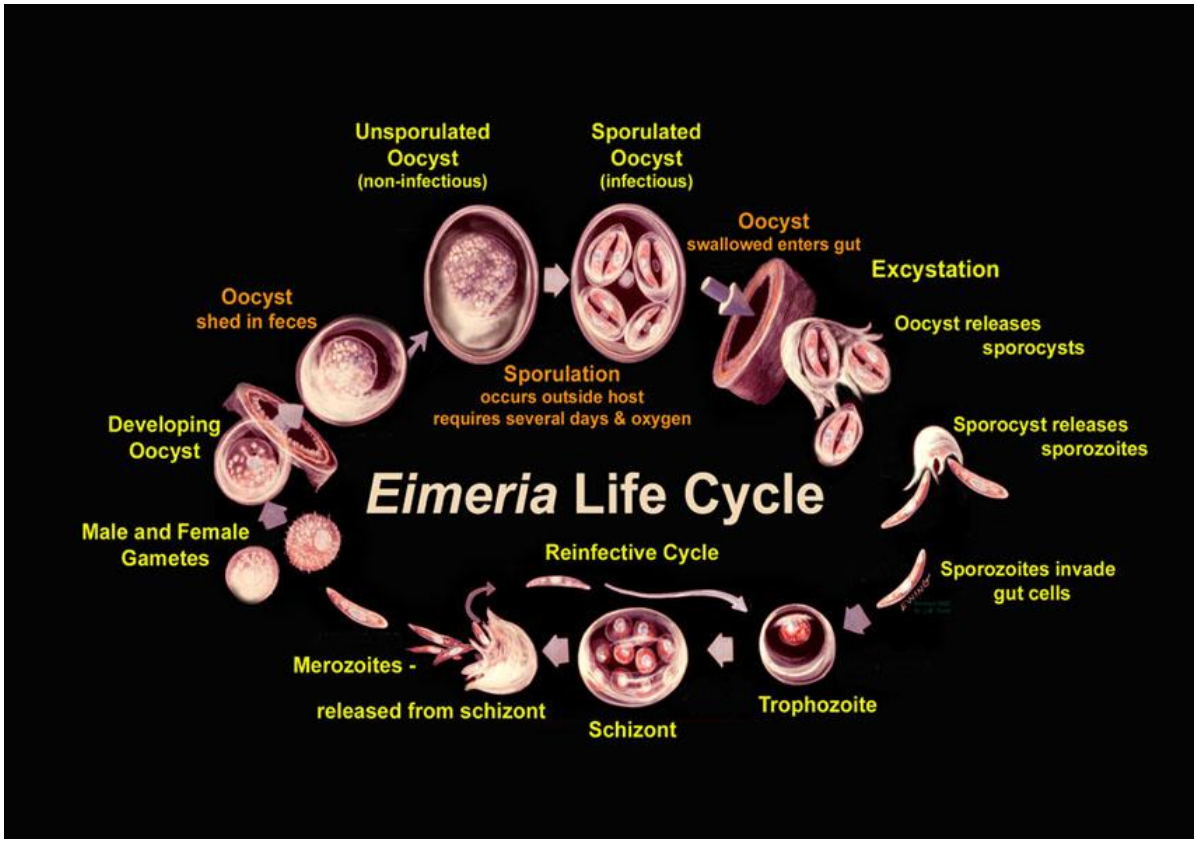

Figure 1: General life cycle of coccidia of the genus Eimeria (http://www.jphmvkpj.gov.my/Parasitology/Para_Gallery.html)

The life cycle of *Eimeria* spp. is shown in Figure 1. Following ingestion, the wall of the oocyst breaks down in the small intestine and releases sporozoites which penetrate the host's intestinal epithelial cells. These sporozoites start their first asexual reproduction in the intestinal epithelial cells, but in some species this first stage is followed by extraintestinal stages that invade liver, spleen and lungs (Box 1977). This seems to depend on species and strain. For example, in canaries, there are two coccidia species, one stays almost exclusively in the gut, while the other always has extraintestinal forms (Box 1977). These extraintestinal stages can be found by the fourth day after inoculation, although in this early stage the intestinal mucosa is more severely affected than the internal organs (Box 1970).

Each generation of asexual reproduction produces multiple merozoites that infect new cells. In passerines, asexual stages can also be found in the blood, where they invade monocytes and lymphocytes (Rommel et al. 2000; Horak et al. 2004). Some of the merozoites in the small intestine (and rarely in other organs) enter new host cells and there transform into gametocytes

(Baker et al. 1996). These gametocytes transform into gametes which fuse and form a zygote which develops into the oocyst. The developing oocysts escape the host's cells and are then passed in the host's faeces. In greenfinches oocysts are shed by most birds for about 80 days, but in some for up to 96 days after infection (Ball, et al. 1998). However, in canaries, Box (1977) found that chronic infections with *Isospora serini* can result in oocyst discharge for as long as 231 days.

Different *Isospora* species can have varying prepatent periods (the period between infection of the host and the earliest time which oocysts can be found in the faeces) and pathogenicity (the ability of a pathogen to produce an infectious disease in an organism). For example, Box (1977) described the life cycles of two *Isospora* species in the canary. *Isospora canaria* develops solely in the epithelium of the small intestine, with three asexual generations and the sexual generation. The prepatent period for this parasite is 4-5 days, and the patency lasts for 11-13 days. In contrast, the first part of the life cycle of *Isospora serini* takes place in mononuclear phagocytes. Five asexual generations have been described developing in this cell type, with two additional asexual and the sexual stages in the intestinal epithelium. The prepatent period for *Isospora serini* is 9-10 days and the infection with this parasite is chronic and long lasting (Box 1977). Dolnik (2006) found that in blackcaps (*Sylvia atricapilla*) infected with *Isospora sylvianthina,* the intensity of the infection in an individual is relatively stable over time, both in the scale of a few consecutive hours and in that of consecutive days. This shows that single measurements of oocyst production, when collected at the proper time of day, can be used to characterize more permanent aspects of an individual´s coccidia infection status.

1.1.4 Identification and diagnosis

Passerine *Isospora* oocysts are commonly subspherical, sometimes spherical or ellipsoidal with a smooth, colourless to pale yellowish, single layered wall, without a micropyle or when sporulated, an oocyst residuum, but usually with one or more polar granules. Typically, the sporocysts are ovoid to lemon shaped, rather thick walled, and possess a Stieda body (Levine 1982; Martinez & Munoz 1998). The Stieda body, which is the plug on one end of the sporocyst, is an important way of differentiating between avian and mammalian *Isospora* spp.

1.1.4.1 Oocyst morphology

The most common method of diagnosing infections with coccidia is by examination of faecal samples. The oocysts of these parasites are separated from the surrounding faecal material by means of flotation, a principle that uses a solution of either a salt or a sugar with a certain specific gravity that allows the oocysts to float and heavier faecal debris to sink (Rommel et al. 2000). Flotation is the most commonly used technique for detecting small parasite eggs as well as coccidial oocysts and other protozoa. Commonly used flotation media are salt solutions ($ZnSO_4$, $ZnCl_2$, or NaCl) or sugar (sucrose) solutions, and the correct specific density is checked by measuring with a hydrometer.

Even among oocysts of the same species there is variability in size and shape of both oocysts and the sporocysts they contain (Levine 1982; Ball et al. 1998)).

Infections with different species, identified using morphology, are common. As an example of this, (Twentyman 2001) found different shapes and sizes in the coccidia oocysts in hihi that she examined (see Appendix 1) and therefore placed them into two main groups based on the general appearance of the oocyst shape in conjunction with a measurement of length-width difference. One group of oocysts was subspherical, while the other group was ellipsoidal.

When sporulated, these oocysts appeared as *Isospora*- type oocysts, containing two sporocysts and each sporocyst containing four sporozoites. As typical for oocysts of the family Eimeriidae, the sporocysts showed Stieda bodies (see Figure 2).

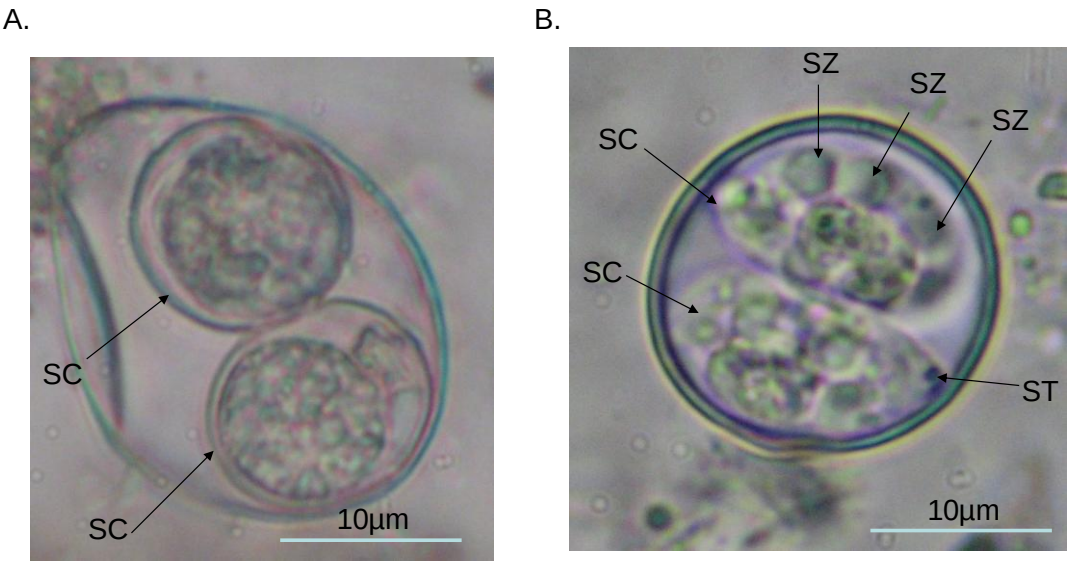

Figure.2: A. Sporulated oocyst from *Isospora sp*. from a cat (400x). B. Sporulated oocyst of a hihi from Mt Bruce NWC (400x) SC- Sporocysts. SZ- Sporozoites. ST - Stieda body Note the absence of Stieda bodies on the sporocysts as typical in mammalian *Cystoisospora spp.* of the family Sarcocystidae. Photographs taken by the author.

1.1.4.2 Diagnosis of extraintestinal stages of coccidia

In species that have no extraintestinal stages, the complete lifecycle occurs in the intestine, while in species that possess extaintesinal stages these can be found in various organs depending on the individual life cycle. In some cases, extraintestinal merozoites, which are the asexual stages of *Isospora,* can be seen in a parasitophorous vacuole within mononuclear phagocytes. They are located close to the nucleus of the host cell. Generally, one merozoite can be seen in each parasitized cell. On ultrastructural examination, the merozoites contain a prominent nucleus, a single mitochondrion, a conoid (function unknown, possibly the organelle used for cell penetration), multiple rhoptries (specialized secretory organelles) with electron-dense contents and numerous elongated micronemes (specialized organelles important for gliding motility and host cell invasion), often confined

to the conoidal region (Sanchez-Cordon et al. 2007). Occasionally, merozoites can be found in hepatocytes and Kupffer cells (Sanchez-Cordon et al. 2007) and this may be used to diagnose infection as early as five days post infection by liver biopsy (Box 1970)

Peripheral blood smears examined for asexual stages in monocytes and lymphocytes are usually negative, although these stages can be found in the heart blood of birds post-mortem as early as 5 days after infection. In these cases, the presence of organisms in the heart blood is associated with the presence of numerous parasites in other extraintestinal tissues (Box 1970).

For the identification of mononuclear cell stages, PCR analysis has become a useful tool. Adkesson et al. (2005) were able to identify 38.3% of their birds examined as positive for *Isospora* spp. using PCR testing of blood from the right jugular vein. PCR is also a helpful tool for identifying *Atoxoplasma/ Isospora* infections from extraintestinal tissue. Because of the parasite's predilection for mononuclear cells, liver and spleen are probably the most valuable tissues for PCR testing at necropsy (Adkesson et al. 2005).

1.1.5 Epidemiology

Most wild birds infected with *Isospora* spp. have subclinical infections and therefore show no detectable symptoms, although there may be a high prevalence of oocyst shedding in the population (Ritchie et al. 1994). For example, in Germany the prevalence of coccidia can be up to 90% in free-living adult blackbirds (Misof 2004). In a New Zealand study a 40% prevalence of coccidia among clutches of song thrushes (*Turdus philomelos*), blackbirds (*Turdus merula*) and starlings was found with no detectable negative effect on the juvenile birds, with nestling weight, tarsus length, plasma carotenoid concentration, clutch size and fledging success measured (Cassey & John 2008).

The effect of subclinical infections on wild host health and reproduction has not been fully established, although decreased reproductive performance, a

general decline of condition and increased susceptibility to other infections might occur. Free-ranging birds only seem to be seriously affected by the disease when confronted with stress, e.g. capture stress (Giacomo et al.1997; Gill & Paperna 2008). For example, stress was a factor in the death of black siskins (*Carduelis atrata*) where 97% died from proliferative visceral *Isospora* infection 2 months after their transport from Bolivia to Italy (Giacomo et al. 1997). In pet and aviary birds, clinical disease and mortality occur primarily in juvenile birds younger than six months and are compounded by additional stressors such as starvation or overcrowding (Zinke et al.2004). In addition, it has been found that greenfinches inoculated with multiple “strains” of *Isospora* developed higher infection intensities than birds infected with a single parasite species (Horak et al. 2006).

1.1.6 Clinical signs

Isospora spp. can cause severe acute infections in songbirds, with proliferative visceral *Isospora* infection (“Atoxoplasmosis”) recognised as a cause of death in captive birds, while free-ranging birds more often show an asymptomatic chronic form of the disease (Box 1970). In various passerine species, coccidia of the *Isospora/Atoxoplasma* group more commonly cause chronic coccidiosis (Box 1970). An explanation for the chronic nature of these infections in passerines without reinfection relates to the extraintestinal lifecycle of some species that have asexual stages in the lymphoid-macrophage cells of internal organs (Box 1970).

In captive birds, coccidian infections are sometimes associated with the syndrome known as “going light” which is characterized by general depression, ruffled feathers, diarrhoea, loss of appetite, weight loss, reduction of pectoral musculature, distended abdomen, dehydration and death four to six days after appearance of the first symptoms (Ball et al., 1998). Sudden death without prior clinical signs is also a possible result of infection (Ball et al. 1998; Dorrestein & Kummerfeld 1998; Martinez & Munoz 1998; Horak et al. 2004; Sanchez-Cordon et al. 2007). In studies using sub-

adult canaries, the mortality due to acute disease was as high as 80% (Dorrestein & Kummerfeld 1998).

Due to the extraintestinal stages in the lifecycle of some coccidia species, the organisms are also able to cause neurological signs, such as loss of balance prior to death (Martinez & Munoz 1998; Sanchez-Cordon et al. 2007), with 30% of sub-adult canaries being affected by this disorder (Dorrestein & Kummerfeld 1998). In chronic infections of canaries (Box 1970), and starlings (Dorrestein & Kummerfeld 1998), the lung is usually the most heavily parasitized organ, what has also been found in starlings. Baker and co-workers also reported an unusual case of pneumonia caused by a coccidian organism in a Northern Cardinal (*Cardinalis cardinalis*) with sexual and asexual stages as well as oocysts seen in lung tissue (Baker et al. 1996).

Coccidia often cause severe pathological changes in the intestine and resulting in nutrient malabsorption. In greenfinches, infection resulted in drastic but transient decreases in serum carotenoids, vitamin E, triglyceride and albumin concentrations and reduced body mass (Horak et al. 2004). Carotenoids participate in immuno-regulation and stimulation, lymphocyte proliferation, free-radical scavenging and detoxification. After an infection, animals are not able to synthesise carotenoids themselves and thus an infection with gastrointestinal parasites can further limit the host´s ability to fight infections (Horak et al. 2004).

There are conflicting findings concerning the degree to which coccidian parasites may influence body condition. No correlation was found in migratory birds (Zinke et al. 2004); on the other hand, a higher level of infection in blue-black grassquit (*Volatinia jacarina*) was found to be associated with a lower body mass and a lower body size index (Costa & Macedo 2005).

1.1.7 Pathology

1.1.7.1 Gross pathology

At necropsy, clinically affected passerines commonly show poor body condition, thickened walls of the intestine (most commonly of the duodenum) and liquid gut contents with excess mucus (Dorrestein & Kummerfeld 1998). In severe cases, haemorrhage of the intestinal mucosa as well as pinpoint foci of necrosis can be found (Dorrestein & Kummerfeld 1998). In cases where the host is infected with coccidian species that possess extraintestinal stages in their lifecycle, extraintestinal findings may include hepatomegaly, with a liver that may show pinpoint necrosis or green colouration due to bile retention as well as discoloration of the spleen and splenomegaly, as found in severe cases in black siskins and canaries (Dorrestein & Kummerfeld 1998; Giacomo et al. 1997). Martinez and Munoz (1998) found blood in the abdomen of a hybrid passerine (*Serinus canarius* x *Carduelis cannabina*) and a liver that was pale yellow in colour. The bowel loops were distended with thickened walls and a creamy white content.

The only native New Zealand passerine affected by coccidia to be studied to date is the hihi/stitchbird. Initial studies of hihi that died of coccidiosis showed a severely distended intestine with extensive thickening of the intestinal wall, numerous foci of necrosis scattered throughout the liver and an enlarged spleen (Cork et al. 1999). Twentyman (2001) found that hihi that died of coccidiosis presented with a variable body condition, ranging from thin to good. The intestines of heavily infected hihi commonly displayed a turgid intestine, a dilated intestinal diameter and a thickened intestinal wall in severely affected birds, the intestine was dilated to twice its normal size over most of its length. Some of the birds had a swollen abdomen, soiled vent and livers that were either smaller than normal in size or swollen and congested; in some cases small spots were visible on the livers.

1.1.7.2 Histopathology

Generally, histopathologic examination has shown marked mononuclear inflammatory cell infiltration of the lamina propria of the intestine and in

species that have extraintestinal stages, of the liver and spleen. The cells are mainly lymphocytes, a few plasma cells and many monocytes (Giacomo et al. 1997; Martinez & Munoz 1998). On histological examination of a case of *Atoxoplasma* in a hybrid passerine the presence of coccidian sexual stages in the intestinal epithelium and the lamina propria were noted (Martinez & Munoz 1998). In infected black siskins, mononuclear infiltrates were also observed in the Lieberkühn crypts, as well as large numbers of oocysts in fresh smears of intestinal contents, mainly in the duodenum and jejunum (Giacomo, et al. 1997). In heavily infected birds, vast numbers of coccidia stages have been observed in the intestinal epithelium (see, for example, Giacomo et al. 1997; Twentyman 2001) and Figure 6.

In severe and fatal cases, the inflammatory response may extend throughout the intestinal mucosa. This was illustrated by the observations of Giacomo et al. (1997) in captive black siskins. They reported a transmural lympho-monocytic enteritis, the mononuclear cell infiltrate extending from the sub-epithelial area to the extraintestinal serosa. The lamina propria and the submucosa were heavily damaged while crypts of Lieberkühn were compressed by the infiltrate to such an extent that there was loss of their central lumen. Mononuclear inflammatory cells invaded the muscularis, disrupting its structure and infiltrating the serosa of the adjacent mesentery. The duodenal and jejunal epithelium were badly damaged by parasites in various stages of their biological cycle, although epithelial necrosis was apparent only when there was a massive infection, particularly in the epithelium of the crypts in the duodenum where the parasites were undergoing merogonic replication. Similar changes were described in severe infections of hihi (Cork et al. 1999).

The livers of hybrid passerines (*Serinus canarius x Carduelis cannabina*) infected with coccidia having extraintestinal stages, showed moderate perivascular inflammatory infiltration that extended into the sinusoids but no abnormalities in other viscera were found, although the spleen was not examined (Martinez & Munoz 1998). Other authors working with different passerines found that the spleen consistently showed signs of activation of

the lymphoid follicles with an overall cellular increase, and the infiltrate into the liver was mainly around portal triads and sometimes centrilobular (Giacomo et al. 1997). In infected captive canaries Sanchez-Cordon et al. (2007) observed microgranulomas in infected livers and a pronounced bile duct hyperplasia with perivascular accumulations of mononuclear cells, particularly in the periportal areas. In the lungs of infected black siskins, intense hyperaemia with numerous mononuclear cells adhering to the walls of the blood vessels has been reported, and the lungs were also generally congested, swollen and infiltrated with exudate (Giacomo et al. 1997).

Ball et al (1998) found merozoites of an *Atoxoplasma* species within parasitophorous vacuoles in the cytoplasm, indenting the nucleus, of leucocytes in the blood vessels in the small intestine of the greenfinch. In Israeli sparrows (*Passer domesticus biblicus*) (Hartert 1904), merozoites were found predominantly in the spleen, but rarely in the peripheral blood, the visceral infections resulting in multifocal necrosis (Gill & Paperna 2008). Early studies of the histopathology of hihi coccidiosis found that all stages of the life cycle were present in the intestinal tissue, but some macrogamonts were seen in the liver and numerous schizonts were observed in the spleen (Cork et al. 1999).

More detailed studies of hihi coccidiosis were made by Twentyman (2001). Histopathlogy revealed all affected hihi had a mild to severe thickening of the lamina propria of the intestine, with large numbers of infiltrating macrophages, which contained schizonts and schizozoites. The lamina propria in these cases also contained proliferating fibroblasts, moderate numbers of lymphocytes, and occasional giant cells. Beside the asexual stages, smaller numbers of coccidian sexual stages were present in the lamina propria, mainly close to the epithelial glands and crypts. In most cases, the intestinal villi were flattened and there was a loss of epithelium. Large numbers of coccidia were present in the hyperplastic intestinal epithelial cells. Macro- and microgametes as well as zygotes and oocysts were recorded. Many epithelial glands contained coccidian parasites in each of the epithelial cells.

In many cases, the livers of the hihi examined by Twentyman (2001) had scattered, sometimes perivascular, excessive lymphoid foci. Occasionally there was a more intense focal inflammatory reaction with involvement of heterophils and macrophages, with inflammatory necrosis present in a few cases. Rarely, sexual and asexual stages of coccidian parasites were associated with these inflammatory foci. The livers of many of these birds also presented with an excessive amount of haemosiderin within both hepatocytes and Kupffer cells.

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1.1.8 Changes in host behaviour and condition as a consequence of infection

Visible behavioural changes caused by infections with endoparasites may only be evident during prepatent, acute and crisis phases of infection when physiological stress reaches the peak. In 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).

Experiments on captive male greenfinches by Horak et al (2004) showed that an infection with isosporan coccidia had severe effects on the physiology and expression of carotenoid based plumage colouration and resulted in drastic but transient decreases in serum carotenoid. It was suggested that the colour of carotenoid based feathers is a sexual selected trait, and in some passerine species more brightly coloured males are known to be favoured by females as mates (Horak et al. 2004). The researchers found that tail feathers of infected birds grown during the experiment in the laboratory contained 52% less carotenoids and also had smaller values of chroma and hue than a control group of medicated greenfinches. The authors suggested that besides being less attractive to females because of their infection, the infected birds would have their general condition weakened by coccidiosis, and therefore might be more susceptible to other pathogens. It was also suggested that this might be a threat to the female, because these males are more likely to infect their partners. Infected males with weakened condition

may also be more vulnerable to predation during breeding and would therefore leave the female alone to care for the young which might threaten breeding success (Horak et al. 2006). A similar finding was recorded in male barn swallows by Saino et al. (1999) who suggested that healthy males are able to invest more of their carotenoids into plumage colouring instead of using them for immunity functions so that bright plumage colour might be a reliable signal for health. As shown by Horak et al. (2001), greenfinch survivors of infection generally had higher values of hue than non- survivors. These results show that carotenoid-based plumage colouration in greenfinches serves as a signal reflecting individual quality in terms of health status and local survival.

To what extent these findings relate to plumage colour in endemic New Zealand passerine species has not been yet investigated, although studies are currently under way (John Ewen, pers. comm.).

1.1.9 Coccidia of the suborder Eimeriorina and the genus *Sarcocystis*

Coccidia of the family Sarcocystidae (phylum Apicomplexa, class Coccidia, order Eucoccidiorida, suborder Eimeriorina) need two hosts to complete their life cycle. The asexual stage forms cysts in skeletal muscle of herbivorous or omnivorous first hosts, the sexual stage is completed in the small intestine of carnivorous animals that devour the first hosts. In domestic chickens, cysts are preferentially formed in the skeletal muscle of head, hip and thighs. The final hosts of *Sarcocystis* sp. in chickens are dogs and cats, where the sexual development takes place in the small intestine (Rommel et al 2000). In passerine birds, the clinical signs may differ between birds and bird species and range from asymptomatic to anorexia, diarrhoea, weakness, dyspnoea, ataxia and death (Harrison & Lightfoot 2006).The diagnosis of a *Sarcocystis* spp infection is made by histopathology of either biopsied or post mortem skeletal muscle (Harrison & Lightfoot 2006). In New Zealand, *Sarcocystis* spp muscle cysts have been found in the New Zealand wood pigeon (McKenna 1998), as well as in black stilt (*Himantopus*

novaezelandiae), brown teal (*Anas chlorotis*), takahe (*Porphyrio hochstetteri*), Fjordland crested penguin (*Eudyptes pachyrhynchus*) (unpublished observations, New Zealand wildlife pathology database).

So far, the organism has only been found in two passerine bird species, North Island saddleback (*Philesturnus carunculatus rufusater*) and robin (*Petroica longipes*) (unpublished observations, New Zealand wildlife pathology database and this thesis).

1.2 Other gastrointestinal parasites

Although this study focuses on coccidia infections in passerines, trematodes, cestodes and nematodes, can commonly be found in passerines (Ritchie et al. 1994; Rommel et al. 2000; Harrison & Lightfoot 2006).

1.2.1 Trematodes

Trematodes, commonly called “flukes”, require at least one intermediate host (e.g. insects, molluscs or other invertebrates) before they are able to complete their life cycle .Infections are mainly asymptomatic but in severe cases they can cause nutritional deficiencies, weight loss and diarrhoea. They can be diagnosed by the detection of eggs in the faeces, although this can be a challenge, as the eggs may be few in number and may not be found by routine faecal examination procedures (Ritchie et al. 1994; Rommel et al. 2000; Harrison & Lightfoot 2006).

1.2.2 Cestodes

Cestodes, commonly known as “tapeworms”, are known to require at least one intermediate host (e.g. an insect) before they are able to complete their life cycle in the bird. Commonly, these worms cause few problems in the bird, but occasional severe infections exist in which these worms can cause nutritional deficiencies, weight loss and diarrhoea. Infections can be diagnosed through finding free eggs (hexacanth larvae with six hooks on the onkosphere) or sometimes free segments (“proglottids”) in the faeces (Rommel et al 2000; Harrison and Lightfoot 2006).

1.2.3 Nematodes

The phylum Nematoda, also known as “roundworms”, is a large and diverse group of worms with various forms, habitats and life cycles, including many parasitic forms. Not all of these parasites will inhabit the intestine, although intestinal forms are the most common. As with other helminth infections of the intestine, heavily infected birds may show malabsorption of nutrients, anorexia, weight loss, diarrhoea and in severe cases, the worms can cause bowel obstruction and death (Rommel et al 2000; Gabrisch 2001; Harrison& Lightfoot 2006).

1.3 Endemic passerine birds of New Zealand

The endemic New Zealand passerines (Figure 3) have an ancient history (Barker et al. 2004). Because the evolutionary relationships of many New Zealand species remain uncertain, this leaves the evaluation of the region's evolutionary distinctness incomplete and possibly underestimated (Driskell et al. 2007).

The information in this section has been summarised from (Higgins 2001; 2002; 2006) unless otherwise stated.

1.3.1 Hihi or stitchbird (*Notiomystis cincta*)

The hihi is a protected and threatened endemic species to New Zealand (see Figure 1A). Formerly, hihi were thought to be a member of the honeyeater (Meliphagidae) family and therefore closely related to tui (*Prosthemadera novaeseelandiae)* and bellbird (*Anthornis melanura*) (Driskell and Christidis 2004; Driskell et al. 2007). However, the results of a recent phylogenetic analysis revealed that hihi have no close living relatives. The closest relatives are the New Zealand wattlebird family (Callaeidae), but these birds separated from the ancestors of the hihi around 33.5 million years ago. Therefore, hihi have now been placed in their own family Notiomystidae (Driskell et al. 2007).

By 1885, this species had completely vanished from New Zealand except a single population on Hauturu Island. Suspected causes of population decline include loss of suitable habitat, the introduction and spread of mammalian predators and diseases that might have been arrived with introduced European birds. Despite several translocations to other offshore islands and mainland islands, hihi have not yet established self sustaining populations. A captive breeding programme at Mt. Bruce National Wildlife Centre (Masterton, Wairarapa, North Island) has also not been entirely successful,

A.

B.

C.

D.

Figure 3: Native New Zealand passerines. A. Hihi (male), B. North Island robin (female), C. Tui , D. Saddleback. Photographs taken by the author.

although the programme produces chicks, the adult and young birds suffer mortality due to aspergillosis (Cork et al. 1999) and coccidiosis (Cork et al. 1999, Twentyman 2001) and therefore the captive population requires continous input of wild birds to be sustainable (Taylor et al. 2005.).

The hihi is around 18cm in length, with males 36.5g- 40g of weight and females around 30g. They feed on nectar, fruit and invertebrates. Like other honey eating birds, hihi have a long tongue with a brush-like tip. They face fierce food competition from more aggressive Tui and Bellbird at the same nectar sources. Hihi nest in cavities such as tree holes and rock crevices.

1.3.2 North Island robin (*Petroica australis longipes*)

The New Zealand robin is a member of the Eopsaltriidae family. There are three subspecies of the New Zealand robin, with each living on one of the main islands of New Zealand, *Petroica australis rakiura* is found living on Stewart Island, *P.a.australis* on the South Island and *P.a.longipes* on the North Island (Figure 1B). North Island robins were once widespread throughout the North Island, but due to habitat degradation and introduced mammalian predators, they are now protected endemic birds with only small populations across the centre of the North Island from Taranaki (but not Mt Taranaki) to the Bay of Plenty and on offshore islands and mainland islands.

New Zealand robins are forest birds around 18cm in length and weigh around 35g. They feed on invertebrates for which they forage on the ground. New Zealand robins prefer mature native forests as habitat, but may also live in tall scrub and introduced pine forest.

1.3.3 Tui (*Prosthemadera novaeseelandiae*)

The tui or Parsons Bird (*Prosthemadera novaeseelandiae*) (see Figure 1C) is one of two endemic species of the honeyeater family (*Meliphagidae*) in New Zealand. There are two subspecies of tui, the rare Chatham Island tui (*P.*

novaeseelandiae chathamensis), which is bigger (males 155 g, females 110g) than the common mainland tui (*P*. *novaeseelandiae)* (body length 27-32 cm; males 120g, females 85- 110g).

The distribution range of tui is throughout the forests and towns of the North, South and Stewart Islands and many offshore islands, they inhabit mixed, often dense, podocarp-broadleaf forest, both in continuous forest tracks as well as in small remnants and regrowth. They also occur in suburbs and frequent parks and gardens.

Tui feed mainly on nectar, fruit and insects, but occasionally take seeds and pollen. Like the other nectar feeding birds in New Zealand, tui play an important role as pollinators of native plants (Heather 1996, Castro & Robertson 1997). However, these birds also utilize plants introduced by humans into New Zealand, such as the widespread planting of flowering trees and shrubs in human settlements and on farmland providing an all year food supply.

1.3.4 North Island saddleback (*Philesturnus carunculatus rufusater*)

The saddleback (see Figure 1D) is a range restricted endemic bird that belongs to the family of wattlebirds (*Callaeidae*) endemic to New Zealand. There are two subspecies, the North Island saddleback (*Philesturnus carunculatus rufusater*) and the South Island saddleback (*P. c. carunculatus).* Characteristic for this family are the colourful fleshy wattles at the gape of the beak, and short, rounded wings which limit their flight. This restriction on flight makes the bird prefer to leap with their strong legs. Other members of this family are the kokako (*Callaeas cincera*) and the extinct huia (*Heterolocha acutirostris*).

The North Island subspecies has a medium size of 25cm, with males around 80g and females around 70g. These birds 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 like tui and hihi a brush tip tongue.

The North Island saddleback is vulnerable to the destruction of natural habitat and introduced mammalian predators, and became extinct on the mainland of the North Island in 1910. A single population survived on Hen Island (Hauraki Gulf, off shore North Island) from where individuals were sourced for several successful translocations to other islands. Currently, the birds survive on offshore and mainland islands, such as Mokoia Island (Lake Rotorua, North Island), and at the Karori Wildlife Sanctuary (Wellington, North Island).

1.4 Native passerine birds of New Zealand

Native passerines originated from Australia, and migrated to New Zealand over the past few hundred years. They were able to establish themselves due to their good acceptance of an environment altered by human activities. The information in this section is given as reviewed by Higgins (2001, 2002, 2006) unless otherwise stated.

1.4.1 Fantail (*Rhipidura fuliginosa*)

Figure 4: Fantail (*R.f.placabilis*) from the North Island of New Zealand. Photograph taken by Dr Isabel Castro.

The fantail is a member of the flycatcher family Rhipiduridae. There are about 10 subspecies of fantail, of which 3 (*R.f.placabilis in the North Island and R.f.fuliginosa, and R.f. penitus on the South Island*) are native to New Zealand. They are widespread and locally abundant, especially on forest edges and in secondary growth scrub habitat. They are one of the most common and widely distributed native birds on the New Zealand mainland and have successfully adapted to the large scale clearance of forest and creation of forest edges and scrub. They feed mainly on invertebrates which are occasionally supplemented by fruit.

Fantails reach up to 16.5 cm, but this includes 8cm of tail. They weigh around 8g. It is a small bird with a small head and bill, the tail is long, and birds from New Zealand have a slightly longer tail than the Australian subspecies. The tail is often fanned for mobility, they are manoeuvrable and use their fanned tail to stop in mid-air and to change direction when fly-catching. Males and females look alike.

Fantails are strongly territorial while breeding, but territories break down in autumn. In winter communal roosts with up to 20 birds can sometimes be seen. They remain in pairs all year and the males retain the same territory each year, however, due to high mortality from predation, few pairs remain together for successive breeding seasons. The breeding season lasts from August to February.

1.4.2 Silvereye (*Zosterops lateralis*)

The silvereye is a member of the Zosteropidae family. These birds are abundant natives in New Zealand. There are at least six subspecies in Australia alone. The Tasmanian subspecies *Z.l.lateralis* migrates to eastern states of the Australian mainland in winter and is the subspecies that colonised New Zealand.

Silvereyes are small birds, approximately 12cm in length and weigh around 11g. Head and upperparts are olive green with a grey back, neck and chest

and a white eye-ring. The under parts are creamy white with pinkish-brown flanks and a white under the tail.

These birds inhabit most habitats with moderate vegetation cover, including shrub- and woodlands, forests, mangroves and also modified habitats like parks and gardens. They have a varied diet, mainly taking fruits and insects, but also nectar, other invertebrates, flowers and seeds. They forage in small groups from the ground up to the canopy and also in the air, using various foraging methods.

1.5 Introduced passerine birds

Passerine birds from Europe were introduced into New Zealand by acclimatisation societies in the late 1800s.
The information in this section is obtained from the reviews of Higgins (2001, 2002, 2006) unless otherwise stated.

1.5.1 Blackbird (*Turdus merula*)

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.

1.5.2 Song thrush (*Turdus philomelos*)

Like the blackbird, the song thrush was also introduced to New Zealand from Europe in the 1800s. The song thrush is member of the family Muscicapidae and the genus *Turdus*, being a close relative to the blackbird. Adult birds

show brown colour on the dorsal side of their body, with a complex facial pattern of dark brown and buff. Breast, flanks and sides of the belly are in cream to off-white colour, with black spots on the chest, both sexes are alike. The song thrush is slightly smaller than the blackbird, measuring around 22cm in length and around 70g in weight. The bird is widespread in New Zealand and commonly inhabits modified habitats, especially parks and gardens, but is also recorded in natural habitats like forests. Song thrushes forage on the ground, often together with blackbirds and starlings (*Sturnus vulgaris).* They take invertebrates, especially snails and earthworms.

1.6 Importance of endemic passerine birds in New Zealand

New Zealand's endemic nectar eating passerines such as the tui and the hihi, and to a lesser degree saddlebacks, fulfil an important role in native plant pollination and seed dispersal in New Zealand.

Island plants often depend on a small set of local pollinators and many of the birds act as such (Cox & Elmqvist 2000). Pollinators are keystone species in an ecosystem, and their population decline has serious consequences for the plant species they pollinate and other animals and plants living on, with or from these plants. For example, after the decline and extinction of a significant number of many bird species in Hawaii, 31 species of the *Campanulaceae* (the bellflower family) have become extinct due to a lack of pollinators (Cox & Elmqvist 2000). In New Zealand, the native mistletoes of the genera *Peraxilla* and *Alepis* have suffered population declines since European settlement, mainly attributable to habitat loss and introduced herbivores like the Australian brushtail possum (*Trichosuris vulpecula*) (Ladley & Kelly 1996). However, the endangered mistletoes of the genus *Peraxilla* are particularly affected as they depend principally on endemic birds that are able to open their flowers. These plants have suffered most of the historical declines in the North Island, due to reduced seed production through inadequate pollination (Murphy and Kelly 2001). In addition, Murphy and Kelly (2001) found that birds other than bellbird, such as the introduced blackbird and the silvereye, do not visit the flowers of the mistletoe. These plants need birds for seed dispersal, it is especially important the fruit skin

(exocarb) is removed by passage through a bird´s gut to enable germination (Ladley & Kelly 1996). *Alepis flavida, P. colensoi* and *P. tetrapetala* are now uncommon on the North Island, and the long term conservation of these plants will require conservation of the endemic honeyeater species tui and bellbirds (Ladley et al.1997) and the recovery of other honeyeating species such as hihi.

1.7 Disease screening for translocation in species recovery programmes

Several approaches for the conservation of endemic New Zealand avifauna have been used by the Department of Conservation over the last 50 years. These methods included reservation of land area for conservation, control and eradication of pests to create predator-free offshore islands and mainland areas that are either predator-free or under intensive pest-control, reintroduction of endangered species as well as threatened species management including captive breeding programmes (Craig et al. 2000). From 1960 to 2000, nearly 260 species transfers of at least 66 animal species have been documented (Craig et al. 2000). More recently, reintroductions and translocations have become the preferred methods in species recovery programmes (Cunningham 1996; Mathews 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).

However, reintroductions and translocations can pose a threat to the pre-existing avifauna through the introduction of pathogens and to the translocated birds through exposure to pathogens already present in the release area and its inhabitants. To avoid this problem, a pre-translocation health screening and surveillance programmes have been established with three main aims: 1. To help to protect the population already living at the target area from introduced pathogens, 2. To increase awareness of possible stress-induced risks to the animals that are to be moved and 3. To assess the risks to the founder group from pathogens endemic to the population in the target area (Cunningham 1996; Leighton 2002; Mathews 2006). Post mortem examination of individuals that die in captivity or shortly

after release to identify the cause of death are a valuable tool in determining the disease risks (Cunningham 1996).

A common problem with endangered species worldwide is the lack of information about pathogens that do or might affect them. Most of the data available to date concentrates on diseases that have possible zoonotic importance, posing threats to livestock or that are able to completely eradicate the population, but there is little knowledge of the parasite and pathogen diversity carried by a particular species in its usual environment (Mathews 2006). A study by Mathews (2006) emphasizes the importance and necessity of establishing baseline data and also highlights the necessity for ongoing surveillance of the translocated animals after their release.

According to (Cunningham 1996), animals housed in captivity could be at risk of parasites and other diseases that are foreign to the particular species, or its site of origin, its destination area or even a combination of above. This risk increases with the duration the animal is kept in captivity and the further away from its natural habitat that it is housed. In the case study presented by (Mathews 2006), it was suggested that affected animals should either be treated or not translocated to minimize threats to species recovery. Diseases of particular concern currently include avian malaria, coccidiosis, salmonellosis and aspergillosis (Alley, 2002).

1.8 Aims of this study

During 2008 and 2009, gastro-intestinal parasites from selected New Zealand endemic, native, and introduced avian species where examined. The primary objectives of this study were to:

1. Establish baseline data of gastro-intestinal parasite infections found in endemic, native and introduced birds from New Zealand.
2. Identify the species of coccidia infecting certain New Zealand passerines as well as create an overview of other gastrointestinal parasites in these birds

3. Develop recommendations for future screening programmes for translocations of endangered bird species in New Zealand.

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Chapter 2. Identification of the organisms causing coccidiosis in hihi (*Notiomystis cincta*) using molecular methods

(This chapter is intended for publication in the New Zealand Veterinary Journal)

Abstract

Coccidian parasites are generally host specific, obligate intracellular protozoan parasites and members of the phylum Apicomplexa. The phylogenetics of coccidian parasites is still in flux due to the advent of molecular techniques which produce different results to those of the more traditional morphometry-based taxonomy. Traditionally the most common Apicomplexans that infect passerine birds are members of the genus *Isospora*. Although coccidian gastrointestinal parasites are important pathogens, particularly for captive populations of native New Zealand songbird species, their prevalence, epidemiology, life cycles and taxonomic relationships are still widely unknown. Likewise, the number of coccidian parasite species infecting a native passerine bird and the possibility that these parasites might have been introduced by non-native passerines remains uncertain. Under natural conditions, coccidian parasites seldom pose a threat, but stressors such as quarantine for translocation, overcrowding or habitat changes may cause an infection outbreak that can severely affect wild native passerines.

Therefore, the purpose of this study was to examine the coccidian parasites infecting the New Zealand endangered hihi or stitchbird (*Notiomystis cincta*) with molecular methods to learn about their taxonomic relationships. PCR and sequencing on extracted DNA from oocysts shed by infected hihi from Mt. Bruce NWC supported the existence of at least two different coccidia species. Similar to prior studies in other countries, preliminary sequencing results of our samples suggest that NZ coccidia are closer related to the genus *Eimeria* than to the phenotypically similar genus *Isospora.* In addition, one liver tissue sample that was examined post mortem

was positive for at least two different coccidia species of the genus *Eimeria* according to sequencing results, and the presence of extraintestinal coccidian stages was proven.

Taken overall, the results of this study could be used to develop an effective PCR as a screening and management tool for health monitoring of hihi and other endangered New Zealand passerines. It also highlights the importance of developing an understanding of coccidian life-cycles, prevalence and taxonomic relationships in order to ensure the best outcomes for breeding and translocated populations.

2.1 Introduction

The hihi is a threatened, protected passerine bird endemic to New Zealand, which has recently been placed in its own family, Notiomystidae (Driskell et al. 2007). After being widespread throughout the North Island, by 1885 this species had completely vanished from New Zealand apart from a single population remaining on Hauturu (Little Barrier) Island. Suspected causes of the decline include a loss of suitable habitat, the introduction and spread of mammalian predators, and diseases that might have arrived with introduced European birds (Higgins 2001). Despite several translocations to other offshore and mainland islands that are free of introduced mammalian predators, hihi have not yet established self-sustaining populations (Taylor et al. 2005) and they survive through intensive management at translocation sites. A captive breeding programme (Taylor et al. 2005), has not been entirely successful. The programme consistently produces chicks, but aspergillosis and coccidiosis are major causes of mortality (Alley et al.1999, Alley 2002).

Previously, the main factor to consider for a translocation site in New Zealand was the absence of introduced predators, but recent research suggests that habitat at the release site should also be given careful consideration (Thorne 2007). The stress of translocation and release into a less than optimal habitat may create an environment for infection by opportunistic pathogens such as *Aspergillus* (Perrott 2001). High bird densities under such conditions will also increase infections with gastrointestinal parasites, particularly coccidia (Ritchie 1994, Harrison and Lightfoot 2006). Coccidia are known to cause serious disease in other wild birds in New Zealand. For example,

coccidiosis has been a cause of mortality in captive adult kiwi (Thompson 1978) as well as crèche-reared juvenile kiwi that are about to be released (Alley 2002).

The sites where translocated hihi have survived, such as Mokoia (Lake Rotoura, North Island) or Tiritiri Matangi (Hauraki Gulf, North Island) Islands, are not pristine habitats but have been re-established after being used by Maori and Europeans for farming during the 1800s and early 1900s. In addition, several of the translocated sites have a considerable level of ecotourism, which may also have an effect on avian inhabitants (Lindsay et al. 2008). These habitats contrast with those on Little Barrier Island which are more pristine. The young, regenerating forests of the translocation sites lack some important features necessary for hihi to complete their life cycle. For example, the hihi are obligate secondary cavity nesters and need older trees with cavities for successful breeding. As a result, the regenerating forests on Mokoia Island and Tiritiri Matangi Island have proven unsuitable for even their short term survival and have required the addition of nest boxes and the use of supplementary food to sustain populations (Castro 1991; Empson 1992; Castro 1993; Armstrong et al. 1999; Castro et al. 2003).

2.1.1 Coccidiosis in the hihi

Systemic coccidial disease has been found to be a serious problem in the captive hihi population at the National Wildlife Centre, where twelve juvenile hihi died of severe coccidian infection during a 5 year period (1994-99). A study by Twentyman (2001) revealed that adult hihi only shed coccidia sporadically, except in cases of poor health or an initial exposure, whereas, juvenile hihi had at least two periods of shedding, one at the nestling stage and one post fledging. These results indicate that coccidia infection is maintained at background level by the adults with oocyst numbers being boosted by the chicks and that oocysts persist in the environment for extended periods. In addition, as efforts to infect other passerines (starlings) with hihi coccidia were unsuccessful, it was suggested that coccidia were host specific (Twentyman 2001).

In a study to determine if disease was an important factor in translocation failures and captive breeding programmes, it was found that coccidiosis caused the death of

at least one hihi chick in 1999 (Cork et al. 1999). The pathological findings, in this case showed both intestinal and extraintestinal lesions. There was a massively distended intestine with extensive thickening of the intestinal wall, as well as numerous foci of necrosis scattered throughout the liver, and an enlarged spleen (Cork et al. 1999).

2.1.2 Study aims

Although coccidia are clearly important for captive and wild populations of hihi, their prevalence, epidemiology, life cycles and even taxonomic relationships are still largely unknown. The main aim of this study was to identify the species of coccidia affecting hihi to provide a basis for improving our understanding of the epidemiology, pathology and life cycle of the disease.

2.2 Material and methods

2.2.1 Sample collection

In this study, faecal samples from Mt. Bruce National Wildlife Centre (NWC) , located in Masterton, Wairarapa, North Island (lat. 42° 43' 0 S long. 171° 19' 60 E) were received by the parasitology laboratory in the Institute of Veterinary, Animal and Biomedical Sciences (IVABS) at Massey University between the years 2007 and 2009. At Mt. Bruce NWC, most samples were collected by staff during the morning feed rounds between 8 and 10 am, and either taken from the roost boxes or from a sheet of black polythene placed under the feed stations. Whenever possible, individual samples were collected, particularly from the adult birds which were often kept by themselves. In the case of nestlings, pooled samples consisting of 4-6 single droppings were collected. The initial faecal flotation and examination of the samples was undertaken by Barbara Adlington, senior technician in the laboratory. Between 23/11/2007 and 28/10/2009, 136 faecal samples from a population of six adults and pooled samples of eleven nests were submitted. In the laboratory, the samples were either processed within 24 hours or stored at 4°C until examination. Oocysts were concentrated using standard flotation procedures using 33% $ZnSO_4$, specific gravity

1.20 (Bowman 1995). Approximately 74% of 66 samples from adults and ~56% of pooled samples from chicks were positive for oocysts. Fifteen of the positive samples were made available to the author for molecular work, and these were frozen at -18°C until processing. Unfortunately the identity of the individual birds or pooled samples which yielded positive results were not recorded. Details of the history and management of hihi at Mt. Bruce over this period were provided by Darren Page, Department of Conservation aviculturalist. The author carried out a post mortem examination on a dead bird from Mt Bruce NWC, and tissue samples were taken for histology and molecular work. In addition, archived hihi tissues stored by IVABS were also used for DNA extraction in this study.

2.2.2 Pathology

One hihi from Mt Bruce NWC died over night in 2009 at the New Zealand Wildlife Health Centre and was received chilled. The bird was weighed and the body condition assessed and recorded using a 1-9 scale (Appendix 2). The bird was then examined using a standard necropsy technique (Appendix 3). Samples of pectoral muscle and all internal organs were collected for histology (fixed in 10% buffered formalin for a minimum of 48 hours) and both pectoral muscle and liver were freshly frozen for later PCR analysis. Intestinal contents were collected for parasitological examination.

Representative areas of all tissues and lesions were selected. While trimming, special attention was given to the presence of pathological changes in the tissues selected for blocking. The tissues were embedded in paraffin, and then cut at 4 µm before staining with Haematoxylin and Eosin and examination under a light microscope.

2.2.3 DNA extraction

DNA was extracted from fresh tissue and paraffin embedded stored tissues using the Qiagen DNeasy blood and tissue kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. DNA was also extracted from oocysts isolated from faecal samples using the Qiagen QIAamp DNA Stool Mini Kit (Qiagen, Düsseldorf,

Germany) according to the manufacturer's instructions with the following minor modifications. The frozen samples were thawed and the oocysts were concentrated by centrifugation at 150g for 5 minutes (W.E. Pomroy, pers. comm.). The supernatant was carefully removed leaving less than 0.5ml remaining in the microcentrifuge tube. In order to break open the walls of the oocysts, the samples were ground for 2 min with micropestles before the Qiagen Stool kit protocol was applied. As the Qiagen Stool kit was developed for mammalian faecal samples which have PCR inhibitors in the faeces different from those of birds (which contain uric acid as the main inhibitor), the InhibitEX tablet included in the stool kit was not used (S. Trewick, pers. comm.). All purified DNA samples were stored at -20ºC until used for PCR analysis.

2.2.4 PCR for the identification of passerine coccidian parasites

Previously published primer sets CoC1/CoC2, P3/P5 and CocciA/CocciB were chosen based on their successful use in other studies of coccidia isolated from passerine birds and chickens (Table 1) (Schnitzler et al.1998; Carreno & Barta 1999; Schrenzel et al., 2005). Avian host primers AH1/AH2 were also used to amplify a region of the mitochondrial small subunit 18S gene as a control for DNA extraction and quality (Table 1) (Schrenzel et al., 2005).

Table 1: Overview of the apicomplexan 18s ribosomal subunit primers used to amplify a section of the ribosomal subunit gene of coccidia in hihi

Target	Primer Name	Sequence	Product Size	Reference
Apicomplexan 18S	CoC1 CoC2	5'AAGTATAAGCTTTTATACGGCT-3' 5'CACTGCCACGGTAGTCCAATAC-3'	295bp	(Schrenzel, et al., 2005)
Avian host 18S	AH1 AH2	5'-CGCCTGTTTATCAAAAACAT-3' 5'-CCGGTCTGAACTCAGATCACGT-3'	720bp	(Schrenzel, et al., 2005)
Coccidia 18S	P3 P5	5'GGAAGCTTATCTGGTTGATCCTGCCAGTA-3' 5'GGGATCCTGATCCTTCCGCAGGTTCACCTC-3'	~1200bp	(Carreno & Barta, 1999)
Isospora robini 18S	CocciA CocciB	5'-CCAGGTCCAGACATGG-3' 5'-CTTGCGCCTACTAGGC-3'	~600bp	(Carreno & Barta, 1999)

The PCR reactions for the detection of coccidian DNA using primer sets Coc1/Coc2 or for the detection of avian host DNA using primer sets AH1/AH2 consisted of 25 µl reaction volumes consisting of 5-10 ng template DNA, 10X PCR Buffer, 2.5 mM $MgCl_2$, 0.4mM of each dNTP, 0.25 µM of each forward and reverse primer, and 1U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Water was added up to 25µl volume. The PCR reaction conditions were as follows: Initial denaturing at 95°C for 5 min followed by 40 cycles of 95°C for 45 sec, 54°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min.

The PCR reactions for the detection of parasite DNA using primer sets P3/P5 or CocciA/CocciB consisted of 50 µl reaction volumes consisting of 5-10 ng template DNA, 10x PCR Buffer, 1.5 mM $MgCl_2$, 0.2mM of each dNTP, 0.5 µM of each primer, and 1U of Platinum Taq DNA polymerase (Invitrogen). Water was added up to 50 µl volume. The PCR reaction conditions were as follows: Initial denaturing at 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min with a final extension at 72°C for 10 min. All reactions included a positive control consisting of *Neospora* DNA derived from cell culture at IVABS, Massey University provided by Dr. L. Howe, that showed a strongly positive reaction when used with the primers to identify coccidia mentioned above. In all reactions, a negative control was also used, consisting of a blank sample containing only sterile autoclaved Millipore water.

To confirm successful product amplification, 10µl of each PCR product was run on a 1.5% agarose gel (Invitrogen) containing ethidium bromide, with the exception of the products run with the P3/P5 primer set which were run on a 0.5% agarose gel (Invitrogen). A 100bp ladder was used to measure amplicon size (Promega Corporation, Madison, Wisconsin). The gels were run at 100V for 45 minutes and visualized under UV light and photographed

2.2.5 Sequencing and phylogenetic analysis

All coccidia positive PCR amplicon samples were purified using PureLink PCR purification kit (Invitrogen) and subjected to automatic dye-terminator cycle sequencing by BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing

kit and the ABI3730 Genetic Analyser (Applied Biosystems Inc., Foster City, California, USA) at the sequencing facility of the Allan Wilson Centre/ Massey University/ Palmerston North as well as at the facility of the University of Waikato/ Hamilton to confirm genomic sequence. The original amplification primers were used in the sequencing process.

Coccidia sequences of approximately 100-300 base pairs, depending on primer sets, obtained from the hihi were compared to those of other published sequences available from Genbank, using BLAST (Basic Local Alignment Search Tool) available from the NCBI database. Alignments were performed using Clustal W with gaps ignored (Higgins et al. 1994) and a phylogenetic tree generated using a Jukes-Cantor distance model and neighbour joining method in Geneious™ (Biomatters, Auckland, New Zealand), Bootstrap values were generated from 1000 cycles. The sequence divergence between and within the different lineages was calculated using a Jukes-Cantor model of substitution implemented in the program PAUP* 4.0 Beta version 10 (Swofford 2002).

2.3 Results

2.3.1 Pathology

The dead adult hihi from Mt Bruce NWC was in poor body condition of two out of nine and showed moderate distension of the small intestine up to a diameter of 6mm. Histopathology revealed multiple foci of proliferating coccidian gametocytes in the mucosal epithelium of the small intestine. Schizonts and released merozoites were also present in the lamina propria (Figure 5). Two apparently morphologically different types of micro- and macrogametes as well as schizonts could be identified. One type was smaller than the other, and also stained more darkly basophilic than the second type which had larger more eosinophilic stages. Severe thickening of the lamina propria by infiltrating macrophages was also present in these regions (Figure 5). Extraintesinal coccidial schizonts were also found in the liver (Figure 6 and 7), in both hepatocytes and Kupffer cells, and in the spleen. The extraintestinal stages of coccidia were associated with foci of necrosis (Figure 6).

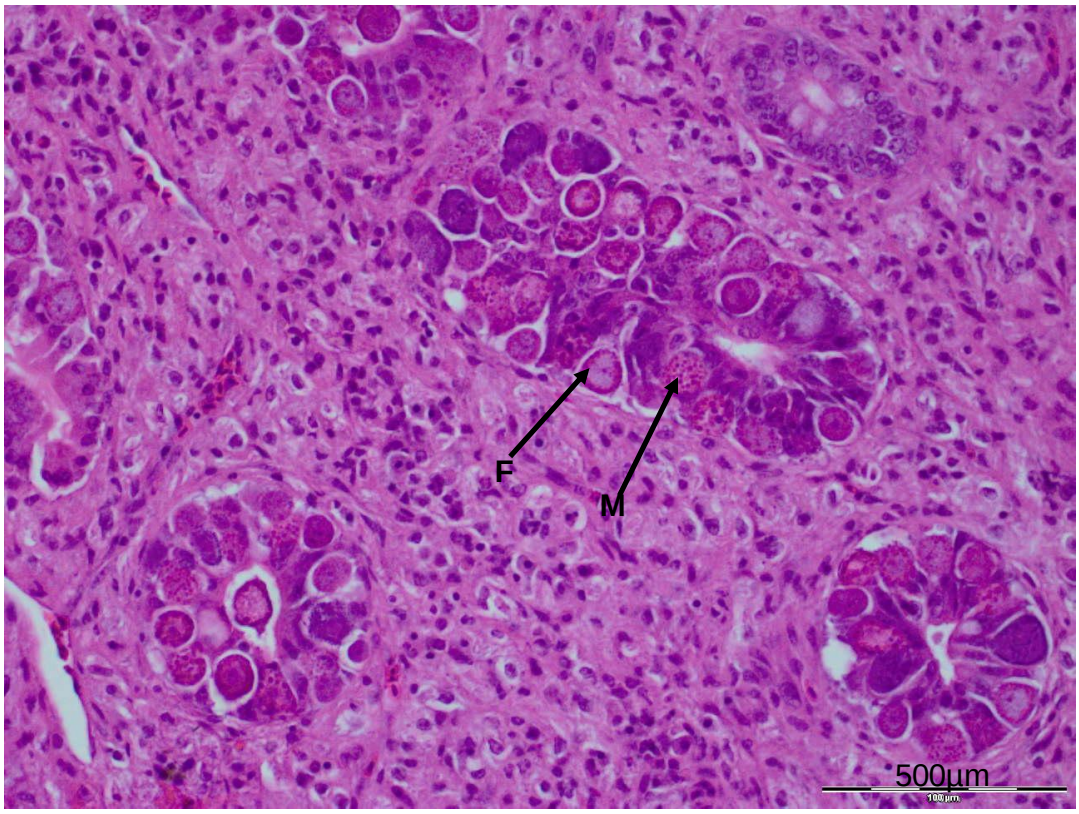

Figure 5: Photomicrograph of the duodenum of a hihi showing sexual stages (F- macrogamete, M-microgamete) of coccidia in the epithelial cells. H&E (400x). Photograph taken by the author.

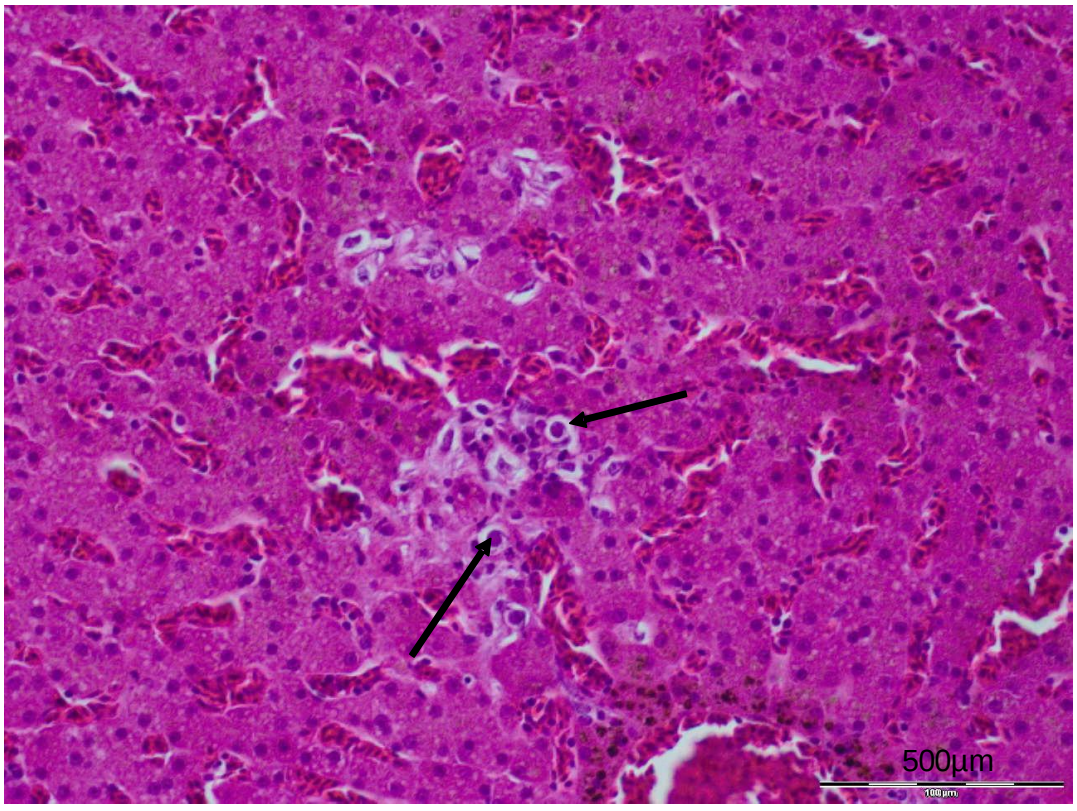

Figure 6: Photomicrograph of the liver of a hihi showing a focus of necrosis associated with structures resembling coccidia schizonts (arrows) H&E (400x). Photograph taken by the author.

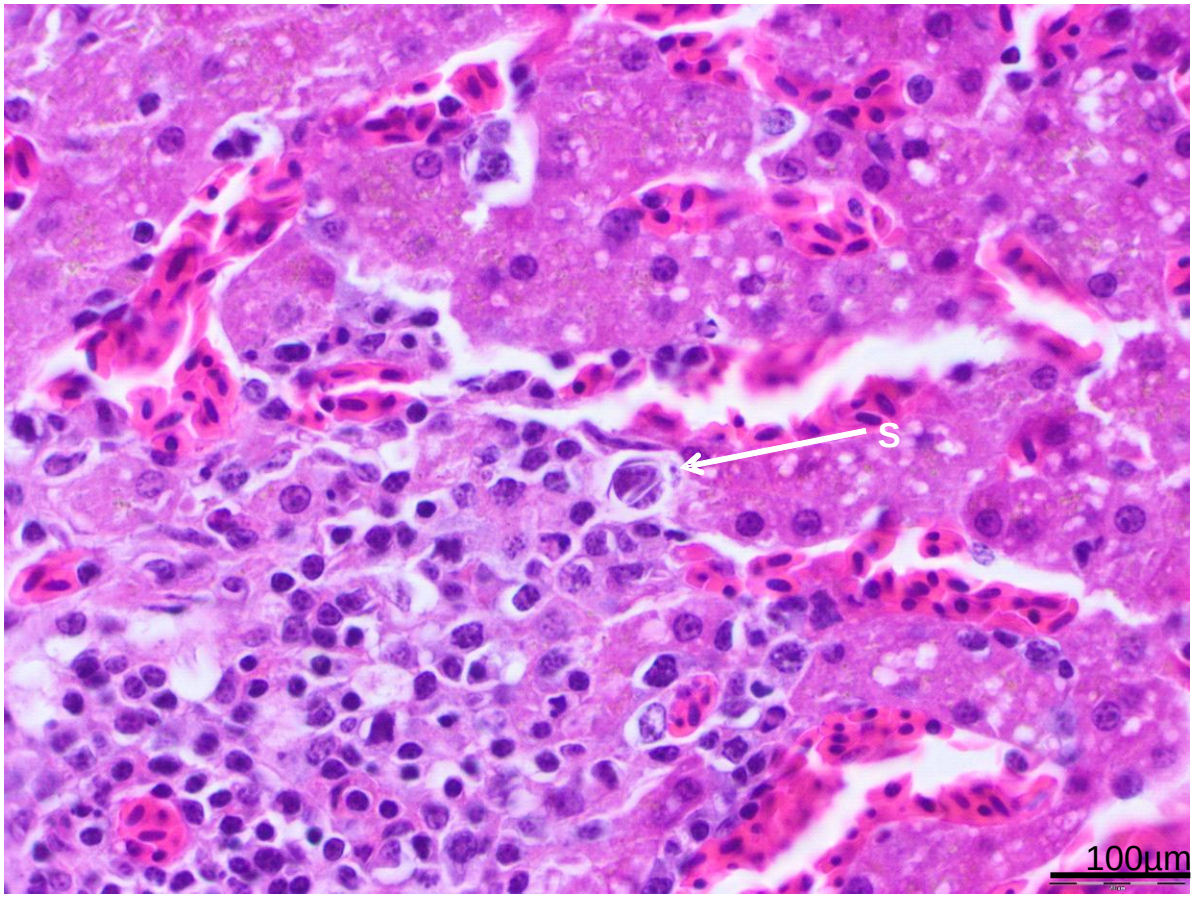

Figure 7: Photomicrograph of the liver of a hihi showing intra cytoplasmic structures resembling schizonts (S) at the periphery of an inflammatory focus. H&E (400x). Photograph taken by the author.

2.3.2 Sequencing and phylogenetic analysis

Coccidial DNA from oocysts was successfully amplified by PCR, using the primers COC1 and COC2, from 4 of the 15 (26.7%) faecal samples. Of these four samples, designated “Hihi1”, “Hihi2”, “Hihi new” and “Hihi Waikato”, three (20%), namely “Hihi2” (177 base pairs), “Hihi Waikato” (108bp) and “Hihi new” (60bp), could be sequenced successfully. The sequence of the sample designated “Hihi new” was too short with only 60 base pairs and also from a highly conserved region of the 18S ribosomal subunit gene and therefore did not provide useful information or present any new findings, and so was not included in the results below. In addition, the liver tissue sample that was taken from the hihi from Mt Bruce NWC examined post mortem was positive for at least two different coccidial species of the genus *Eimeria* according to sequencing results, and the presence of extraintestinal coccidial stages (presumably schizonts) was proven.

The results of the NCBI BLAST search and phylogenetic analysis also suggested the presence of two different species of coccidia in the hihi. The “Hihi Waikato” sequence had 100% sequence identity with other members of the *Eimeria* genus, including coccidia from rodents (e.g. *Eimeria leucopi* , GenBank AF339491*, E. arizonensis ,* GenBank AF307878*, E. peromysci ,* GenBank AF339492) as well as an *Isospora robini* isolate from a North American robin (GenBank AF080612) (Figure 8). In contrast, the isolate “Hihi2” did not closely relate to other members of the genus as the results of the BLAST search revealed only 94% similarity to an environmental *Eimeria* clone (GenBank- EU044776) as well as a 93% similarity to different *Eimeria acervulina* and *E. tenella* spp in poultry (GenBank EF210324, EF210323, EF210326). As a result, the “Hihi2” isolate was placed on a unique location among the *Eimeria* genus on the phylogentic tree (Fig.8). The divergence analysis revealed that the “Hihi2” isolate had a sequence divergence of 48% when compared to the isolate “Hihi Waikato”, supporting the existence of at least two different coccidial species.

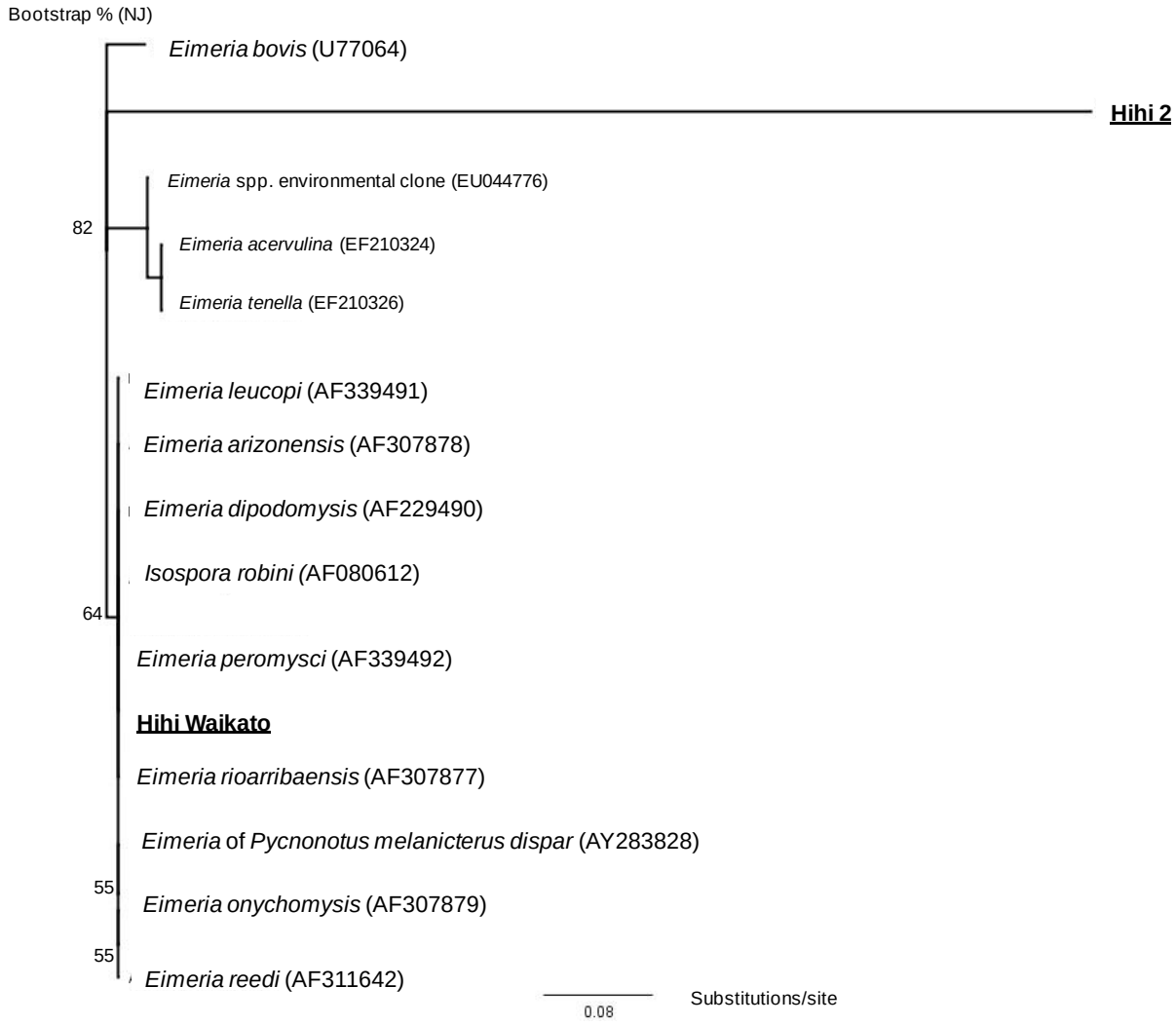

Figure 8: Phylogenetic analysis of *Eimeria spp*. isolated from hihi from Mt Bruce NWC. Neighbour joining phylogeny of the 18S small subunit ribosomal gene. The tree is rooted using *Eimeria bovis*. Numbers above the branches indicate bootstrap support based on 1000 replicates. Names of the lineages (when available) and Genbank accession numbers of the sequences are given after the species names of the parasites.

2.4 Discussion

This investigation has genetically identified possible species of the coccidia affecting hihi. The results have documented the phylogenetic relationship between coccidian species infecting the hihi for the first time, and shown that in addition to the presence of an eimeriid species, a new species of eimeriid coccidia was identified. This discovery is not unexpected and supports the findings of Twentyman (2001) who postulated the presence of more than one coccidial organism in hihi based on oocyst morphology. Despite these results, these findings have to be regarded with caution, because in the short region of the 18S gene amplified with the COC1/2 primers,

there is not enough information to draw firm conclusions (Barta, J., pers. com.). The experiments described in this thesis were an initial research to determine possible genera of coccidia involved for providing a backbone to create further primer sets. Additional primer sets will have to be developed to amplify further regions of the 18S gene what would provide more specific phylogenetic identification.

Studies in chickens have revealed a variety of different species of coccidia infecting a single host species, and even a single individual (Augustine 1996, 1999).Additional studies in canaries (*Serinus canarius*) (Box 1977), greenfinches (*Carduelis chloris*) (Cringoli & Quesada 1991; Ball et al. 1998) and other passerines (Barta et al. 2005; Dolnik et al. 2009) have also shown at least two different species of coccidia infecting a host. The current findings are also in agreement with those of Dolnik et al. (2009) who suggested that mixed infections with different coccidia haplotypes are rather common in the wild. Moreover, Dolnik et al. (2009) found the simultaneous presence of oocysts belonging to four different haplotypes in faeces of free-ranging blackcap (*Sylvia atricapilla)* (GeneBank accession numbers FJ269357-59 and -62). These lineages showed a 92% similarity with *Eimeria* spp.in chickens (*Gallus gallus*) when compared with sequences in GeneBank (Dolnik, et al. 2009). Taken together, the results suggest a high diversity of coccidia species present in passerine birds. It is therefore possible that these parasite lineages possibly interact and compete for resources in the host.

The current results together with the observations of Twentyman (2001) who examined sporulated coccidia of hihi and found they displayed a Stieda body in their sporocysts in most cases, support Carreno and Barta's (1999) classification of coccidial parasites. It placed species with tetrasporozoic, diplosporocystic oocysts possessing Stieda bodies in their sporocysts, as found in birds, into the genus *Isospora*, family Eimeriidae (Carreno & Barta 1999). Whereas, tetrasporozoic, diplosporocystic oocysts without Stieda bodies in their sporocysts, as found in mammals, should be placed into the genus *Cystoisospora*, family Sarcocystidae, as described by Carreno and Barta (1999). Previously, the term *Atoxoplasma* was used to describe coccidia with obligate blood stages. After examining oocysts from the genus "*Atoxoplasma*", associated with passerines, Barta et al. (2005) suggested that

"*Atoxoplasma*" can be used as a synonym for the genus "*Isospora*" in birds, due to their genetic similarity

Unfortunately, identification of the extraintestinal coccidia present in the liver of the hihi examined in this study did not produce conclusive results because there were several species of coccidia present. The development and use of specifically designed primers for each species are necessary to sequence single species. Therefore, further molecular examinations, such as gene cloning, will have to be done to discriminate between the extra intestinal species present in these birds.

The results of this study and future studies on the life cycle of extraintestinal coccidia will help better understand the life cycle of coccidia in hihi and other passerine birds in addition to providing a basis for developing improved methods for diagnosis, treatment and management of affected birds. This is of particular importance when trying to identify infected birds prior to translocations. Birds with extraintestinal stages of coccidia cannot be identified with certainty because the parasite may not be found in faecal samples. Furthermore, birds undergoing extraintestinal infections may not be treated with usual orally administered drugs, such as Toltrazuril, successfully. This complicates the interpretation of disease screening results at the time of translocation and as a result the unidentified infected birds may be translocated and released. It is also possible that infected hihi carry these parasites for several months to a year. For example, greenfinches can carry coccidia for 80 days (Ball et al. 1998) and in chronically infected canaries for at least 231 days (Box 1977) and are able to shed oocysts at anytime during that period, infecting other birds at the translocation sites. Thus translocated birds that were negative on faecal examination pre-release might begin shedding oocysts post-release, indicating that faecal examination alone is not a reliable determinant of infective status. In the future it may also be useful to examine hihi blood for the presence of coccidial extraintestinal stages with molecular methods. For example, Dolnik et al. (2009) demonstrated that at least some haplotypes of *Isospora* spp found in their research had extraintestinal stages in the blood which did not appear as oocysts in the faeces until much later. The ability to screen hihi blood could therefore provide a management method to diagnose coccidiosis in birds that are not currently shedding oocysts in their faeces.

Taken overall, the results of this study highlight the importance of developing an understanding of coccidial life-cycles, prevalence and taxonomic relationships in order to ensure the best outcomes for health monitoring of breeding and translocated populations.

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Chapter 3. Coccidia species in endemic, native, and introduced New Zealand passerine birds

(This chapter is intended for publication in the Journal of Wildlife Diseases)

Abstract

The relationship between host and parasite is ancient and like any other birds in the world, New Zealand native passerine birds are hosts to a whole variety of gastrointestinal parasites, in particular coccidia. Coccidian parasites are generally host specific, obligate intracellular protozoan parasites and members of the phylum Apicomplexa. The phylogenetics of coccidian parasites are still in flux due to the advent of molecular techniques which produces different results to those of the more traditional morphometry-based taxonomy. Traditionally the most common Apicomplexans that affect passerine birds are members of the genus *Isospora*. Although coccidian gastrointestinal parasites are important pathogens, particularly for captive populations of native New Zealand songbird species, their prevalence, epidemiology, life cycles and taxonomic relationships are still widely unknown. Likewise, the number of coccidian parasite species infecting a native passerine bird and the possibility that these parasites might have been introduced by non- native passerines remains uncertain. Under natural conditions, these parasites seldom pose a threat, but stressors such as quarantine for translocation, overcrowding or habitat changes may cause an infection outbreak that can severely affect wild native passerines.

Therefore, the purpose of this study was to examine the coccidian parasites affecting the New Zealand native and introduced passerines. We examined 345 faecal samples of six native (tui, North Island saddleback, North Island robin, silvereye and fantail) and one non native (blackbird) passerine species. The overall prevalence of coccidian infection in the New Zealand bird species examined was 21- 38%; 21% in North Island robin, 38% in tui and 25% in saddleback. The sample size of blackbirds was too small to establish an accurate prevalence.

Using molecular methods, at least 7 new genetically distinct coccidia species in the examined birds were identified, with three distinct sequences obtained from tui, one each from saddleback and North Island robin, as well as two from blackbirds. Similar to prior studies in other countries, preliminary sequencing results of our samples suggest that NZ coccidia are more closely related to the genus *Eimeria* than to the phenotypically similar genus *Isospora.* The results of these studies will be valuable in guiding management options for the host species and in providing baseline data for health monitoring.

3.1 Introduction

Endangered endemic New Zealand passerine species only survive today on off-shore and mainland islands that are free of introduced mammalian predators where surviving individuals have been translocated. In many cases these environments are no longer natural, pristine habitats as they have been used for forestry and farming by both Maori and Europeans over the last 1000 years. As a result, these environments are highly disturbed and contain young, regenerating forest which might not be the ideal habitat for the translocated native birds. In addition, several conservation islands, such as Tiritiri Matangi, are ecotourism sites which may also have a notable effect on the resident and translocated bird populations (Lindsay et al. 2008).

Although low levels of coccidia are common and harmless in most bird species (Ritchie et al. 1994, Jakob-Hoff 2001), serious infections are known to cause serious disease in several captive endemic birds in New Zealand. For example, coccidia have caused mortality in captive (Thompson 1978) as well as crèche-reared juvenile kiwi that were about to be released (Alley 2002). Systemic coccidial disease has been found to be a serious problem in the captive hihi population at the Mt. Bruce National Wildlife Centre (Masterton, Wairarapa, North Island) (Twentyman 2001). During a two-year study Twentyman found that 100% of the nestlings suffered from it with five young hihi dying. In 2002, there was a disease outbreak in South Island saddleback (*Philesturnus c. carunculatus*) that wiped out more than 50% of the population on Motuara Island. Coccidiosis together with avian malaria and avipox

infection were implicated in these mortalities. This outbreak was associated with a high population density after the carrying capacity of the island was reached (Hale 2009). Reasons for these mortalities are likely to be twofold: Firstly, any level of captivity poses a form of stress on the birds and secondly, the birds are often confined to small areas and kept in larger groups than in the wild, which increases the chances of ingesting large numbers of coccidial oocysts. In addition, the stress of translocation and release in a less than optimal habitat, as well as exposure to opportunistic pathogens, such as *Aspergillus,* which prefer highly disturbed habitats (Perrott 2001), may predispose immunologically challenged birds to infection with gastrointestinal parasites, such as coccidia. Taken together, all these factors may, in certain circumstances be more than enough to endanger the reintroduction success of susceptible passerine species.

Wild populations of introduced bird species in New Zealand seem to show a similar high prevalence of coccidiosis to that described in other countries. Recent studies described a 40% prevalence of coccidia among clutches of song thrushes (*Turdus philomelos*), blackbirds (*Turdus merula*) and starlings (*Sturnus vulgaris*) in New Zealand, with no detectable negative influence on the juvenile birds, according to the measured parameters of nestling body mass, tarsus length and plasma carotenoid concentration (Cassey and John 2008).

3.1.1Study aims

Although coccidiosis may in some circumstances be an important cause of mortality in endemic New Zealand passerines, the prevalence, epidemiology, life cycles and even taxonomic relationships of the organisms involved are still largely unknown. In this study, an attempt is made to establish baseline data on coccidian parasites found in endemic, native and introduced birds in New Zealand. This knowledge will help to develop screening programmes for translocations of endemic bird species in New Zealand and provide a greater understanding of the effects of parasitism and on population dynamics.

3.2 Material and methods

3.2.1 Study sites

3.2.1.1 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 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 the maori introduced pacific rats, kiore (*Rattus exulans*) and dogs (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 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).

3.2.1.2 Other sample sources

While Mokoia was the sampling site for most native birds, dead specimens of introduced birds such as blackbirds, as well as not endangered endemic birds such as tui, were obtained from other sites in the North Island. The main source for dead birds, especially introduced ones, was Palmerston North, Manawatu (40° 21' S, 175° 37' E). One tui was received from near Masterton in the Wairarapa (40° 57' S, 175° 39' E). One faecal sample as well as one dead saddleback were received from a captive population from Orana Park in Christchurch (43°28′ S, 172°27′ E) that is derived from the Mokoia population. Seven dead tui were received from Raoul Island

(29.27°S 177.92°W), the largest island in the Kermadec group approximately 750-1000km north-north-east of New Zealand.

3.2.2 Capture, handling and faecal sampling

Seven trips were made for capturing and sampling of birds on Mokoia Island, in January 2007, May 2007, October 2007, March 2008, November 2008, December 2008 and February 2009. Birds were caught in mist nets set up next to the tracks on Mokoia Island as well as at the only two streams occurring on the island (Figure 9). North Island saddlebacks and robins were attracted to the net with the help of recorded songs of their own species. Other species were caught by placing the mist nets at sites frequented by the birds to increase the chances of capture. For example tui and saddleback were known to frequent streams in search of bathing opportunities and mist nets were placed there, as well as across flight paths towards feeding trees used at the time by tui. Blackbirds were caught by placing nets close to the ground and across narrow stretches of the bush tracks. Care was taken to make sure the net was always under tension and not entangled in vegetation. The bags for restraining the birds were used only once on each side and washed 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 bird had the following measurements and information taken: weight, tarsus length, wing length, body condition, presence of a brood patch in females and wattle size in saddlebacks. 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 was estimated according to the method described by Melville in the “New Zealand bird bander’s manual, DOCDM-285890” (In press) (Pectoral muscle scores (0-3); (Melville 2007). Initially the pectoral muscle scores only were used, with the modification that scores were recorded on a scale of 1-4. On later trips to Mokoia, a fat score which measures the amount of fat in the thoracic inlet of the bird, was also used to assess body condition score, using a scale of 0 to 4 (Melville 2007).

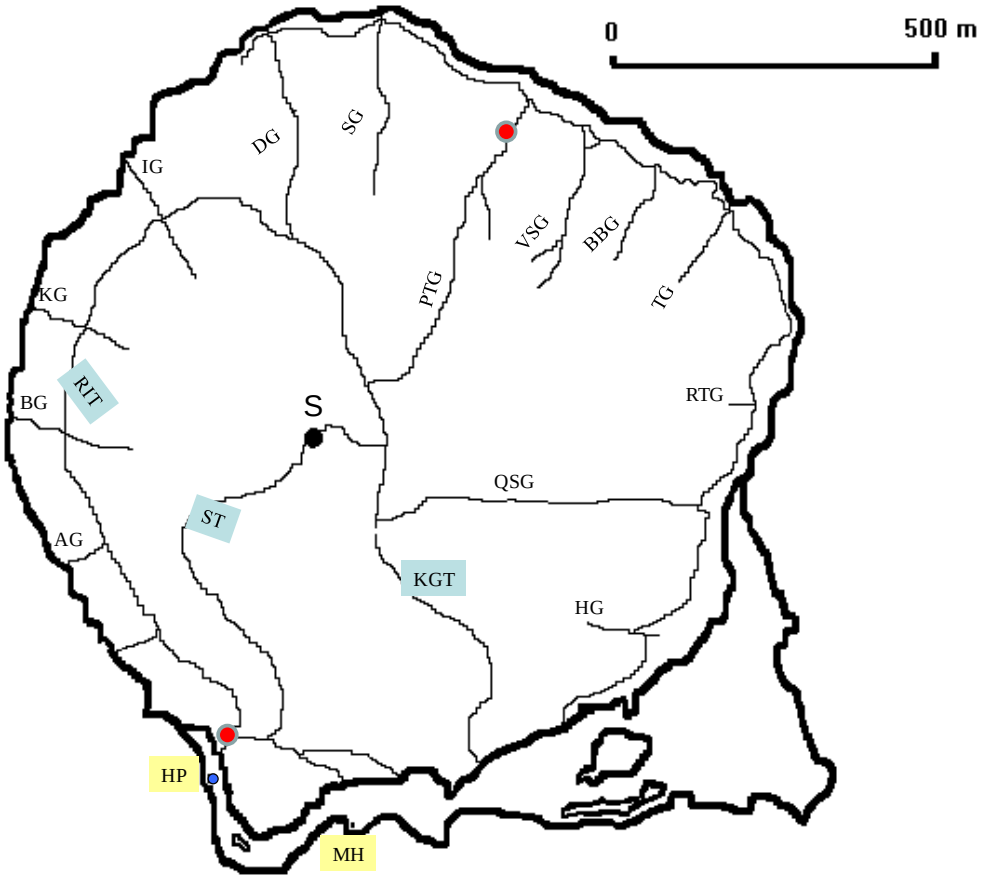

Figure 9: Map of Mokoia Island showing the **main tracks** (Blue labels: RIT = Round the Island track; ST = Summit track; KGT = Kumara God track); **focal sites** (cream labels (HP = Hinemoa's hot pool; and MH = Maori hut); **gullies** (AG = A gully; AG = A gully; BG = B gully; KG = K gulley; IG = I gulley; DG = D gulley; SG = Steep gulley; PTG = Pine tree gulley; VSG = Victoria Street gulley; BBG = Blackberry gulley; TG = Trough gulley; RTG = Rat tube gulley; QSG = Queen Street gulley; HG = Hidden Gulley); and the **summit (S)** (black dot). The two red coloured dots mark the sites of the two natural streams of the island.

Faecal samples for the different species were collected as follows: saddleback and robin faecal samples were obtained from the holding bags in which the birds were restrained as they frequently defecated in the bag. This was less common with tui so they were released from the mist net and placed inside a cardboard box (measuring 31x25x22cm) containing a perch, for 10 minutes after being released from the mist net. This was enough time for the birds to defecate. Aluminium foil was placed in the bottom of each box to facilitate the collection of the faecal sample. The foil was changed after each bird. Faecal samples from all species were collected in 1.5ml microcentrifuge tubes and refrigerated at 4ºC until examination in the laboratory.

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

3.2.3 Flotation, microscopic examination and oocyst-count

The collected faecal samples were either examined within 24 hours or stored at 4°C until examination. Cooling the samples or processing them as quickly as possible is standard procedure to minimise developmental changes that may affect identification. Faecal samples were placed into a sieve and weighed using a digital scale. Then the sieve was placed over a metal bowl and rinsed with ZnSO4 to wash the small materials including parasite's eggs. The contents of the bowl were then transferred into a glass test tube. The tube was filled to the rim with flotation solution (saturated $ZnSO_4$ solution at a specific density of 1.32) until a positive meniscus was achieved (modified after Bowman 1995). A square microscope cover slip was applied with care to avoid including any air bubbles. The glass tube and cover slip were centrifuged for 5 minutes at 300g, after which the cover slip was removed and placed on a previously labelled microscope slide that was then examined at 100x magnification. After examination, the coccidia were washed off the slide into a 1.5ml microcentrifuge tube and frozen for later genetic identification. In the case of blackbirds, the abundance of positive samples made it possible to collect single oocysts for PCR, because the process of collecting single oocysts carries a high risk of losing the oocyst in the process. Single oocysts were identified on the slide under 100x magnification, PBS was added to the slide to allow the extraction of the oocyst using 100µl pipette, and transferred into a 1.5 ml plastic tube. For native birds a more conservative approach was used and groups of 10-20 oocysts were collected. In cases where a sample contained fewer than 10 oocysts, the sample was not used for PCR.

For sporulation, a small amount of faeces that was positive for coccidia on microscopy, was placed into a 2ml Eppendorf tube with a drop of 2% H_2SO_4 to prevent bacterial overgrowth and left at room temperature for a week. Twice a day the tube was opened to allow oxygen into the tube. After a week, the sample was checked for successful sporulation and the oocysts were measured and photographed. The measurements were made using calibrated ocular micrometer

scales at 400x magnification. With few exceptions, at least 20 oocysts were measured from each type of oocyst from each bird species.

3.2.4 Pathology

Each dead bird was weighed and the body condition score was assessed and recorded using a 1-9 scale (Appendix 2). The bird was then examined using a standard necropsy technique (Appendix 3). Samples of pectoral muscle and all internal organs were fixed in 10% buffered formalin for histology and both pectoral muscle and liver were frozen for later PCR analysis if required. The cranium was opened with sharp scissors to expose the brain to formalin. Collected tissues were fixed for a minimum of 48 hours before trimming and blocking.

Intestinal contents were collected for parasitological examination. Any adult internal parasites found were fixed in 70% ethanol.

While trimming representative areas of all tissues any visible lesions were selected for processing. The fixed tissues were embedded in paraffin, and then cut at 4 µm before routine staining with haematoxylin-and-eosin for examination under the microscope.

3.2.5 DNA extraction

DNA was extracted from individual oocysts from blackbirds and from groups of 10-20 oocysts in the other birds, samples using the Qiagen QIAamp DNA Stool Mini Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions with the following minor modifications. The frozen samples were thawed and the oocysts were concentrated by centrifugation at 150g for 5 minutes. The supernatant was carefully removed leaving less than 0.5ml in the microcentrifuge tube. In order to break open the walls of the oocysts, the samples were then ground for 2 minutes with micropestules, before the Qiagen stool kit protocol was applied. As the Qiagen stool kit was developed for mammalian faecal samples which have different PCR inhibitors in the stool from those seen in birds in which uric acid is the main inhibitor. The InhibitEX tablet included in the kit was not used (Trewick, S., pers. comm.). All extracted DNA samples were stored at -20ºC until used for PCR analysis.

3.2.6 PCR for the identification of passerine coccidian parasites

Previously published primer sets CoC1/CoC2, BSEF/BSER, P3/P5 and CocciA/CocciB were chosen based on their successful use in other studies of coccidia isolated from passerine birds and chickens (Table 2) (Schnitzler et al. 1998; Carreno & Barta 1999; Schrenzel et al. 2005). Avian host primers AH1/AH2 were also used to amplify a region of the mitochondrial small subunit 18S gene as a control for DNA extraction and quality (Table 2) (Schrenzel et al. 2005).

Table 2: Overview of the apicomplexan 18s ribosomal subunit primers used to amplify a section of the ribosomal subunit gene of coccidia in New Zealand passerines

Target	Primer Name	Sequence	Size of Product	Reference
Apicomplexan 18S	CoC1 CoC2	5'AAGTATAAGCTTTTATACGGCT-3' 5'CACTGCCACGGTAGTCCAATAC-3'	295bp	(Schrenzel et al., 2005)
Eimeria ITS1 (18S)	BSEF BSER	5'CTGTGAATTCATCGGA-3' 5'ATCGCATTTCGCTGCGTCCT-3'	~600bp	(Schnitzler, Thebo, Mattsson, Tomley, & Shirley, 1998)
Avian Host 18S	AH1 AH2	5'-CGCCTGTTTATCAAAAACAT-3' 5'-CCGGTCTGAACTCAGATCACGT-3'	720bp	(Schrenzel, et al., 2005)
Coccidia 18S	P3 P5	5'GGAAGCTTATCTGGTTGATCCTGCCAGTA-3' 5'GGGATCCTGATCCTTCCGCAGGTTCACCTC-3'	~1200bp	(Carreno & Barta, 1999)
Isospora robini 18S	CocciA CocciB	5'-CCAGGTCCAGACATGG-3' 5'-CTTGCGCCTACTAGGC-3'	~600bp	(Carreno & Barta, 1999)

The PCR reactions for the detection of parasitic DNA using primer sets Coc1/Coc2 or for the detection of avian host DNA using primer sets AH1/AH2 consisted of 25 µl reaction volumes consisting of 5-10 ng template DNA, 10X PCR Buffer, 2.5 mM $MgCl_2$, 0.4mM of each dNTP, 0.25 µM of each forward and reverse primer, and 1U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA. Sterile autoclaved millipore water was added up to 25µl volume. The PCR reaction conditions were as follows: Initial denaturing at 95°C for 5 min by 40 cycles of 95°C for 45 sec, 54°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min.

BSEF and BSER are an *Eimeria* genus specific primer set (Schnitzler et al. 1998). For this, the PCR reactions consisted of 25 µl reaction volumes containing 5-10 ng template DNA, 10X PCR Buffer, 1 mM $MgCl_2$, 0.1mM of each dNTP, 0.2 µM of each primer, and 1U of Platinum Taq DNA polymerase (Invitrogen). Water was added up to 25 µl volume. The PCR reaction conditions were as follows: Initial denaturing at 95°C for 5 min followed by 35 cycles of 95°C for 50 sec, 50°C for 40 sec, and 72°C for 1 min with a final extension at 72°C for 10 min.

The PCR reactions for the detection of parasitic DNA using primer sets P3/P5 or CocciA/CocciB consisted of 50 µl reaction volumes consisting of 5-10 ng template DNA, 10X PCR Buffer, 1.5 mM $MgCl_2$, 0.2mM of each dNTP 0.5 µM of each primer, and 1U of Platinum Taq DNA polymerase (Invitrogen). Water was added up to 50 µl volume. The PCR reaction conditions were as follows: Initial denaturing at 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min with a final extension at 72°C for 10 min. All reactions included a positive control consisting of *Neospora* DNA derived from cell culture at IVABS, Massey University provided by Dr. L. Howe. Controls showed a strongly positive reaction when used with the primers to identify coccidia mentioned above. In all reactions, a negative control was also used, consisting of a blank sample only containing sterile autoclaved Millipore water.

To confirm successful product amplification, 10µl of each PCR product was run on a 1.5% agarose gel (Invitrogen) containing ethidium bromide, with the exception of the products run with the P3/P5 primer set which were run on a 0.5% agarose gel (Invitrogen). A 100bp ladder was used to measure amplicon size (Promega

Corporation, Madison, Wisconsin). The gels were run at 100V for 45 minutes and visualized under UV light and photographed

3.2.7 Sequencing and phylogenetic analysis

All coccidia positive PCR amplicon samples were purified using PureLink PCR purification kit (Invitrogen) and subjected to automatic dye-terminator cycle sequencing by BigDye^{TM} Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyser (Applied Biosystems Inc., Foster City, California, USA) at the sequencing facility of the Allan Wilson Centre/ Massey University/ Palmerston North as well as at the facility of the University of Waikato/ Hamilton to confirm genomic sequence. The original amplification primers were used in the sequencing process.

Analysis of coccidia sequences of approximately 100-300 base pairs depending on primer sets, obtained from the saddlebacks, tuis, robins and blackbirds were compared to those of other published sequences available from Genbank. Alignments were performed using Clustal W with gaps ignored (Higgins et al., 1994) and a phylogenetic tree generated using a Jukes-Cantor distance model and neighbour joining method in Geneious^{TM} (Biomatters, Auckland, New Zealand), Bootstrap values were generated from 1000 cycles. The sequence divergence between and within the different lineages was calculated using a Jukes-Cantor model of substitution implemented in the program PAUP* 4.0 Beta version 10 (Swofford 2002).

3.2.8 Statistics

A Kendall correlation (Kendall 1938) was used to evaluate the relationship between faecal oocyst counts and date of sampling trip, weight and body condition. The relationship was explored using first data for the individual trips and then grouping the data according to season. Seasons were defined as: summer (Dec, Jan, Feb), autumn (Mar, Apr, May), and spring (Sep, Oct, Nov). Data for oocyst counts was not normally distributed and a Box-Cox transformation was carried out. The resulting transformed data was normally distributed (Anderson- Darling normality test A^2 =

0.562; p-value = 0.140). Body condition was estimated by dividing the weight of the birds (g) by the tarsus length (calculated as an average of three measurements).to control for body size. Weight and body condition were normally distributed (Anderson- Darling normality test $A^2 = 0.970$; $p = 0.218$ and $A^2 = 0.193$; $p = 0.890$ respectively). Other studies of Coccidia have shown that oocyst shedding occurs in the afternoon. I did not record the time of faecal collection for my samples and therefore negative samples may represent false negatives. For this reason, only samples positive for coccidia were included in the analysis. A single bird was caught twice in the same trip and for this bird I used the average of the oocyst counts.

For all the tests and analyses described in this study, the program WinSTAT® for Microsoft Excel version 2007.1, copyright 2007 Robert K. Fitch, was used.

3.3 Results

3.3.1 Faecal flotation and microscopy

The results of the faecal examination of 345 samples from New Zealand passerines including 6 native and 3 introduced species are shown in Table 3. North Island saddleback and North Island robins had similar rates of coccidian infection 21% and 25% respectively while tui had and a higher rate of 38%.

Table 3: Results of the examination of 345 faecal samples of New Zealand passerines for *Coccidia*

Family	Species	Common Name (E,N,I)	Sampling site	Number sampled	Coccidia positive by:		Coccidia sequences obtained
					Flotation	PCR	
Callaeidae	*Philesturnus carunculatus*	Saddleback (E)	Mokoia	233	58/233 (24.9%)	7/ 47 (14.9 %)	1/7
Meliphagidae	*Prosthemadera novaeseelandiae*	Tui	Mokoia	42	16/42 (38.1%)	7/15(46.67%)	3
			Mainland	7			
			Raoul Island	7			
Eopsaltriidae	*Petroica australis longipes*	NI Robin (E)	Mokoia	38	8/38 (21%)	2/3	1/2
Monarchidae	*Rhipidura fuliginosa*	Fantail	Mainland	1	1/1		
Zosteropidae	*Zosterops lateralis*	Silvereye	Mainland	2	2/2		
Muscicapidae	*Turdus merula*	Blackbird	Mokoia	3	1/3		2
			Mainland	8	3/8	2/3	
	Turdus Philomenos	Song thrush	Mainland	1			
Passeridae	*Passer domesticus*	House Sparrow	Mainland	2			
Cuculidae	*Chrysococcyx lucidas*	Shining cuckoo	Mainland	1			
Total				**245**	**89**	**18**	**7**

3.3.2 Data and Statistics of sampling on Mokoia

The data of the North Island saddleback on Mokoia Island was used for statistical analysis (Table 4).

Table 4: Descriptive statistics of the data of North Island saddleback in 7 trips to Mokoia Island. n= number of birds, body condition evaluated by division weight/average tarsus length

Variable	n	Minimum	Maximum	Mean	Std. deviation
Trip	51	1.000	7.000		
body cond.					
Wt/avts	51	1.347	2.037	1.668	0.139
coccidia	51	2.708	11.479	6.351	2.080
Wt Bird	51	60.000	90.000	76.099	6.872

There was a significant correlation between trips and oocyst count (Kendall test coefficient of determination (KCC) = 0.092; p=0.004) with higher counts in 2007 and lower in 2009 regardless of season (Figure10).

When data was examined seasonally, there was a significant effect of season on oocyst shedding with higher counts in autumn when compared to summer and spring (KCC = -0.166; p = 0.043).
Correlations between oocyst counts and weight and body condition were not significant (KCC = 0.001, p = 0.788 and KCC =- 0.014, p = 0.208 respectively).

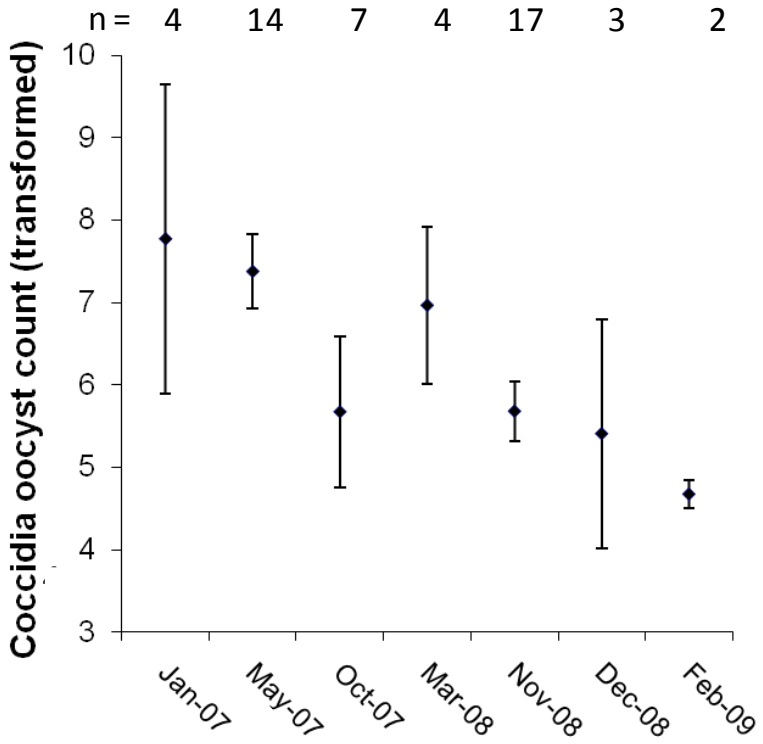

Figure 10: Oocyst faecal counts in saddleback from Mokoia compared to time of year sampled. n= number of examined samples per trip. Bars showing the standard error and the means are included.

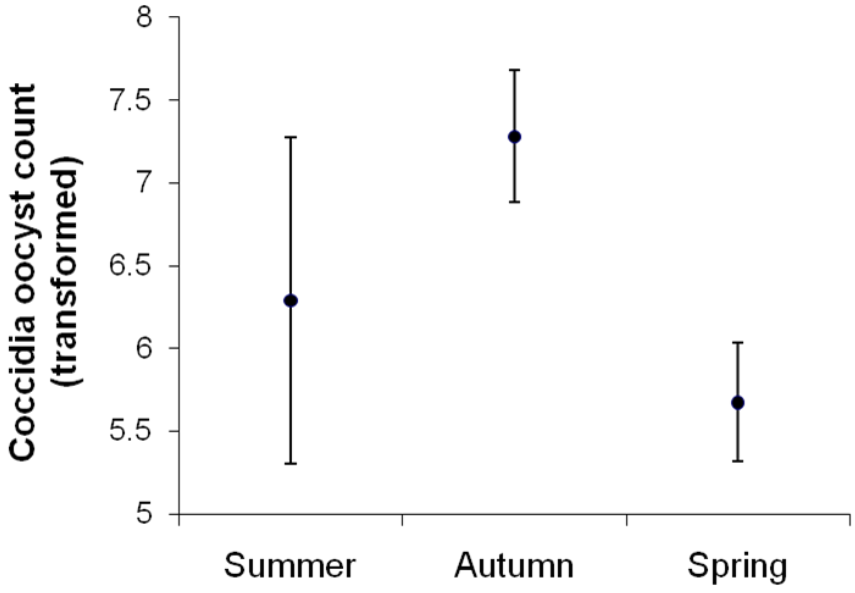

Figure 11: Oocyst faecal counts in saddleback from Mokoia compared to the season of sampling. Bars showing the standard error and the means are included.

3.3.3 Additional findings on a tui from Mokoia

In the study presented here, one juvenile tui captured twice in the period of one week in March 2008 was found to have a high faecal count of 72339 oocysts per gram faeces. Although not weighed the second time so as not to cause additional handling stress, its body condition score was only 1 as opposed to 3 at the first time it was caught.

3.3.4 Oocyst morphology

Oocysts recovered from faecal samples obtained from both silvereyes and North Island robins showed a consistent spherical morphology (Table 5), whereas those from the other birds included more than one morphological type. Tui samples contained subspherical and ellipsoidal oocysts (Figure 12). Saddleback from Mokoia also had subspherical and ellipsoidal oocysts (Table 5). Ellipsoidal oocysts were also encountered in fantail, while blackbirds had subspherical oocysts (Figure 13). In several individual birds, oocysts with different shapes and sizes were found, indicating current infection with different species of coccidia (Table 6). Oocysts from all species were sporulated successfully, except from North Island robin. Therefore all measurements and observations on robin oocysts were performed only on unsporulated oocysts.

After sporulation, the saddleback, tui, silvereye and blackbird oocysts resembled *Isospora*- type oocysts, containing two 2 sporocysts and each sporocyst contained four 4 sporozoites (Figure 13). However, the sporulated oocysts collected from fantail were similar to *Caryospora*- type oocysts, containing 1 sporocyst with up to 8 sporozoites. Additionally, as is typical for oocysts of the family Eimeriidae, all the sporocysts also had Stieda bodies (Figure 14).

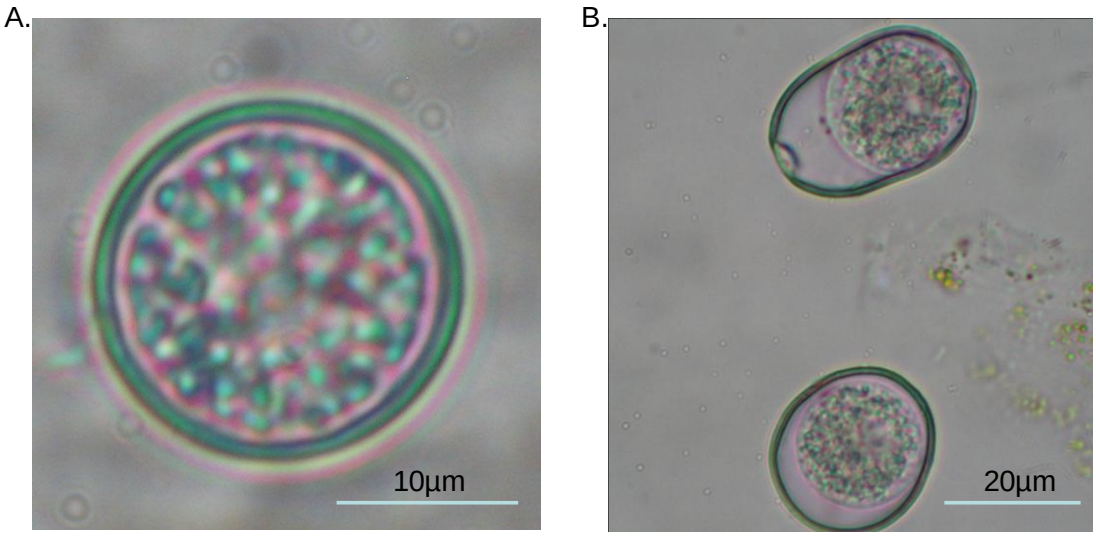

Figure 12: Unsporulated oocysts from A. North Island robin B. Tui; Note the two different types (ellipsoidal and subspherical). Both birds were sampled on Mokoia Island (400x). Photographs taken by the author.

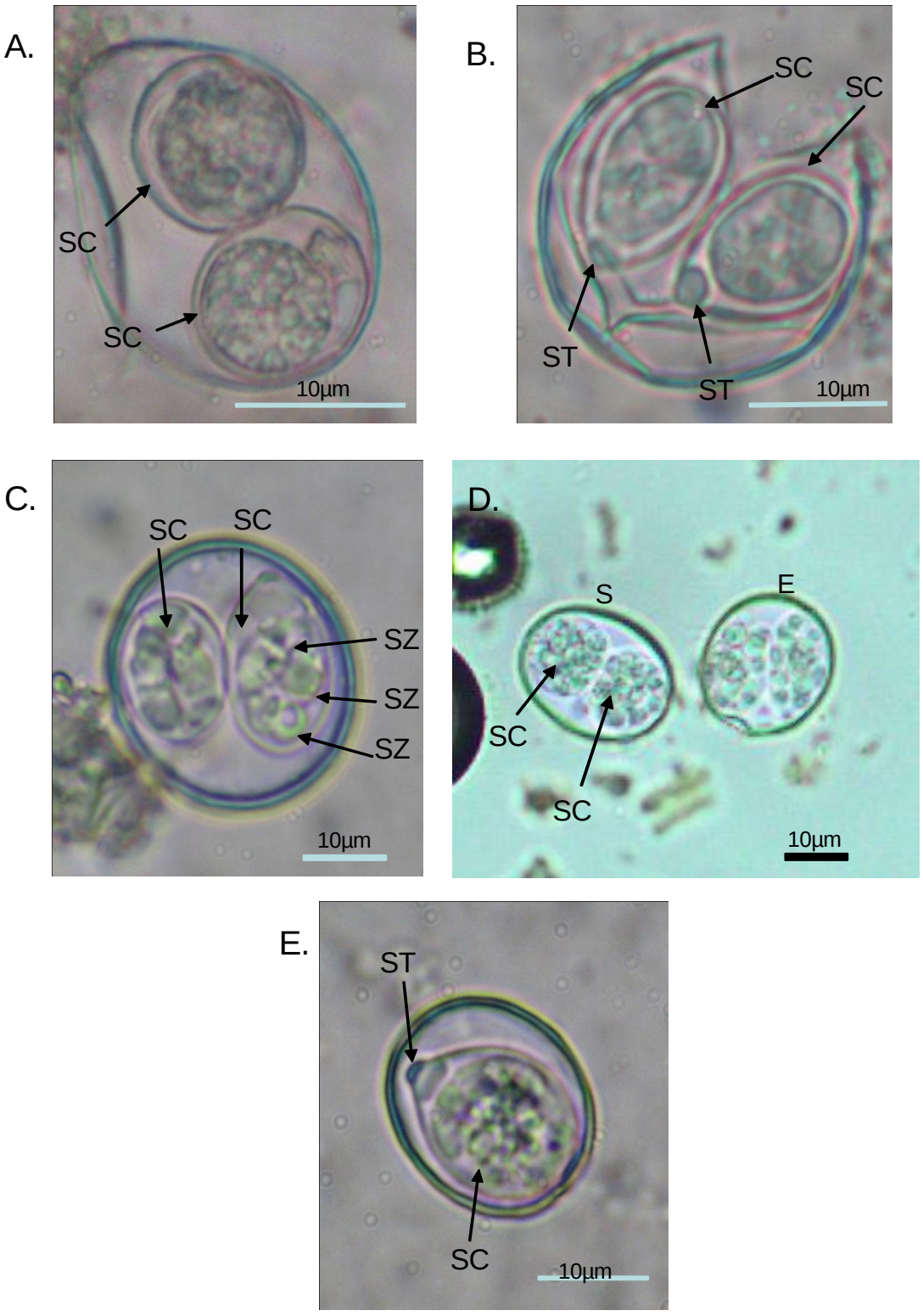

Figure 13: Sporulated oocysts obtained from A; Domestic cat (for comparison). Note the absence of Stieda bodies on the sporocysts as typical of mammalian *Isospora spp.* of the family Sarcocystidae. B. Silvereye (partly ruptured oocyst wall). C. North Island saddleback. D. Tui, two oocysts with immature subspherical (S) and ellipsoid (E) sporocysts. E. Fantail. 400x SC- Sporocyst SZ- Sporozoite ST- Stieda body. Photographs taken by the author.

	North Island saddleback (SS)			Tui (E)			North Island robin (S)		
	Length	Width	L/W ratio	Length	Width	L/W ratio	Length	Width	L/W ratio
N	20	20	20	26	26	26	16	16	16
Mean	19.73	18	1.11	28.65	23.69	1.22	17.81	17.66	1.01
SD	1.83	2.24	0.13	2.67	2.54	0.15	1.33	1.20	0.04
SE Mean	0.41	0.5	0.03	0.52	0.50	0.03	0.33	0.30	0.01
Min	15	15	1	20	16	1	15	15	1
Max	25	20	1.33	30	25	1.56	20	20	1.14

	Fantail (E)			Blackbird (SS)			Silvereye (S)
	Length	Width	L/W ratio	Length	Width	L/ W ratio	Diameter
N	21	21	21	18	18	18	14
Mean	19.64	17.32	1.14	19.03	17.22	1.15	27.14
SD	1.06	0.72	0.10	1.25	1.90	0.10	1.14
SE Mean	0.23	0.16	0.02	0.30	0.45	0.03	0.31
Min	17.5	15	1	17.5	15	1	25
Max	21.25	18.75	1.42	20	20	1.3	28.75

Table 5: Descriptive statistics of unsporulated oocysts measured in µm. (E) ellipsoidal, (S) spherical, (SS) subspherical

	North Island Saddleback (SS)			North Island Saddleback (E)			Silvereye (S)	Tui (with spherical sporocysts)		
	Length	Width	L/W ratio	Length	Width	L/W ratio	Diameter	Length	Width	L/W ratio
N	12	12	12	12	12	12	9	11	11	11
Mean	24.79	22.5	1.12	19.17	15.83	1.21	28.19	25.69	22.5	1.14
SD	1.29	3.02	0.20	1.23	1.23	0.09	1.55	1.52	1.85	0.06
SE Mean	0.37	0.87	0.06	0.36	0.36	0.03	0.52	0.46	0.56	0.02
Min	22.5	15	1	17.5	15	1.14	25	22.5	17.5	1.1
Max	27.5	25	1.67	20	17.5	1.33	30	27.5	25	1.29

	Tui (with ellipsoidal sporocysts)		
	Length	Width	L/W ratio
N	11	11	11
Mean	27.05	23.07	1.18
SD	1.16	2.12	0.11
SE Mean	0.35	0.64	0.03
Min	25	20	1.05
Max	28.75	26.25	1.44

Table 6: Descriptive statistics of the Spherical (S) and Ellipsoidal (E) and subspherical (SS) sporulated oocysts, measurements in µm

3.3.5 Sporocyst morphology

The sporocysts of blackbirds and silvereyes were elliptical in shape, sporocysts in the saddleback and tui showed elliptical as well as subspherical sporocysts (Table 6, Figure 13).

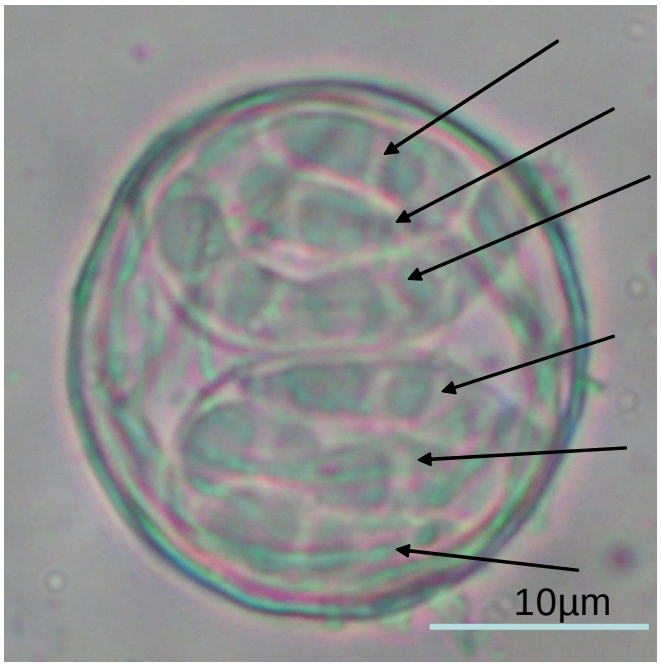

Figure 14: Sporulated oocyst from a silvereye from Massey campus/ Manawatu. Note the sporozoites in the sporocysts (arrows). (400x). Photographs taken by the author.

3.3.6 Pathology

Thirty-two birds were examined post mortem, including six blackbirds, 14 tui and five saddleback, two song thrushes, two silvereyes, two sparrows and one shining cuckoo (*Chrysococcyx lucidas*).

Four cases examined at necropsy presented with a severely dilated gallbladder associated with a trematode infection (three saddlebacks and a song thrush). No gross abnormalities due to gastrointestinal parasites were observed in the remaining birds, but on histology, one saddleback and one tui, both from Mokoia Island, had a moderate number of sexual coccidial stages in the duodenum, causing no apparent inflammatory reaction (Fig. 15 and 16)

	North Island saddleback			North Island saddleback			Tui (spherical sporocysts)		
	Length	Width	L/W ratio	Length	Width	L/W ratio	Length	Width	L/W ratio
N	20	20	20	22	22	22	22	22	22
Mean	15.75	10.38	1.53	8.75	7.05	1.26	13.92	11.25	1.25
SD	2.00	1.22	0.19	1.50	0.99	0.22	1.24	1.16	0.16
SE Mean	0.45	0.27	0.04	0.32	0.21	0.05	0.26	0.25	0.04
Min	12.5	7.5	1.2	7.5	5	1	12.5	10	1
Max	20	12.5	3	12.5	7.5	1.67	15	12.5	1.5

	Tui (ellipsoidal sporocysts)			Blackbird			Silvereye		
	Length	Width	L/W ratio	Length	Width	L/W ratio	Length	Width	L/W ratio
N	22	22	22	27	27	28	20	20	20
Mean	18.24	10.45	1.76	13.33	8.89	1.51	18	11.56	1.57
SD	1.37	1.13	0.24	2.08	1.27	0.28	1.69	1.14	0.20
SE Mean	0.29	0.24	0.05	0.40	0.24	0.05	0.38	0.25	0.04
Min	15	7.5	1.4	10	7.5	1	15	10	1.33
Max	20	12.5	2.33	15	10	2	20	12.5	2

	Fantail		
	Length	Width	L/W ratio
N	21	21	21
Mean	16.78	12.44	1.35
SD	1.51	0.74	0.13
SE Mean	0.33	0.16	0.03
Min	12.5	10	1
Max	18.75	13.75	1.56

Table 7: Descriptive statistics of the Sporocysts, measurements in µm

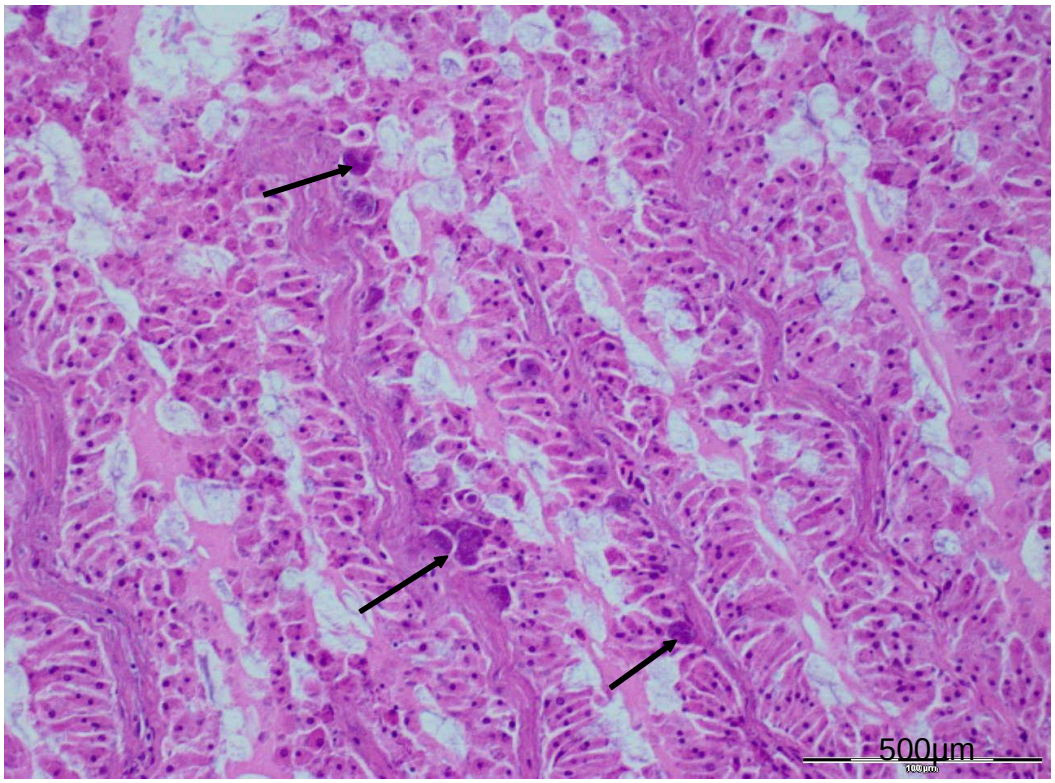

Figure 15: Sexual stages of coccidia in the duodenal epithelium of a tui (arrows) H&E (400x). Photograph taken by the author.

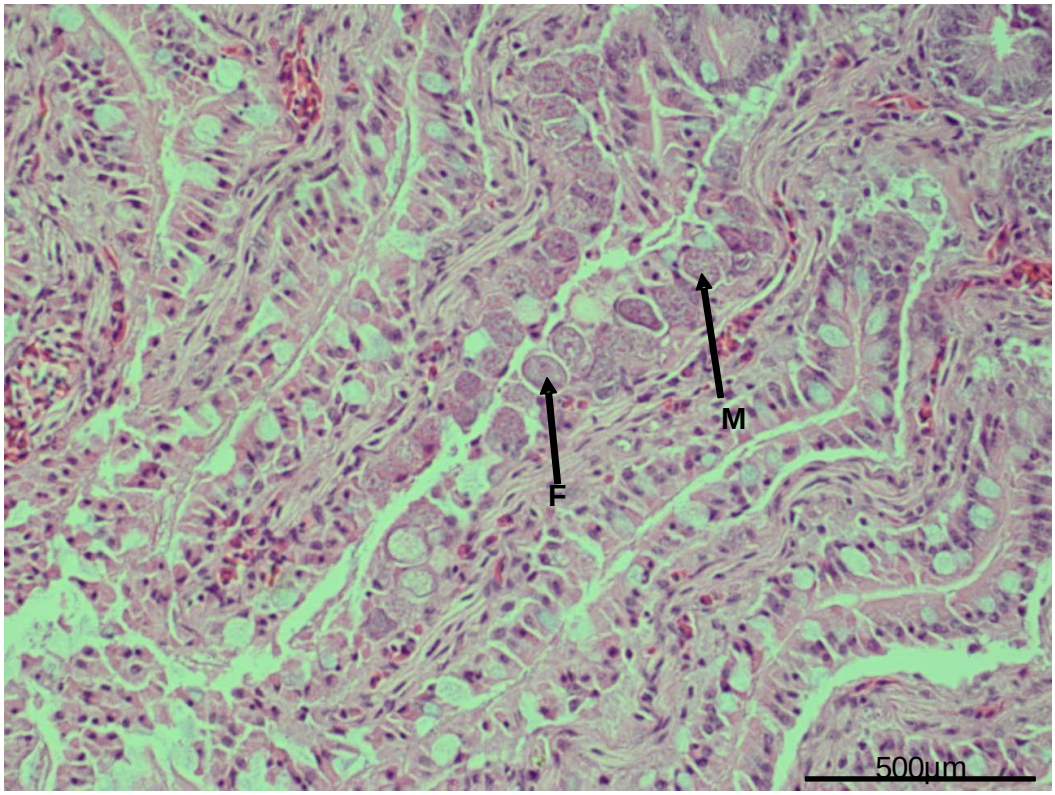

Figure 16: Sexual stages of coccidia in the duodenal epithelium of a blackbird from Mokoia Island H&E (400x). F- macrogamete, M- microgamete. Photograph taken by the author.

3.3.7 Sequencing and phylogenetics

3.3.7.1 COC1 and COC2 primer sets

Individual or groups of around 10-20 coccidia ooysts DNA were amplified by PCR, using the primers COC1 and COC2 from 7 of the 15 (46.67%) tui faecal samples that were positive for coccidia on flotation. Of these 7 positive samples, 3 (20%) could be sequenced with conclusive results and were subjected to a BLAST search on the NCBI database. The results revealed that one isolate, “Tui 1” (223 base pairs), had 99% sequence identity to different *Eimeria* spp in bats (GenBank AF307876) and rodents (GenBank AF307879, AF307878) as well as 98% sequence identity to *Isospora* and *Atoxoplasma* spp. in passerine birds of North America (GenBank AF080612) and Asia (GenBank AY283829, AY283828). The second isolate, “Tui 4” (205bp), had 94% similarity to *Eimeria* spp. in bats (GenBank AF307876) as well as a 93% similarity to *Isospora* and *Atoxoplasma* spp. in North America (GenBank AF080612) and Asia (GenBank AY283829, AY283828).

A multiple alignment was performed using Clustal W on 205bp of the 18S small subunit ribosomal gene of the two coccidia isolates “Tui1” and “Tui4” and other *Eimeria* species available from GenBank. A phylogenetic tree was constructed using the neighbour joining method which resulted in the isolate “Tui 1” clustering with *Eimeria* from rodents from North America (GenBank AF307879, AF307878) as well as passerines from North America (GenBank AF080612) and Asia (GenBank AY283829, AY283828) (Fig.20) as suggested by the BLAST search results. Tui 1 had a sequence divergence with other sequences, it clustered with of 0.95% to 1.4%, so they were relatively closely related. However, the sequence from the “Tui 4” isolate was placed on a unique location among the *Eimeria* genus (Figure 17). As a result, ”Tui 4” appears to have no direct genetic relationship with other published coccidia species found so far in passerines as well as chickens and other birds worldwide. The divergence analysis showed a sequence divergence of 5.6% between the isolates “Tui 1” and “Tui 4”. Tui 4 also showed a sequence divergence of 5%- 6.2% to the other sequences Tui 1

clustered with. This suggests that two distinct species of *Eimeria* are present in tui.

Unfortunately, the CocciA/B primers amplify a different region of the 18S gene from the Cocci1/2 primer set. Therefore, a direct comparison between the two below mentioned Tui coccidia, “Tui1” and “Tui4”, and “Tui Waikato” cannot be made.

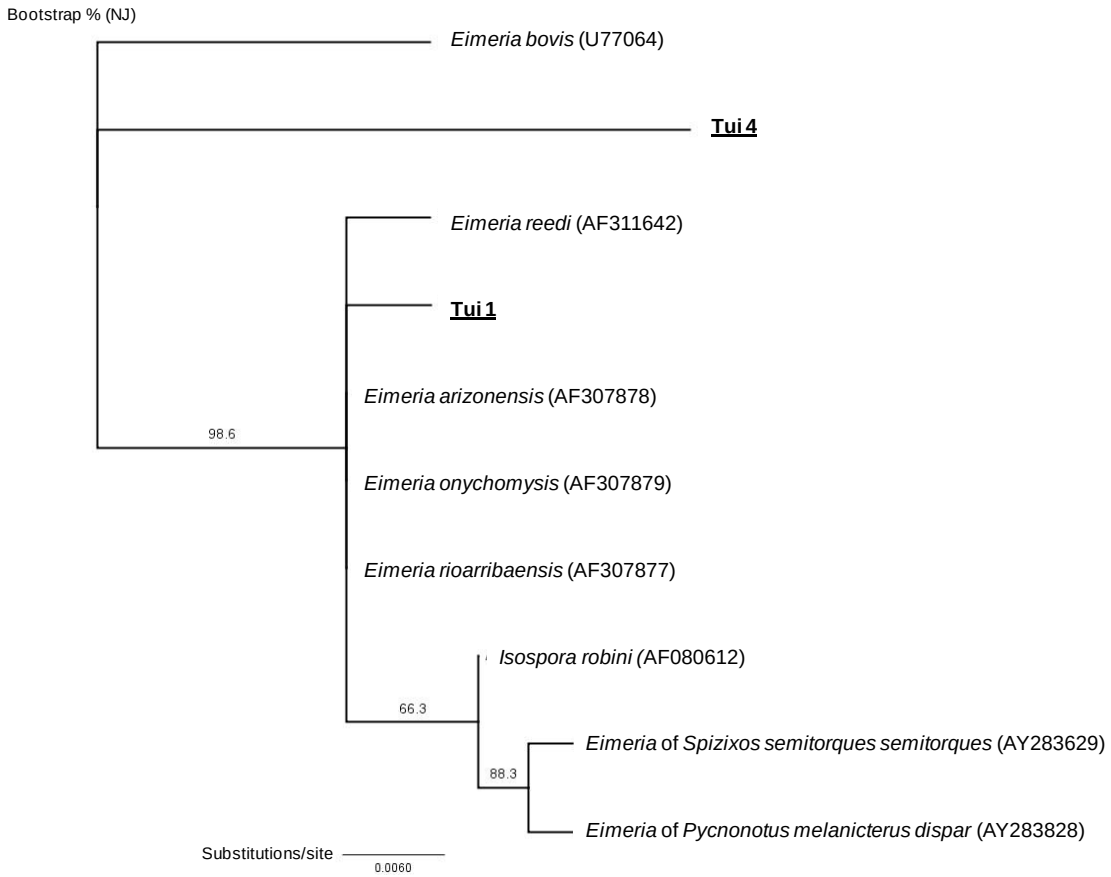

Figure 17: Phylogenetic analysis of *Eimeria spp*. isolated from **tui** from Mokoia Island using the primers COC1/2. Neighbour joining phylogeny of the 18S small subunit ribosomal gene. The tree is rooted on a linage of *Eimeria bovis*. Numbers above the branches indicate bootstrap support based on 1000 replicates. Names of the lineages (when available) and GenBank accession numbers of the sequences are given after the species names of the parasites.

3.3.7.2 CocciA and CocciB primer sets

The limited amount of sample material did not allow PCR using these primers on many samples, as most material was already used with the COC1/2 primers. However, coccidia DNA was successfully amplified with PCR from 1/1 saddleback, 1/1 tui and 2/2 (100%) blackbird faecal samples using the primers CocciA and CocciB. The coccidia isolates’ “NI saddleback 44” (378bp), “Tui Waikato” (260bp), “BB Waikato” (227bp), and “BBP3”

(314bp) sequences were subjected to a BLAST search on the NCBI database. The results revealed that the coccidia isolated from saddleback and tui were most closely related to coccidia of the *Eimeria* genus in North American rodents with 97% similarity (e.g.GeneBank AF 339489, AF 313642) and North American passerines with 96% similarity (e.g. GeneBank AF 080612).

However, a BLAST search on the NCBI database showed the closest related coccidia to the blackbird sample “BB Waikato” was an unidentified environmental sample *Eimeria* clone (Genebank number BF024854) with 96% genetic similarity. The BLAST search on the second blackbird sample P3 showed an identity of 97% to an Eimeriidae environmental sample clone EF024854.1 as well as a 97% identity to an Orchitophryidae environmental sample clone FE024426.1 and a 93% identity to a *Monocystis agilis* AF213515.

A multiple alignment was performed using Clustal W on 260bp of the 18S small subunit ribosomal gene of the four New Zealand coccidia isolates and some other *Eimeria* species available from the GenBank database. A phylogenetic tree was constructed which clustered the sequence of the “NI saddleback 44” isolate with the “Tui Waikato” isolate (Figure 18). Both the “NI saddleback 44” and “Tui Waikato” isolates clustered on a separate branch from the *Eimeria* species from rodents (e.g.GenBank AF324214, AF339491), chickens (GenBank EU025116) and North American robin (GenBank AF080612) (Figure 18). Sequence divergence analysis revealed a difference of 1.9% between the “Tui Waikato” and “NI saddleback 44” isolates. This suggests that although there is some limited sequence variation, the coccidia from tui and saddleback, both sampled on Mokoia Island, are quite similar. The divergence analysis also showed that the tui and saddleback isolates had a 2.8% and 3.8% divergence, respectively, from *Eimeria* in North American rodents (*Eimeria reedi,* GenBank AF311642) as well as a 4.3% and 4.8% divergence, respectively, to *Isospora robini* (GenBank AF080612) isolated from a North American robin. In addition, there was a large

sequence divergence of 63% with an *Atoxoplasma* species isolated from Northern house sparrows (GenBank AY331573).

The blackbird P3 sample had a sequence divergence of 68.1% to the Tui Waikato sample as well as a divergence of 67.6% to the saddleback sample, the divergence to the blackbird Waikato sample was 67%. The two blackbird samples also cluster on different clades of the tree, showing that they might be distinctly different species. While the BB Waikato sample is closest related to an uncultured eimeriid environmental clone EF024854, with a sequence divergence of 2.4%, the blackbird P3 sample shows a 68% divergence to the *Atoxoplasma* sp. AY331573 it clusters closest with on the tree, showing that it is quite different to the other species found so far.

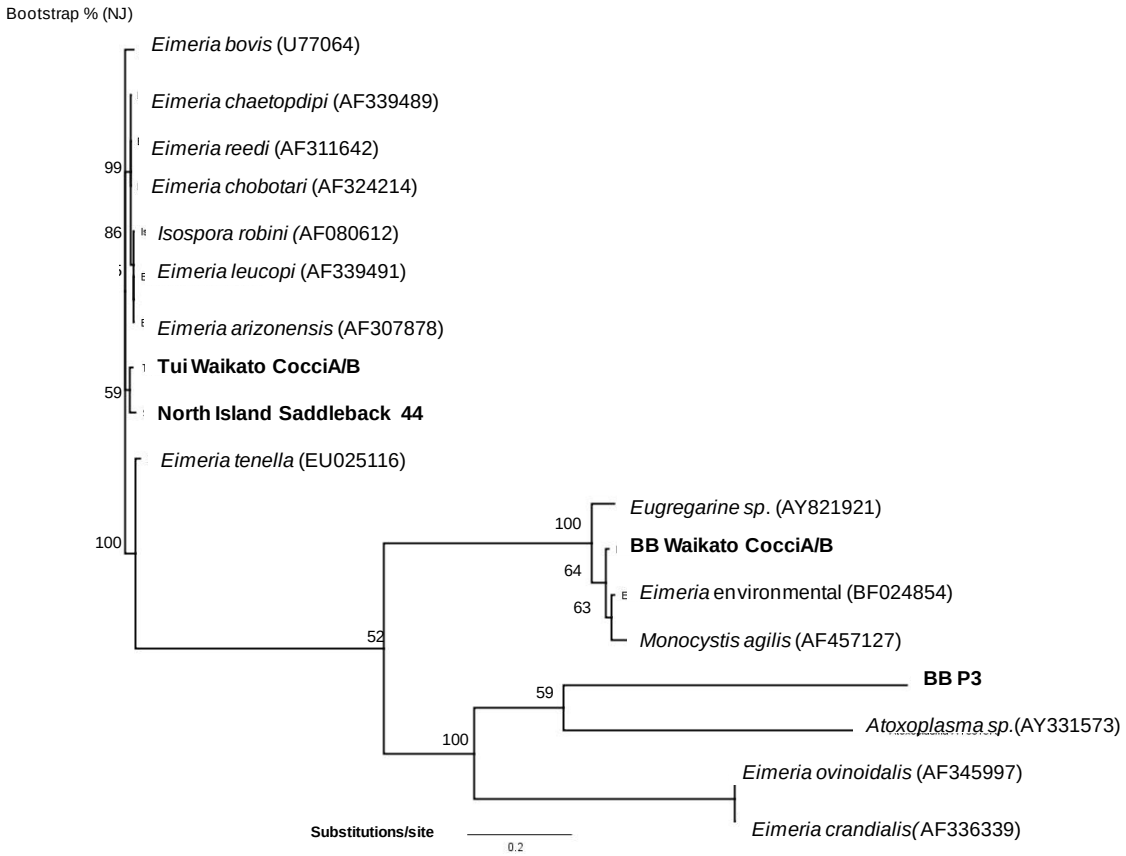

Figure 18: Phylogenetic analysis of *Eimeria spp*. isolated from blackbird, North Island saddleback and tui. Neighbour joining phylogeny of the 18S small subunit ribosomal gene. The tree is rooted on a linage of *Eimeria bovis*. Numbers above the branches indicate bootstrap support based on 1000 replicates. Names of the lineages (when available) and Genbank accession numbers of the sequences are given after the species names of the parasites.

3.3.7.3 BSEF and BSER primer set

Coccidia DNA was amplified by PCR, using the BSEF and BSER primers, from two of the three samples from North Island robin, but only one sample could be sequenced with conclusive results, a piece of 104 base pair length. A BLAST search on the NCBI database revealed a 95% sequence identity to *Atoxoplasma* spp. from sparrows (GenBank AY331573, with 96% sequence coverage) as well as 98% sequence identity (ranging from 77-82% sequence coverage) with to a group of *Eimeria* spp. in poultry (GenBank FJ449586, FJ449688).

A multiple alignment was performed using Clustal W on the 104 bp of the ITS1 18S small subunit ribosomal gene of coccidia isolated from the faecal sample of North Island robin from Mokoia and some other *Eimeria* species available from GenBank. A phylogenetic tree constructed using neighbour joining method resulted in the sequence of *Eimeria* DNA from the North Island robin being placed on a unique location among the *Eimeria* genus (Figure 19). As a result, the isolated coccidia clusters with "*Atoxoplasma*" from northern house sparrows (AY331573) but are different from *Eimeria* species isolated from chickens worldwide (Figure 19).

The findings using multiple alignment might not be consistent with the results from the BLAST search, but this can be explained with the lower sequence coverage in the *Eimeria* species compared to the *Atoxoplasma* species, while the *Atoxoplasma* has a coverage of 98% that matches 95% of the robin sequence, only 77-82% of the sequence in *Eimeria* spp. is covered by the robin sequence.

Sequence divergence results support the findings in the tree, with "*Atoxoplasma*" from northern house sparrows (AY331573) having a divergence of 6.1% from the North Island robin sample, and between 14-16% divergence to the *Eimeria* species from poultry (examples- *Eimeria maxima* FJ449686 to North Island robin 15.2% and *Eimeria acervulina* FJ449688 14.1%).

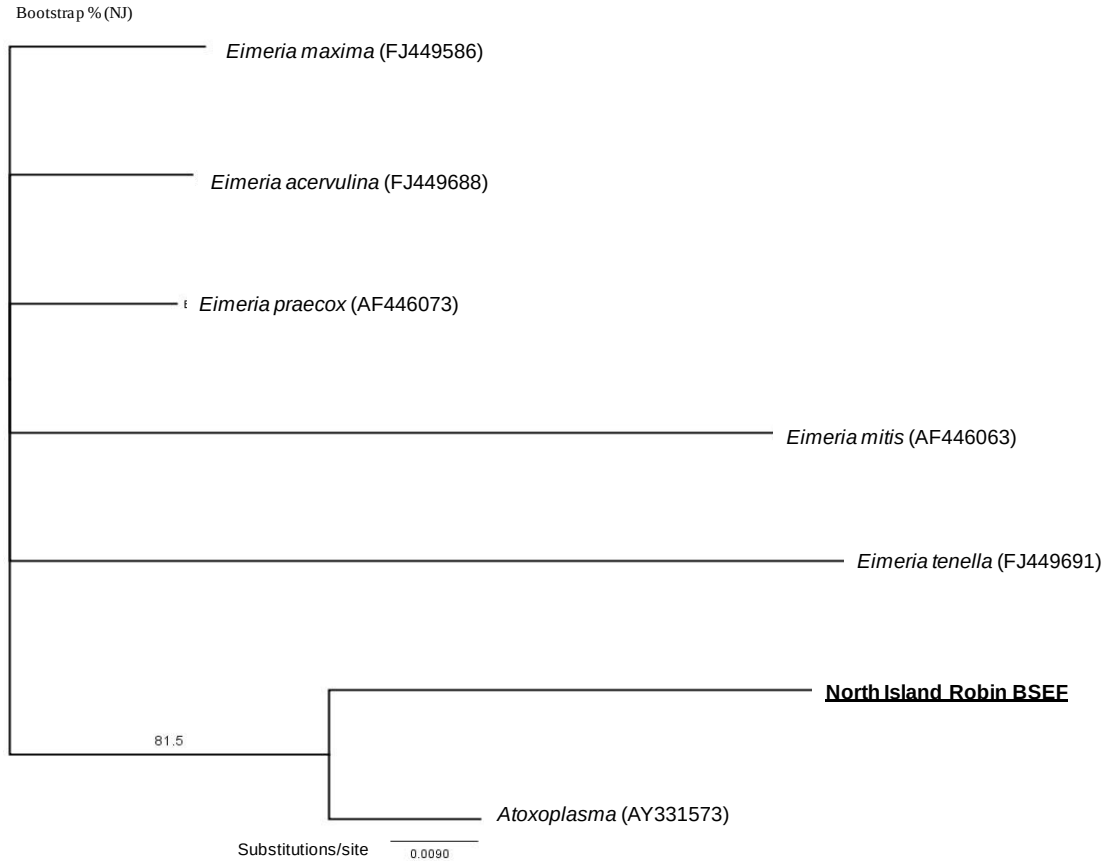

Figure 19: Phylogenetic analysis of *Eimeria spp*. Isolated from North Island robin. Neighbour joining phylogeny of the 16S small subunit ribosomal gene. The tree is rooted on a linage of *Eimeria bovis*. Numbers above the branches indicate bootstrap support based on 1000 replicates. Names of the lineages (when available) and GenBank accession numbers of the sequences are given after the species names of the parasites.

3.3.7.4 Fantail and silvereye

To date, PCR has been unsuccessful in amplifying coccidia from faecal samples from this group of birds using any of the primers listed in Table 2. Further primer sets are currently under evaluation.

3.4 Discussion

The overall prevalence of coccidian infection in the New Zealand bird species examined was 21- 38%; 21% in North Island robin, 38% in tui and 25% in saddleback. The sample size of blackbirds was too small to establish an accurate prevalence. This result is different to findings elsewhere in the world, where a higher prevalence of coccidia in passerines can be found, for example 89% in greenfinches (*Caruelis chloris*) in Estonia (Horak et al. 2004)

and 90% in blackbirds in Germany (Misof 2004). Previous studies in blackbirds, song thrushes and starlings in New Zealand found a prevalence of 40% in fledglings (Cassey 2008). Large numbers of coccidian oocysts were found in saddleback that were translocated by Thorne (2007), although the prevalence in these birds was not recorded.

There was a significant correlation in oocyst shedding at different times of the year, especially when comparing oocyst counts in autumn vs. spring/ summer and between the years (2007 vs. 2009). This observation might be connected to the physiological stress during the breeding season, which might cause greater susceptibility towards any parasite infection and will be evident at the end of the breeding season (i.e. autumn) (Ritchie 1994; Adkesson et al. 2005; Harrison 2006). No significant difference was found in the relationship between oocyst counts and both weight and body condition of the birds, which is possibly due to limited data concerning age and sex of the birds examined. Age and sex have various effects on weight and body condition which were not evaluated in this study, and so these results would need to be viewed in the context of age and sex of the birds to gain more reliable information.

Other studies have shown that the most important source of variation in oocyst counts is the time of day of faecal sampling (Brawner 1999, Lopez et al. 2007). Since the faecal samples were obtained at different times of day in the present study, the results presented here have to be regarded with caution. Coccidian oocysts are shed in a diurnal cycle, with faecal counts highest in the late afternoon, like studies in blackbirds (Misof 2004; Cassey 2008), dark-eyed juncos *(Junco hyemalis)* (Hudman, Ketterson et al. 2000), greenfinches (Brown, Ball et al. 2001) and other passerines (Lopez, Figuerola et al. 2007; Cassey 2008) have shown. Although it is not known if coccidia in passerines native to New Zealand follow the same diurnal cycle, this does seem likely. Therefore, an important field for future study would be to determine if saddleback and other native New Zealand passerines also show diurnal shedding of coccidian oocysts. One way to avoid wrong assumptions would be to ignore faecal counts altogether and just note the

presence or absence of coccidia oocysts in the sample, as done by Cassey (2008).

In the study presented here, one juvenile tui captured twice in the period of one week was found to have a high faecal count of oocysts. Although not weighed the second time so as not to cause additional handling stress, its body condition score was only 1 as opposed to 3 at the first time it was caught. This suggests that young birds might be affected by the parasites, whereas in adult birds infection is usually asymptomatic. Dorrestein (1998) reported that acute disease due to coccidia is the most common parasitic disease in juvenile canaries in breeding aviaries in Germany, with a mortality rate of up to 80%.

The methods used in this research, namely faecal flotation and the Qiagen stool kit (as recommended by Schrenzel et al. 2005), were sensitive enough to obtain parasite DNA even from low parasite counts. This finding is contrary to the opinion of Dolnik et al. (2009) who described a low efficiency for the Qiagen stool kit and regarded it as unsuitable for single cell DNA extraction. In the present study, it was possible to extract DNA even from few oocysts, and in several cases even single oocysts.

Morphological differences did exist in the oocysts of the faecal samples of individual birds, particularly in the tui and saddleback, and this supports the genetic findings which indicated several different species of coccidia in these birds. In future, more samples of individual birds containing oocysts should be collected, and single sporulated oocysts with documented morphology should be used for PCR analysis and sequencing. If this is done, it would be possible to link the morphology of the oocysts with their phylogeny. A similar study has been done before by Dolnik et al (2009) who isolated single oocysts from the faeces of wild blackcaps (*Sylvia atricapilla*), photographed them, isolated DNA and obtained the sequence .These authors also took blood samples and analysed the extraintestinal coccidial stages found in the blood to relate their findings to the different oocysts shed in the faeces. By

doing this, it was possible to determine which species of coccidia had extraintestinal stages in their life cycle.

Formerly, all coccidian parasites with two sporocysts ("diplosporocystic") in their oocyst have been placed together in the genus *Isospora* due to their morphological similarity. Our findings confirm those of Carreno & Barta (1999) who have postulated that diplosporocystic coccidian parasites should be placed in different genera, or even families, due to their relationships based on sequence homologies in the genes of the small subunit rRNA. These authors suggested that species with diplosporocystic, tetrasporozoic oocysts possessing Stieda bodies in their sporocysts should be placed in the genus *Isospora*, family Eimeriidae. These species have been described in the faeces of many birds. Diplosporocystic, tetrasporozoic oocysts without Stieda bodies in their sporocysts, as found in mammals, should be placed in the genus *Cystoisospora*, family Sarcocystidae. More recently, Barta and co-workers, after examining oocysts from the genus "*Atoxoplasma*" in passerines, suggested that the term "*Atoxoplasma*" should be a synonym for the genus "*Isospora*" in birds, due to their genetic similarity (Barta et al. 2005). Sporulated coccidia from the New Zealand passerines examined in this study all showed a Stieda body on their sporocysts. So they too are distinguishable from mammal "*Isospora*" that lack a Stieda body. The nucleic acid sequences derived from coccidial parasites of New Zealand passerines confirm the value of this morphological feature since all species it which it was found were closely related to species of the family Eimeriidae. The family Eimeriidae contains parasites infecting all classes of vertebrates, including reptiles, birds and mammals.

The taxonomy of the coccidia examined in this thesis has to be regarded with caution, because in the short region of the 18S gene amplified with the COC1/2 primers, there is not enough information to draw firm conclusions (Barta, J., pers. com.). The experiments described in this thesis were an initial research to determine possible genera of coccidia involved for providing a backbone to create further primer sets. Additional primer sets will have to be developed to amplify further regions of the 18S gene what would

provide more specific phylogenetic identification. In addition, the similarities of the coccidia found in this study with rodents and bats in other parts of the world have also to be viewed with caution. There has not been enough work done on coccidia and their genetic relationships in general and much less so in birds, and the database available so far is far from complete. Therefore, the sequences found in this study could only be compared with the sequences available so far, which consist of data from only a few studies.

In the sequence from blackbird sample P3, a BLAST search revealed a close sequence identity with “gregarine-like” species. These protozoa can be found infecting invertebrates like molluscs and earthworms, which are part of the diet of the examined birds. Indeed, during the faecal examination stage of this research, oocysts of these organisms were found in samples from blackbirds. The finding of these “gregarine-like” sequences in the blackbird might be possible due to co-purification of gregarine oocysts with the coccidian oocysts (Barta, J., personal communication). Therefore, the sequence found in the sample of blackbird P3 might not be of the coccidia infecting the bird.

The study described in this chapter is a first step in understanding coccidian parasitism in New Zealand passerines and it is evident that there is a range of species yet to be identified. As we know from chickens, a variety of different species of coccidia can infect a single host species, and even a single individual (Augustine 1996; 1999), a phenomenon that seems to be repeated in passerine birds around the world, as shown in the studies of Box (1977), Cringoli and Quesada (1991), Ball et al. (1998) Barta et al. (2005) and Dolnik et al. (2009). It would have been surprising to find a different situation in passerine birds native to New Zealand. However, the significance of coccidian infections in New Zealand birds remains to be established.

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Chapter 4. Helminths in endemic, native and introduced passerine birds in New Zealand

(This chapter is intended for publication in the New Zealand Journal of Zoology)

Abstract

Disease screening and management are becoming an important part of wildlife management worldwide. However, knowledge of diseases affecting individual species is lacking and hinders management decisions. In New Zealand, native birds are expected to undergo a disease screening programme and a quarantine period prior to translocation. There is little knowledge of the taxonomy, prevalence and epidemiology of diseases caused by internal parasites such as cestodes, trematodes or nematodes. These parasites possess the ability to inflict major damage to their avian hosts and limit their reproduction and survival, especially in a crowded and stressful environment such as a quarantine facility. Therefore their impact may result in translocation failure.

The research undertaken in this study is a preliminary investigation into the spectrum of helminth parasites in New Zealand passerine birds, using faecal samples from live birds as well as samples taken post mortem. We examined 358 faecal as well as tissue samples of seven native (Hihi or stitchbird, tui, North Island saddleback, North Island robin, silvereye, fantail) and three non native (blackbird, song thrush, house sparrow) passerine species.

The study revealed a new occurrence of a trematode (Dicrocoeliodea) and a cestode in the North Island saddleback, a new cestode and trematode in tui and previously undescribed *Capillaria* nematodes in hihi and tui. Although species identification of these helminths still has to be performed, it is likely that this is the first record of trematodes in native birds as well as three cestode species in tui and saddleback.

4.1 Introduction

4.1.1 Helminths in Passerine Birds

Of the thousands of bird species known today, only a few hundred species and their parasites have been studied in detail. Even fewer studies have been done on passerine birds and their parasites, however, publications from all over the world have documented an extraordinary spectrum of helminths inhabiting the passerine hosts so far examined (Binder 1971, Cooper and Crites 1976; Misof 2005).

In the wild, most infections are mild and birds suffer few ill effects. There are exceptions and, particularly in captivity, serious infections may sometimes occur. Heavily infected birds may show malabsorption of nutrients, anorexia, weight loss, and diarrhoea and in severe cases, the worms can cause intestinal obstruction and death (Ritchie et al. 1994; Rommel et al. 2000; Harrison and Lightfoot 2006).

Most of the work has been done on the identification of passerine helminths in the Northern hemisphere. In the United States, papers have been published describing the helminth fauna of some passerines in different states.

Among the earliest studies was that of Ballard and Olsen (1966) who found that of the 70 starlings collected in northeastern Colorado, 48 (68.6%) were infected with helminths. Three species of cestode, two species of acanthocephalan as well as two species of nematode were identified. In a study of helminth parasites of 49 juvenile starlings from South Bass Island, Ohio (Cooper & Crites 1975), 47 (95.9%) were infected with helminths. Four species of trematodes, three species of cestode, four species of nematodes and one acanthocephalan species were found.

Roderick and Johnson (1971) found that 88.2% of a sample of starlings (*Sturnus vulgaris*) residing in southeastern Kansas were infected by three different species of cestodes, two acanthocephalan species and one nematode. The most common helminth found in the 128 host specimens was the acanthocephalan *Prosthorhynchus formosus*.

Cooper and Crites (Cooper and Crites 1976) also worked on helminths of the North American robin *Turdus migratorius*, and found eleven different species of helminth parasites in 62 of the 68 (91.2%) birds examined. The helminth species identified were one trematode, three cestodes, one acanthocephalan as well as six nematodes.

More recent work has involved the helminth fauna of the genus *Turdus* during its autumn migration in the Spanish region of Granada (Diaz and Hierro 2004), where seven different species of helminths were found: two cestodes, four trematodes as well as one species of acanthocephalan. In a study of the helminth fauna of passerines in the Netherlands (Borgsteede et al. 2000), 210 birds of 47 species were collected, with 138 (65.7%) being infected with helminths. Of these birds, 22.4% were infected with trematodes, 39% with cestodes, 45.2% with nematodes and 17.1% with acanthocephalans. Twenty-nine species of helminths were identified, among them eleven species of trematode, five cestode, eleven nematode and two acanthocephalan. Most of these findings were new records for the Netherlands, showing that until recently not much had been known about the biodiversity of helminth parasites in that country.

In her doctoral thesis, Misof (2005) examined the helminth fauna of blackbirds (*Turdus merula*) in Germany comparing three different years, 1996-1998. She found that three helminth taxa commonly occurred in blackbirds, namely nematodes of the genus *Capillaria*, cestodes and acanthocephalans. *Capillaria* were present in 56% of the birds and were therefore the most prevalent helminths, cestodes were found in 38% of the birds, and Acanthocephalans in 28%. In 1971, (Binder 1971) conducted a similar study on blackbirds in Germany, but in his study, the cestode *Dilepis undula* and the nematode *Porrocaecum ensicaudatum* were the most prevalent helminths.

The helminth fauna of the red-crested cardinal (*Paroaria coronata*) in Brazil (Mascarenhas et al. 2009) was studied in 40 birds that were necropsied. 45% of these birds were parasitized, with nematodes the most common helminth parasite in 22.5% of the birds. Fourteen helminth species were identified, eight nematodes, four trematodes, and one cestode as well as one acanthocephalan.

4.1.2 Helminths in Captive Passerines

In passerines that are commonly kept as pets, helminths are also known and presented mainly in veterinary publications like Stacey Gelis' paper on Gouldian finches (*Erythrura gouldiae*)(Gelis 2003). Gelis (2003) mentions that nematodes and cestodes are occasionally found in these birds, but less often than in other finches. Ascarids may cause weight loss and sudden death and are mainly found in the small intestine. *Acuaria* spp. infect the koilin layer of the gizzard and may cause wasting and undigested seed may be found in the droppings. Cestodes are occasionally seen causing diarrhoea, emaciation, increased appetite and death due to intestinal obstruction.

4.1.3 Helminths in New Zealand Passerines

The checklists created by Weekes (1982) and McKenna (1998) constitute the bulk of the work done on helminths of New Zealand passerines. These authors listed helminths in introduced passerines and passerines kept as pets, with the silvereye (*Zosterops lateralis lateralis*) and the North Island saddleback (*Philesturnus carunculatus rufusater*) being the only native species included. Further findings have been added by McKenna in 2005 with *Capillaria* spp. in kokako (*Callaeas cinerea*) and in 2006 with the finding of *Syngamus trachea* in hihi (*Notiomystis cincta*) (McKenna 2005 and 2006). The prevalence and epidemiology of diseases caused by internal parasites such as cestodes, trematodes and coccidia are unknown.

In New Zealand, only two members of the genus *Turdus* have been introduced into the country, namely the blackbird (*Turdus merula*) and the song thrush (*Turdus philomenos*). In blackbirds in New Zealand, three species of nematode (*Capillaria* sp., *Porrocaecum ensicaudatum, Syngamus trachae*) and four species of cestode (*Anomotaenia verulamii, Aploparaksis dujardinii, Dilepsis undula*, *Hymenolepis serpentulus*) are known according to McKenna (1998). In the song thrush two species of nematodes (*Capillaria* sp., *Porrocaecum ensicaudatum*) and three species of cestodes (*Anomotaenia verulamii, Aploparaksis dujardinii, Dilepsis undula*) are on record (McKenna 1998).

None of the other different species of helminths found in starlings overseas, except *Syngamus tracheae* have been reported in New Zealand starlings (McKenna 1998) to date. This includes a group of common helminths found overseas such as the acanthocephalans, like *Prosthorhynchus formosus*, that have never been documented in passerines in New Zealand.

Although helminths rarely cause problems, there have been examples of a severe impact of helminths on native New Zealand hosts. One is the report of nematodes of the ascarid family, genus *Porrocaecum,* that have been found to cause death in saddleback due to their migration into the intestinal wall resulting in septic peritonitis (Alley et al. 2007).

4.1.4 Translocations and health screening in New Zealand

Endangered endemic New Zealand passerines only survive today on off-shore and mainland islands that are free of introduced mammalian predators. The environments, which the birds now occupy on these islands, are in many cases no longer pristine habitats since they were formerly used by Maori and Europeans for farming and mainly contain recently regenerating forest. Some of these sanctuary islands, such as Tiritiri Matangi, are used for ecotourism (Lindsay et al. 2008).
The stress of capture and release into a less than optimal habitat, as well as the pressure by opportunistic pathogens like *Aspergillus,* which prefer highly disturbed habitats (Perrott 2001), could increase susceptibility to gastrointestinal parasites in translocated birds. Although helminths are mostly harmless to their hosts, under certain conditions like the crowded conditions in a quarantine aviary before translocation, they may have a negative effect on the birds' condition. One reason for the high rates of infection is the difficulty in avoiding faecal contamination of food sources in the crowded conditions of a captive or quarantine facility. The variety and abundance of parasite species infecting free-living native passerine species and the possibility that some may have been introduced from non-native passerines is therefore in need of investigation.

4.1.5 Aims of this paper

In this study, we aimed to establish some baseline data on the helminths found in native and introduced birds in New Zealand. This knowledge may help in future screening programmes for translocations of rare birds in New Zealand and allow the creation of a diagnostic and de-worming regime for captive and quarantined birds.

The birds examined in this study included samples of four endemic birds, the tui (*Prosthemadera novaeseelandiae*), the North Island saddleback (*Philesturnus carunculatus rufusater), the* hihi (or stitchbird) *(Notiomystis cincta)* and the North Island robin (*Petroica australis longipes*). One species, the silvereye, *Zosterops lateralis*, is a recent self-introduced species from Australia while the blackbird (*Turdus merula*) and the song thrush (*Turdus philomenos*) are recent European introductions.

An aspect that should not been overlooked is that most parasites in New Zealand native birds are part of the native faunal diversity of New Zealand, a part that is poorly documented and understood. Since many of the hosts are endangered, these parasites may also be at risk of disappearing before they have been studied, and in some cases may disappear before we are aware of their existence.

4.2 Material and methods

4.2.1 Study sites

4.2.1.1 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 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 the maori introduced pacific rats, kiore (*Rattus exulans*) and dogs (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 by 2001. Mokoia Island is covered with regenerating broadleaf-podocarp forest (Andrews 1992; Perrot 2000; Armstrong et al. 2002; Christensen 2007). Nectar sources for honeyeaters 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).

4.2.1.2 Other sample sources

While Mokoia was the sampling site for most native birds, dead specimens of introduced birds such as blackbirds, as well as not endangered endemic birds such as tui, were obtained from other sites of the North Island. The main source for dead birds, especially introduced birds, was Palmerston North, Manawatu (40° 21' S, 175° 37' E). One tui was received from near Masterton in the Wairarapa (40° 57' S, 175° 39' E). One faecal sample as well as one dead saddleback were received from a captive saddleback population at Orana Park in Christchurch (43°28′ S, 172°27′ E) that was derived from the Mokoia population. Seven dead tui were received from Raoul Island (29.27°S 177.92°W), the largest island in the Kermadec group approximately 750-1000km Nothnortheast of New Zealand. Hihi faecal samples from Mt. Bruce National Wildlife Centre (NWC), located in Masterton, Wairarapa, North Island (lat. 42° 43' 0 S long. 171° 19' 60 E) were routinely examined by the parasitology laboratory in the Institute of Veterinary, Animal and Biomedical Sciences (IVABS) at Massey University and results were shared with the author. Formalin fixed tissue samples archived by IVABS (Massey University) of 29 saddleback (27 North Island saddleback, two South Island saddleback) were also examined grossly and histologically for trematodes.

4.2.2 Capture, handling and faecal sampling

Seven trips were made for capturing and sampling of birds on Mokoia Island, in January 2007, May 2007, October 2007, March 2008, November 2008, December 2008 and February 2009. Birds were caught in mist nets set up next to the tracks on Mokoia Island as well as at the only two streams occurring on the island (Figure 20). North Island saddlebacks and robins were attracted to the net with the help of recorded songs of their own species. Other species were caught by placing the mist nets at sites frequented by the birds to increase the chances of capture. For example tui and saddleback were known to frequent streams in search of bathing opportunities and mist nets were placed there, as well as across flight paths towards feeding trees used at the time by tui. Blackbirds were caught by placing nets close to the ground and across narrow stretches of the tracks. Care was taken to make sure these nets were always under tension and not entangled in vegetation. The bags for restraining the birds were used only once on each side and washed and dried at the end of each day. The birds were carefully placed into these bags by hand and hands were disinfected after handling each bird, to reduce the spread of transmissible diseases.

After capture, each bird had the following measurements taken and information recorded: weight, tarsus length, wing length, body condition, presence of a brood patch in females and wattle size in saddlebacks. 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 was estimated according to the method described by Melville in the “New Zealand bird bander’s manual, DOCDM-285890” (In press) (Pectoral muscle scores (0-3); (Melville 2007). Initially the pectoral muscle scores only were used, with the modification that scores were recorded on a scale of 1-4.

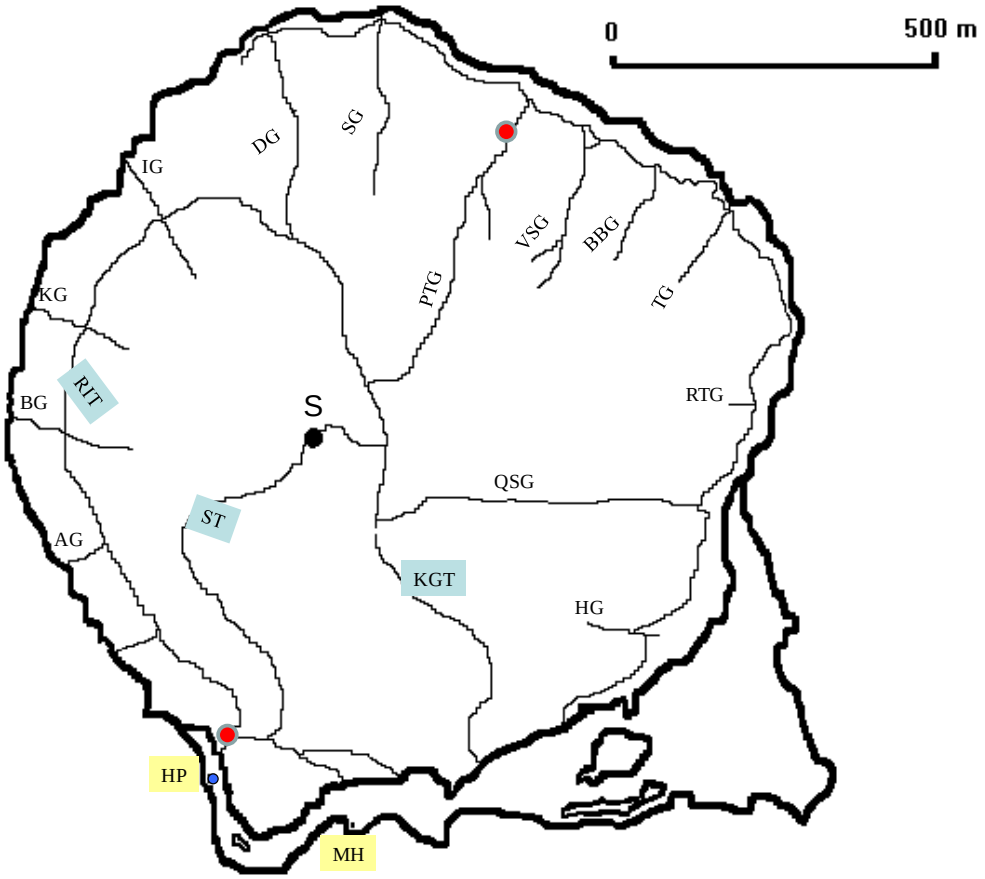

Figure 20: Map of Mokoia Island showing the **main tracks** (Blue labels: RIT = Round the Island track; ST = Summit track; KGT = Kumara God track); **focal sites** (cream labels (HP = Hinemoa's hot pool; and MH = Maori hut); **gullies** (AG = A gully; AG = A gully; BG = B gully; KG = K gulley; IG = I gulley; DG = D gulley; SG = Steep gulley; PTG = Pine tree gulley; VSG = Victoria Street gulley; BBG = Blackberry gulley; TG = Trough gulley; RTG = Rat tube gulley; QSG = Queen Street gulley; HG = Hidden Gulley); and the **summit (S)** (black dot). The two red coloured dots mark the sites of the two natural streams of the island.

On later trips to Mokoia, a fat score which measures the amount of fat in the thoracic inlet of the bird, was also used to assess body condition score, using a scale of 0 to 4 (Melville 2007).

Faecal samples from all species were collected in 1.5ml microcentrifuge tubes and refrigerated at 4ºC until examination in the laboratory. Saddleback and robin faecal samples were obtained from the holding bags in which the birds were restrained as they frequently defecated in the bag. This was less common with tui so they were released from the mist net and placed inside a cardboard box (measuring 31x25x22cm) provided with a perch for 10 minutes after being released from the mist net. This was enough time for the birds to defecate. Aluminium foil was placed in the

bottom of each box to facilitate the collection of the faecal sample. The foil was changed after each bird.

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

4.2.3 Flotation, microscopic examination and oocyst count

The collected faecal samples were either examined within 24 hours or stored at 4°C until examination. Cooling the samples or processing them as quickly as possible is standard procedure to minimise developmental changes that may affect identification. Faecal samples were placed into a sieve and weighed using a digital scale. Then the sieve was placed over a metal bowl and rinsed with ZnSO4 to wash the small materials including parasite's eggs. The contents of the bowl were then transferred into a glass test tube. The tube was filled to the rim with flotation solution (saturated ZnSO4 solution at a specific density of 1.32) until a positive meniscus was achieved (modified after Bowman 1995). A square microscope cover slip was applied with care to avoid including any air bubbles. The glass tube and cover slip were centrifuged for 5 minutes at 300g, after which the cover slip was removed and placed on a previously labelled microscope slide which was then examined at 100x magnification.

4.2.4 Pathology

Thirty-two birds were examined post mortem, including six blackbirds, 14 tui and five saddleback, two song thrushes, two silvereyes, two sparrows (*Passer domesticus*) and one shining cuckoo (*Chrysococcyx lucidas)*. Each dead bird was weighed and the body condition score assessed and recorded using a 1-9 scale (Appendix 2). The bird was then examined using a standard necropsy technique (Appendix 3). Samples of pectoral muscle and all internal organs were fixed in 10% buffered formalin for histology and both pectoral muscle and liver were frozen for later PCR analysis if required. The cranium was opened with sharp scissors to expose the brain to

formalin. Collected tissues were fixed for a minimum of 48 hours before trimming and blocking.

Intestinal contents were collected for parasitological examination. Any adult internal parasites found then were fixed in 70% ethanol.

While trimming representative areas of all tissues, any visible lesions were selected for histological processing. The fixed tissues were embedded in paraffin, and then cut at 4 µm before routine staining with haematoxylin and eosin for examination under a light microscope.

4.2.5 Statistics

An independent-samples t-test was used to the relationship between length and length/width ratios of cestode eggs found in saddleback from Mokoia Island and Orana Park. The data referring to the cestode egg sizes is given as average ± SD (standard deviation). Analyses were carried out using WinSTAT® for Microsoft Excel version 2007.1, P-values of <0.05 were considered significant.

4.3 Results

Over the duration of the study period, faecal samples from 358 New Zealand passerines were collected. These were obtained from 10 different species; 4 endemic, 2 native and 4 introduced. Trematode eggs were found in 16 (4.5%), cestode eggs were found in 29 (8.1%) and *Capillaria* spp. eggs in three (0.8%) individual birds (Table 8). In addition, 29 archived tissue samples of saddleback were examined, 3 (10.3%) had trematodes in their gallbladders.

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Table 8: Results of the examination of 358 faecal samples of New Zealand passerines for helminths

Family	**Species**	**Common name**	**Origin**	**Numbers sampled**	**Trematodes**	**Cestodes**	***Capillaria***
Callaeidae	*Philesturnus carunculatus*	Saddleback	Mokoia	233	13 (5.6 %)	23 (9.9%)	0
		Saddleback	Orana Park	1	1	1	Other nematodes
		Saddleback (archived material from all over North Island)	North Island	27	2	-	-
		South Island saddleback (archived material)	South Island	2	1	-	-
Meliphagidae	*Prosthemadera novaeseelandiae*	Tui	Mokoia	56	0	0	4 (7.1%)
		Tui	Mainland	7	0	3 (42.9%)	2 (28.6%)
		Tui	Raoul Island	7	3 (42.9%)	-	-
Eopsaltriidae	*Petroica australis longipes*	Robin	Mokoia	38	-	1 (2.6%)	-
Notiomysitae	*Notiomystis cincta*	Hihi	Mt. Bruce NWC	136	-	-	42 (not all of individual birds)
Muscicapidae	*Turdus merula*	Blackbird	Mokoia	3	-	-	-
		Blackbird	Mainland (Palmerston North)	8	-	2 (25%)	-
	Turdus philomenos	Song thrush	Mokoia	1	1	-	-
Monarchidae	*Rhipidura fuliginosa*	Fantail	Mainland (Palmerston North)	1	-	-	-
Zosteropidae	*Zosterops lateralis*	Silvereye	Mainland (Palmerston North)	2	-	-	-
Passeridae	*Passer domesticus*	House sparrow	Mainland (Palmerston North)	2	-	-	-
Cuculidae	Chrysococcyx lucidas	Shinning cuckoo	Mainland (Palmerston North)	1	0	0	0
		totals		387	21 (5.4%)	29 (7.5%)	48 (12.4%)

4.3.1 *Capillaria* spp. sensu lato

The only nematode eggs identified were those of *Capillaria* spp which were found in 6 of 56 (9.33%) tui samples. In addition, *Capillaria* were found in 37 of 66 (56.1%) of individual faecal samples from hihi routinely examined in the IVABS parasitology laboratory and in 4 of 70 (5.7%) pooled samples from juvenile hihi. The faecal samples from hihi were obtained in the years 2007-2009 from 6 adult birds (66 individual samples) as well from 11 nests in 70 pooled samples. The mean faecal count in tui was 77.7eggs/ g and in hihi it was 8589 eggs/g in individual samples; however, one hihi had an extremely high egg count of >77000, eggs/g. A review of the records of the IVABS parasitology laboratory, which examined hihi faecal samples from Mt Bruce NWC in both years, show that the highest faecal counts for *Capillaria* were in the period between January and March, with mostly negative results in the remaining months of the year.

4.3.1.1 Morphology

The *Capillaria* spp. eggs found had an appearance typical of the group, with the eggs from hihi being more rounded than the eggs from the tui (Figure 21). Eggs from hihi had a mean length of 68.6 µm (66.3- 71.3 µm; n = 7) and a width of 29.3µm (27.5-31.3 µm; n = 7) (see Table 9). *Capillaria* eggs from tui were not measured.

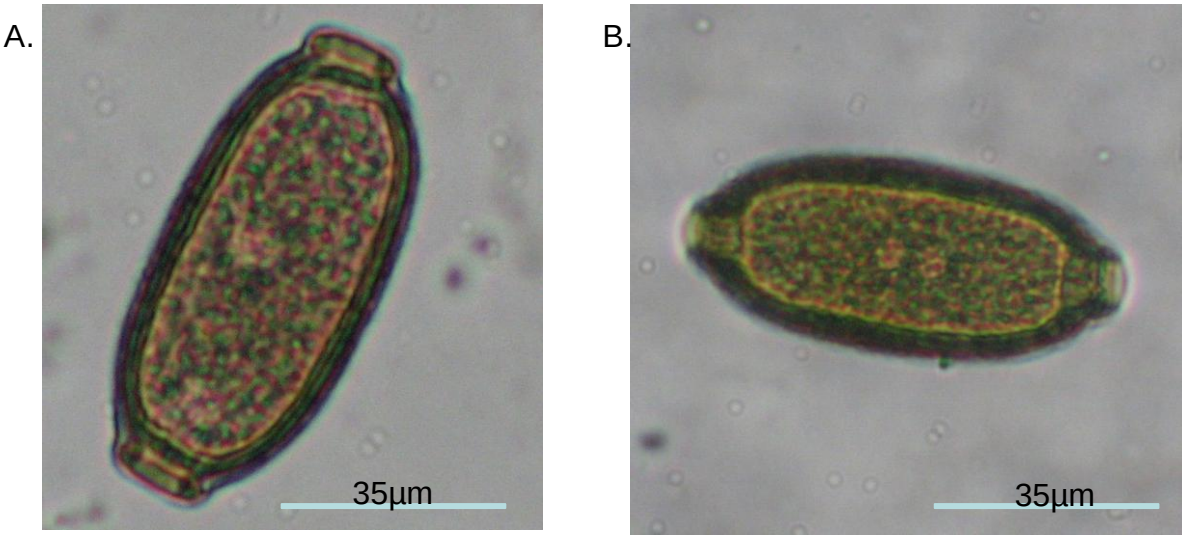

Figure 21: A. *Capillaria* spp. egg of a tui. B. *Capillaria* spp. egg from a hihi from Mt Bruce NWC (400x). Photographs taken by the author.

Table 9: Descriptive statistics of the *Capillaria* eggs recovered from hihi from Mt Bruce NWC, Measurements are given in µm

	Length	Width	Length/ width ratio
N	7	7	7
Mean	68.57	29.29	2.35
SD	1.97	1.22	0.16
SE Mean	0.74	0.46	0.06
Minimum	66.25	27.5	2.16
Maximum	71.25	31.25	2.60

4.3.2 Cestodes

Cestodes eggs, identified using faecal flotation and microscopy, were found in 1/ 38 (2.63%) North Island robin, 3/56 (5.4%) tui, 23/232 (9.9%) North Island saddleback, 3/136 (2.2%) hihi and 2/11 (18%) blackbird faecal samples.

4.3.2.1 Morphology

Cestode eggs of tui, blackbird and saddleback from Orana Park were observed to have a delicate transparent sheath around them in most cases ("oncospheral envelope"). Cestode eggs from saddleback on Mokoia did not show this sheath (Figure 22). All cestode eggs contained clearly visible hexacanth larvae. The eggs found in blackbirds were subspherical and approximately 30µm in length and 24µm in width (Table 9). Eggs from tui were subspherical and 29.4µm in length (22.5-42.5µm) and 25.8µm in width (21.3-30µm). Cestode eggs from Mokoia saddleback were subspherical with a length of 43.91µm (37.5-57.5µm) and a width of 36.1 µm (30-42.5 µm). Eggs from non-Mokoia saddleback were ellipsoid and 65.5 µm (40-87.5 µm) in length and 37.1 µm (32.5-42.5 µm) in width (Table 10). Cestode eggs from North Island robin were not measured.

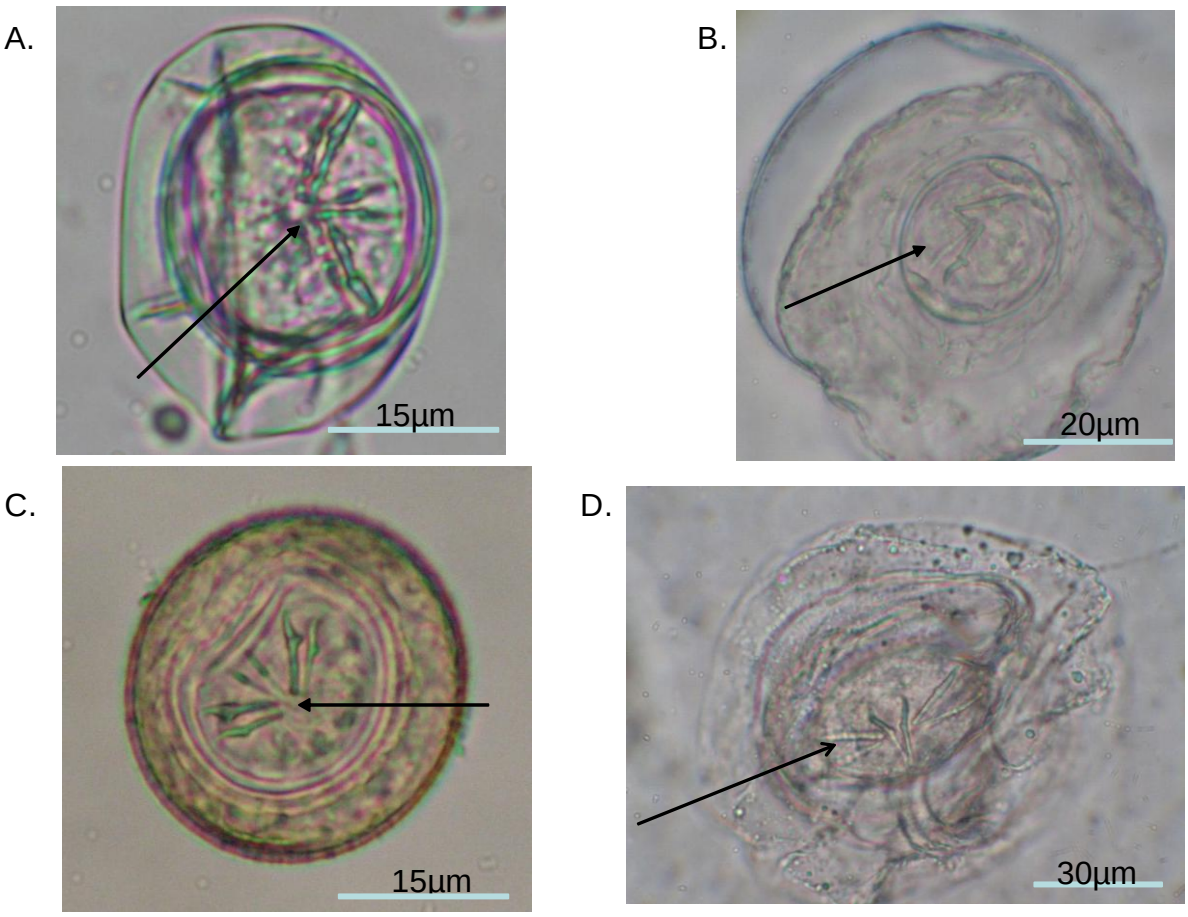

Figure 22: Cestode eggs (400x) Note the hexacanth larvae inside (arrow). A. Blackbird, B. Tui, C. North Island saddleback (Mokoia), D. North Island saddleback (Orana Park). Photographs taken by the author.

Table 10: Descriptive statistics of cestode eggs from North Island saddleback and tui, measurements are given in µm

	Saddleback Mokoia Length	Saddleback Mokoia width	Saddleback Mokoia length/ width ratio	Saddleback Orana Park length	Saddleback Orana Park width	Saddleback Orana Park length/width ratio	Tui length	Tui width	Tui length/ width ratio
N	16	16	16	20	20	20	14	14	14
Mean	43.91	36.10	1.22	65.5	37.31	1.76	29.38	25.8	1.15
SD	5.91	3.41	0.15	13.92	2.30	0.37	4.75	2.62	0.25
SE Mean	1.48	0.85	0.04	3.11	0.52	0.08	1.27	0.7	0.07
Minimum	37.5	30	1.07	40	32.5	1.07	22.5	21.25	0.87
Maximum	57.5	42.5	1.64	87.5	42.5	219	42.5	30	1.89

There was a significant difference in the length of eggs from Mokoia birds (43.91±5.91) when compared to Orana Park (n=20, M=65.5, SD=13.92) (independent-sample t-test t = -5.921845354, n= 16, 20; p = 1.087×10^{-6}) (Figure 23). There was also a significant difference in the length/width ratios of eggs from Mokoia birds (1.22±0.15) and Orana Park birds (1.76±0.37) (t-test t = -6.265862682, n=16, 20; p= 3.17×10^{-6}) (Figure 24). These findings are a further indication that the cestode eggs found in the two different locations belong to two different species.

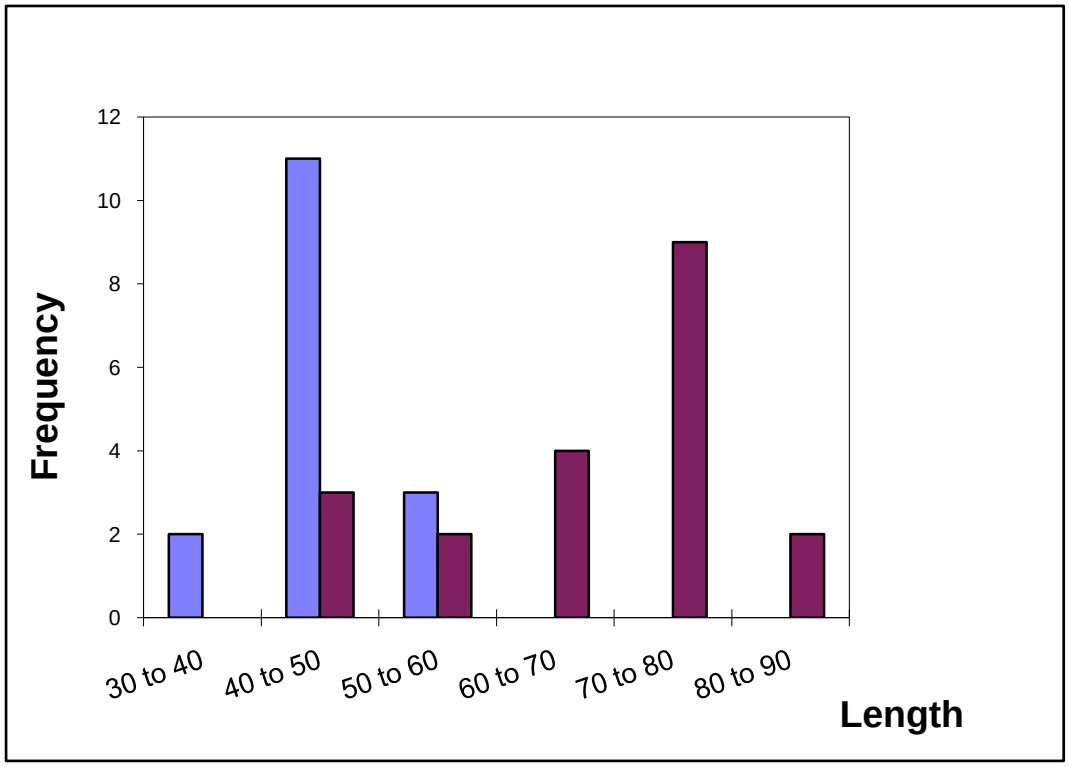

Figure 23: Comparison of the length of cestode eggs of North Island saddleback from Mokoia (blue) and Orana Park (purple)

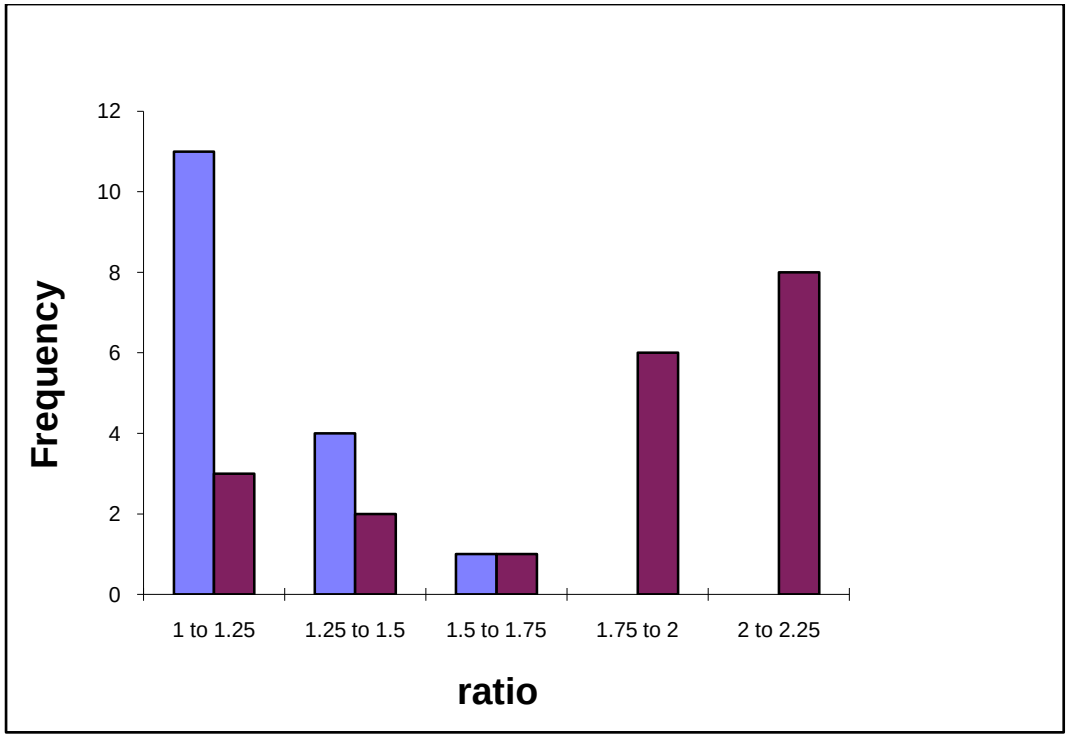

Figure 24: Comparison of the length/width ratio of cestode eggs of North Island saddleback from Mokoia (blue) and Orana Park (purple)

4.3.3 Trematodes

Faecal flotation and microscopy revealed 3/56 (5.36%) tui and 13/232 (5.6%) North Island saddleback faecal samples contained trematode eggs. No trematode eggs were recovered from any of the other species examined. Trematode worms were recovered from the large intestine of 3/7 (42.9%) tui from Raoul Island examined post mortem and from the gallbladders of 3 dead North Island saddlebacks (two from Mokoia, one from Orana park/ Christchurch) and from the gallbladder of a song thrush from Palmerston North. In three of the 29 (10.3%) archived saddleback samples examined, trematodes were also found in the gallbladders.

4.3.3.1 Morphology

The trematodes recovered from saddleback gallbladders were adults that were up to 4mm in length and had a prominent ventral sucker (Figure 25A).The numerous adult trematodes recovered from the tui large intestine were roughly triangular in shape up to 2mm long (Figure 25B).

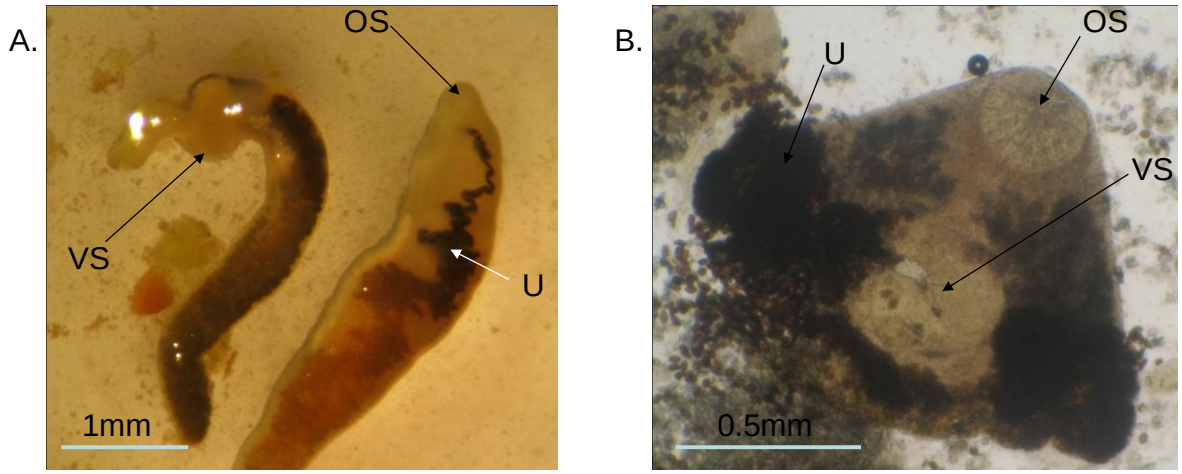

Figure 25: A. Adult trematodes from the gallbladder of a North Island saddleback B. Adult trematode found in the large intestine of a tui. OS- Oral sucker BS- Ventral sucker U- Uterus. Photographs taken by the author.

The eggs from both tui and saddleback species were the typical ellipsoidal shape of trematode eggs with an operculum at one end (Figure 26B). The eggs of the saddleback trematode were more rounded than the longer ones from the tui and contained clearly visible miracidium larvae. These larvae were not visible in the eggs from tui. The eggs from tui were average 21.8µm in length (12.5-25 µm; n=17) and 13µm in width (10-20 µm; n= 17) (see Table11). The eggs from saddleback were on average 42.2 µm in length (25-47.5 µm; n=19) and 19.74 µm in width (10-23.8 µm; n=19) (see Table11).

The trematodes in the thrush were only found on histology, the faecal examination was negative, and the eggs could therefore not be measured.

Figure 26: Trematode eggs (400x) A. Tui B. North Island Saddleback/ Mokoia Island. M- Miracidium larvae O- Operculum. Photographs taken by the author.

Table 11: Descriptive statistics of the trematode eggs from tui and North Island saddleback, measurements are given in µm

	Tui length	Tui width	Tui length/ width ratio	Saddleback length	Saddleback width	Saddleback length/ width ratio
N	17	17	17	19	19	19
Mean	21.84	12.96	1.74	42.24	19.74	2.22
SD	2.80	2.11	0.35	6.05	3.19	0.68
SE Mean	0.68	0.51	0.08	1.39	0.73	0.16
Minimum	12.5	10	0.63	25	10	1.47
Maximum	25	20	2.25	47.5	23.75	4.75

4.3.3.2 Pathology

Three dead saddlebacks and one song thrush were collected during the study period. One of the saddlebacks died from unknown causes during blood sampling on Mokoia Island, another was found dead on Mokoia and the third, from Orana Park, died after a duodenal rupture and peritonitis. The song thrush was found dead on State Highway 2 north of Palmerston North after being hit by car. All birds appeared to be in good body condition. The bird from Orana Park was heavily parasitized with a range of cestodes, trematodes and nematodes and the gallbladder was distended. The gallbladder in all three birds contained several adult trematodes and their eggs (Figure 28-30). The gallbladder in the saddleback that died during blood sampling on Mokoia measured 11x 4x 5mm, the gallbladder of the saddleback found dead on Mokoia measured 9x 6x 4mm and the gallbladder of the thrush measured 10x 4x 4mm. The gallbladder of the saddleback from Orana Park was not measured.

In addition, three more saddleback with infected gallbladders were found in archived material. The first of these birds was from 2004 and came from Napier/ Hawkes Bay and was in poor body condition and presented with aspergillosis as cause of death. The gallbladder measured 8.2x 5.3x 3.1mm. The second bird was found in 2006 and was translocated from Mokoia Island to Bushy Park Reserve. The bird was in moderate body condition and died of trauma. The gallbladder measured 9.5x 6.4x 3.1mm. The third bird was a South Island saddleback found 2007, the gallbladder measured 5.5x 2.1x 2.3mm. The gallbladders of all these birds contained several adult trematodes and their eggs.

On histology, the livers of these birds showed a mild chronic cholangiohepatotitis with disseminated foci of inflammation of mainly lymphocytes and plasma cells associated with the bile ducts. The bile ducts in affected areas showed epithelial hyperplasia and contained necrotic debris. There was mild haemosiderosis in the liver as well as focal areas of bile pigment accumulation in sinusoids. The gallbladder of these birds

contained pale basophilic shales of sloughed epithelium, the remaining epithelium was folded and hyperplastic. In the part of the gallbladder closest to the liver, there were numerous hyperplastic glands secreting excess basophilic mucus. The lesions were most severe in the saddleback that died during bloodsampling, although the other birds showed similar histological changes.

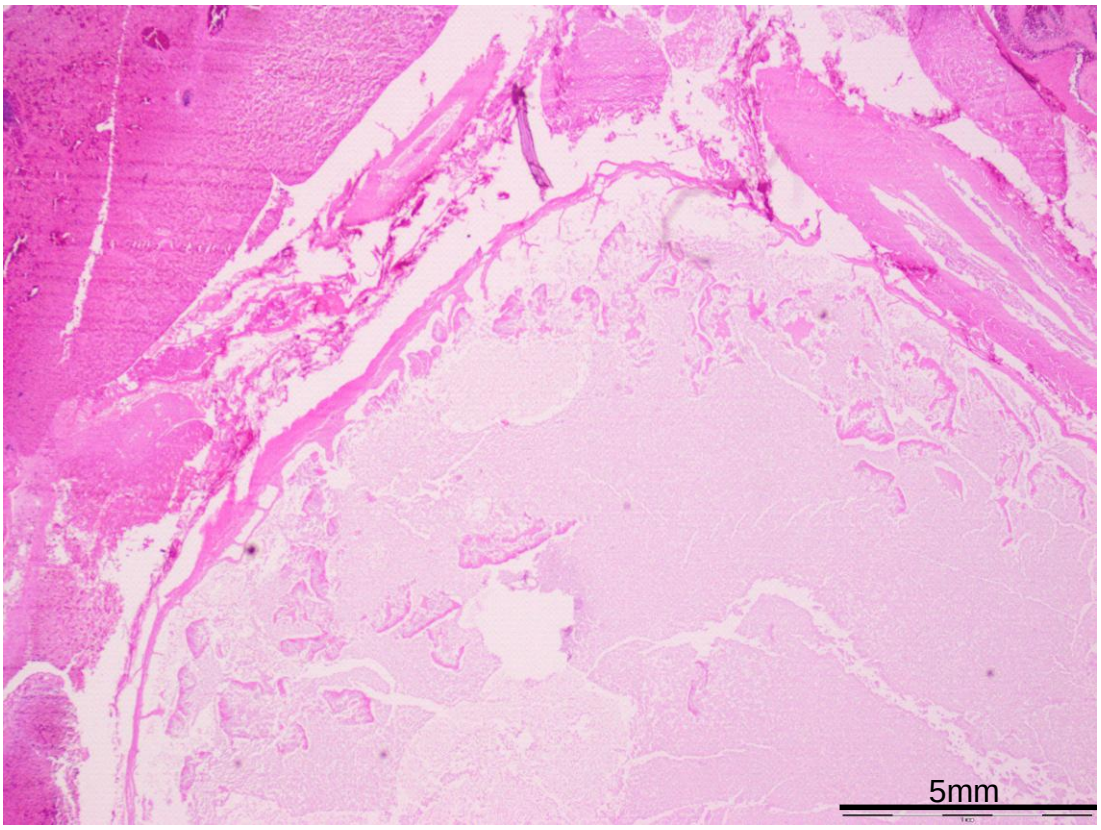

Figure 27: Distended gallbladder of a North Island saddleback infected with trematodes. The lumen contains excess mucus and sloughed epithelial cells. H&E (40x). Photograph taken by the author.

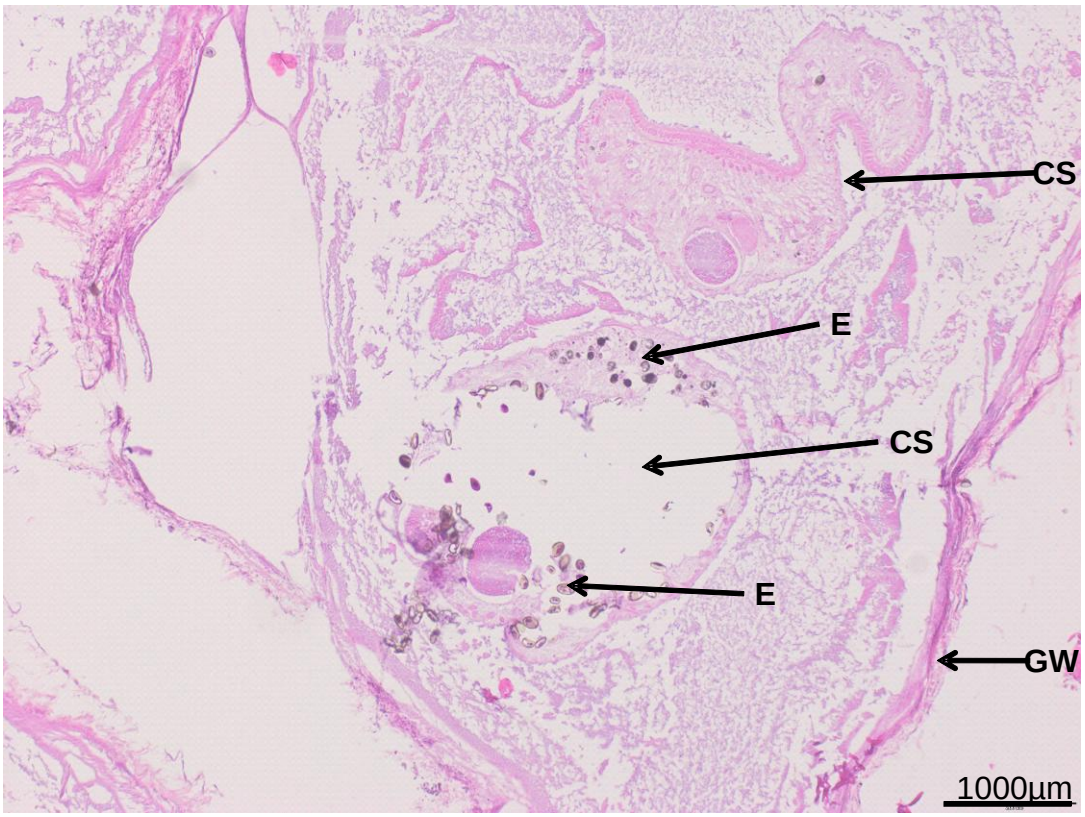

Figure 28: Distended gallbladder of a North Island saddleback, showing multiple cross sections (CS) of trematodes containing eggs (E); GW- wall of the gallbladder H&E (100x). Photograph taken by the author.

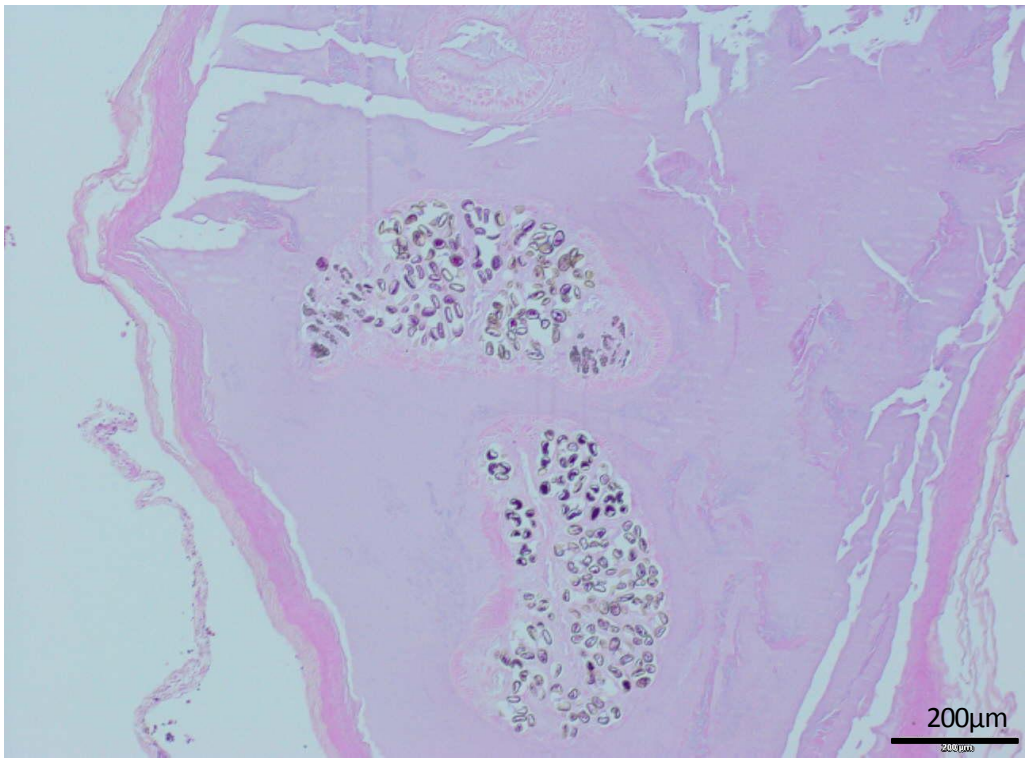

Figure 29: Adult trematodes containing numerous eggs in the gallbladder of a north island saddleback from Mokoia. H&E (400x). Photograph taken by the author.

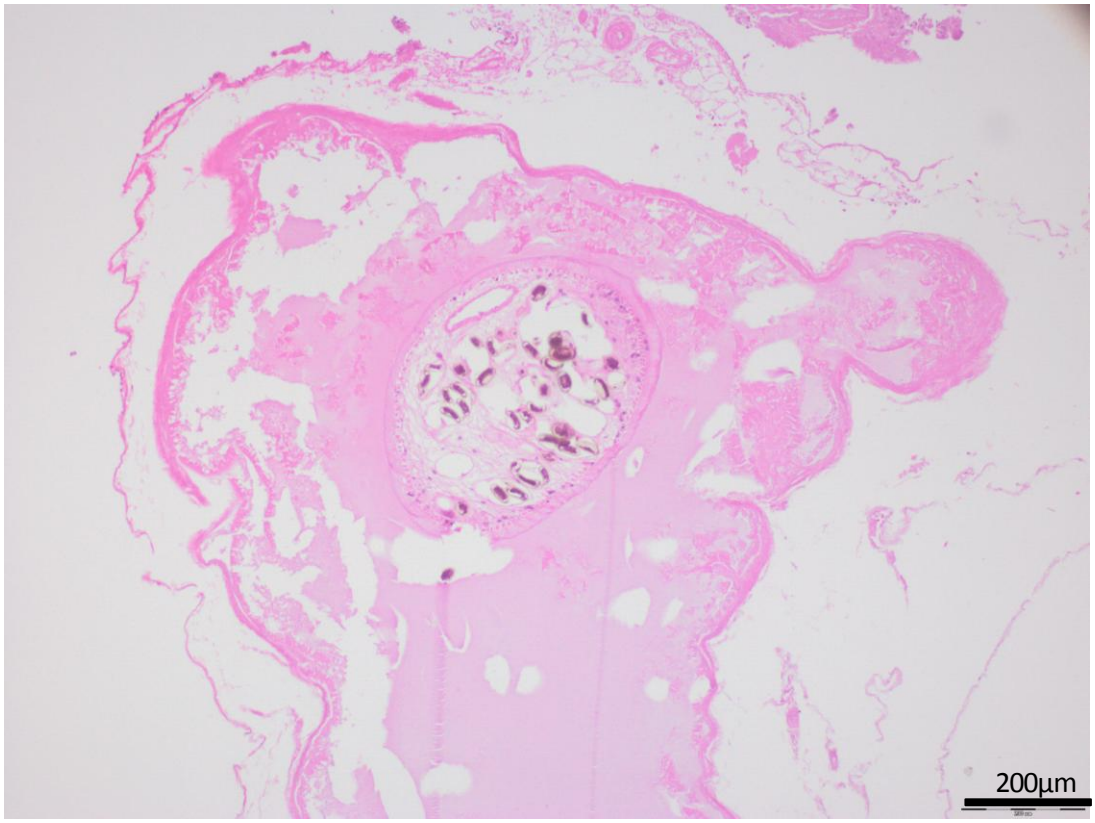

Figure 30: Adult trematodes and eggs in the gallbladder of a song thrush from Palmerston North. H&E (400x). Photograph taken by the author.

4.4 Discussion

The research undertaken in this study is a preliminary investigation into the spectrum of helminth parasites in New Zealand passerine birds. The earlier parasite checklists (Weekes, 1982; McKenna 1998) recorded a few parasites of New Zealand passerines, and although we documented helminths in only a small number of species it became clear that there is likely to be a considerable range of species yet to be identified. Although species identification of these helminths still has to be performed, it is likely that this is the first record of trematodes in native birds as well as three cestode species in tui and saddleback.

4.4.1 Nematodes and Cestodes

The nematodes found in tui and hihi have been provisionally identified as belonging to the group of *Capillaria sensu latu*. According to Anderson

(1992), the classification of the Capillariae is one of the most difficult and unsatisfactory in the phylum nematoda. So far, around 300 species have been described in *Capillaria* in *sensu latu*, and five genera are recognized, *Capillaria, Hepaticola, Thominx, Skrjabinocapillaria and Eucoleus.* Therefore, the final identification of the helminths described in this study might prove to be difficult.

Cestodes have been reported to infect passerine birds in New Zealand before and Weekes (1982) listed several cestodes in introduced birds including blackbird and song thrush. A later report by Johnstone and Cork (1993) mentioned tapeworms in a saddleback, but without any closer description. In 1994, Clemance identified the cestodes found in North Island saddleback as *Choanotaenia infundibulum*, a tapeworm of poultry (Clemance, 1993). In the present study, we were able to find two significantly different cestode eggs in saddleback each from the different sites of Mokoia and Orana Park, suggesting that they might be of two different species of cestode. Both of these are unlike the eggs of *Choanotaenia*. So far, both cestodes in saddleback as well as the one found in tui remain unidentified, as complete adult specimens are necessary for identification. Cestode identification is also challenging and might require consultation with international experts.

Cestode eggs from North Island robin were found in the initial part of this study and were not measured, nor were pictures taken. This was because when the initial samples were examined the focus of the study was purely on coccidia. In addition, the finding of cestode eggs in hihi faeces was made during routine examination in the IVABS parasitology laboratory and therefore neither pictures nor measurements were taken. Further studies are therefore required to recover more samples from both these passerine species.

4.4.2 Trematodes

One of the main findings in the current investigation was the discovery of trematodes in the gall bladders of North and South Island saddlebacks and from the large intestine of tui. This is the first record of trematodes infecting passerines in New Zealand. The trematodes found in the gallbladders of the North island saddleback are likely to be Digeneans of the family Dicrocoeliadae, but the worms found in the tui could not be classified so far. Members of the genera *Lutztrema* (formerly *Brachylecithum*) and *Oswaldoia* have been recorded in the biliary system of a variety of passerines (Binder 1970; Carney 1970) and it is possible that the trematodes recorded in this study belong to either of these genera. These trematodes are common in other parts of the world, for example in the Czech republic and Poland 50% of *Turdus merula, T. pilaris, T. philomelos, Sturnus vulgaris and Sylvia atricapilla* were found to be infected with *Lutztrema attenuatum* (formerly *Brachylecithum attenuatum*) (Sitko et al. 2000). The song thrush examined in the present study presented similar pathological findings as the saddlebacks, the worms visible histologically showed similar morphology and size. The faecal examination of the thrush was negative for trematodes, so the eggs were not recovered in the fresh flotation. The finding that trematode eggs can be identified at post mortem examination but not in faecal samples has been noted before by Robertson (2009) who found that the shedding of trematode eggs in black stilts varied between days, but was not related to hour of the day or temperature. Since the trematode infection was found on Mokoia as well as at Orana Park/ Christchurch, both areas inhabited by song thrushes, and in a song thrush in the Manawatu, it is possible that this trematode species is wide spread in New Zealand and may have been introduced and distributed by thrushes.

Nothing is known so far about the life cycle of trematodes in tui and saddleback. Trematodes always require a minimum of two hosts to complete their life cycle; with a vertebrate being the end host. Further research is required to identify the first host or hosts of these parasites which would most likely be molluscs, insects or other invertebrates. In New Zealand there are

more than 1000 species of land slugs and snails, most are endemic (Daugherty et al. 1993) and include enigmatic species such as the giant carnivorous snails of the genus *Powelliphanta*. Along with the native species there are a few introduced pest species. Both of which could potentially be hosts for trematodes. In Australia, for example, all commonly encountered native and introduced land snails were extensively infected with metacercariae (the second larval stage of trematodes) of *Brachylaima cribbi*, a trematode that infects a wide range of birds(Butcher and Grove 2005). Native birds parasitized by this trematode included little ravens (*Corvus mellori*) and emu (*Cromalus novaehollandiae*) while infected introduced species included black birds, starlings and chickens (*Gallus gallus*). This raises interesting questions about the lifecycles of these worms, particularly in tui which do not normally feed on the ground and are therefore unlikely to ingest any molluscs harbouring trematode larvae. As saddleback often forage for invertebrates close to the ground there is a high likelihood of ingesting infected molluscs. They have also been observed eating slugs on Mokoia (Alana Smith, personal communication). Despite this, the prevalence and diversity of trematodes infecting saddleback on Mokoia is low. The reason for this might be the low availability of the first intermediate host. A ground level invertebrate survey, currently in progress, indicates a low incidence of molluscs including slugs so far (Alana Smith, personal communication). Due to its small size, climate and volcanic origin, Mokoia is a relatively dry island. This might restrict the diversity and number of slugs and snails available to the birds which might in-turn restrict the distribution of trematode species on Mokoia

The eggs of the trematodes found in tui and saddleback also seem to have different development times. The eggs found in saddleback contained clearly visible miracidium larvae whereas larvae were not visible in the eggs from tui. Some trematode eggs develop more rapidly than others and miracidia appear even before the passage through the gut of the host is completed, while in other species the development starts after the eggs are shed from the host (Rommel 2000).

The trematodes in the tui were found in the colon. According to the literature, this is an unusual site and most species either settle in the small intestine or in the caeca and the rectum of birds (Binder 1971, Botcher and Grove 2000, Rommel 2000, Robertson and Alley 2009). The tui in the present study had been dead for several days before the post mortem examination, so this finding might be the result of post mortem changes and the trematodes might originally have been located in the small intestine.

4.4.3 Comparison with findings in Europe

The list of helminth species recovered from native passerines in New Zealand is shorter than that reported in birds from other parts of the world, but there is no obvious reason to believe the number of helminth species present in native birds should be any less. It is therefore likely that more species are still to be found. A different picture has been seen in the introduced bird species. It is worth noting that there is always a chance that animals invading an ecosystem lose some of their parasites ("release") which may increase the invasion success of these introduced species (Prenter et al. 2004).

In blackbirds in New Zealand, three species of nematode and four species of cestode are known according to McKenna (1998). In the song thrush two species of nematode and three species of cestode are mentioned. In comparison, in blackbirds in Germany, a whole range of nematodes has been found, among them three members from the families Syngamidae, Trichuridae and Oxyuridae, as well as the families Ascarididae (*Porrocaecum ensicaudatum*, *P. skrjabinensis*), Spiruridae (*Habronema* spp.) and Acuariidae (*Acuaria spiralis*). The cestodes found in blackbirds in Germany belong to the families Davaineidae (*Fernandezia spinosissima*), Dilepididae (*Dilepis undula, Anomotaenia verulamii, Liga passerum, Choanotaenia spp.),* and Hymenolepididae (*Variolepsis farciminosa, Haploparaxis spec.)*. Trematodes also have been found occasionally, with members of the Dicrocoeliidae (*Oswaldoia petiolatum*), Leucochloridiidae (*Urogonimus macrostomus, Leucochloridium fuscum*) and Brachylaemidae (*Brachylaemus*

sp.). Acanthocephalans have also been found, with the species *Prostorhynchus cylindraceus*, *P. transversus, Centrorhynchus scanensis* and *Sphaerirostris scanensis.* During the annual migration periods, a study of the parasites of the genus *Turdus* has been performed in Spain. Birds of the species *T. philomelos, T. viscivorus and T. iliacus* have been studied in Granada, and 2 species of cestodes, *Dilepis undula* and *Mayhewia serpentulus*, four species of trematodes, *Cyclocoelum mutabile, Cycloprimum exile, Lyperosomum longicauda* and *Brachylaima arcuatus* as well as the acanthocephalan *Prosthorhynchus transversum* parasites have been identified (Diaz and Hierro 2004).

Compared to the helminth fauna found in blackbirds in Germany (Misof 2005), blackbirds in New Zealand have been found to have 40% fewer nematode species and 43% fewer cestode species, and no trematodes and acanthocephalans have been found to date. It is possible, however, that the lack of trematodes and acanthocephalans recorded in New Zealand might be due to insufficient and insensitive diagnostic methods.

The birds examined in this project were infected by parasites from three different groups of helminths of the classes Trematoda (sub-class Digenea) and Cestoda as well as of the phylum Nematoda. Representive samples of the worms found have been documented, measured and preserved for further study. The exact genus and species still need to be identified. The current study has raised the possibility that cross infection of helminths from introduced into native species might occur in some circumstances and in these cases the production of pathological lesions is a possibility. Further work on the exact identification of the helminths involved as well as the knowledge of their life cycles is necessary to understand their importance in the health of passerines in New Zealand.

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Chapter 5.General Discussion

The main aims of this study were to establish baseline data on gastro-intestinal parasites found in some New Zealand passerines, including identifying the species of coccidia infecting these birds as well as recording preliminary data on other gastro-intestinal parasites found. 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 the study a variety of gastrointestinal parasites previously unrecorded in passerine birds in New Zealand were identified. Among these parasites were several different species of coccidia and a variety of helminths yet to be identified. Thus, the overall results of these preliminary studies have made a start in creating an insight into the broad and relatively unexplored field of gastrointestinal parasitology of New Zealand passerines.

5.1 Coccidia

The presence of several different coccidia in each of the New Zealand passerine species is similar to findings in the only bird species that has been studied in depth so far, the chicken (*Gallus gallus domesticus*). In the chicken, a variety of different species of coccidia can infect a single host species, and even a single individual (Augustine 1996; Augustine 1999).

This is also the case in the few passerine species examined so far, for example, canaries (Box 1977), greenfinches (Cringoli and Quesada 1991; Ball, Brown et al. 1998) and several other passerines (Barta, Schrenzel et al. 2005; Dolnik, Palinauskas et al. 2009) have been found to have at least two different species of coccidia infecting them. In this study, phylogenetic analysis has established that, at least two different species of coccidia can be found in the tui, hihi and blackbird. Morphological differences in the sporulated oocysts and the sporocysts found in individual saddlebacks also

support the presence of more than one species; but establishing how many requires more research.

5.2 Helminths

In addition to coccidia, previously unrecorded trematodes were found in the tui and the North Island saddleback, as well as *Capillaria* in tui and hihi, and cestodes in robin, tui and North Island saddleback. Different species of trematodes, cestodes and nematodes infecting passerines have been found worldwide. One group of helminths that is commonly recorded overseas, the Acanthocephalans, was not detected in this study. Two explanations for this absence come to mind. First, the eggs of acanthocephalans are difficult to detect with flotation methods. Second, this study only included a small number of introduced birds which are known to be infected with these helminths overseas. Further studies using other diagnostic methods should be carried out to determine if acanthocephalans are present in New Zealand.

The observed 5% infection rate with trematodes in saddleback is low compared to infection rates of European birds of up to 50% (Sitko, Okulewicz and Noga, 2000). In addition the diversity of trematodes we found in saddleback is not as large as in passerines in other parts of the world, where several different worm species are found in one bird species. This finding might be associated with the difficulty in detecting trematode eggs with flotation methods, as well as the timing of faecal collection. Robertson (2009) observed that the shedding of trematode eggs varies between days in black stilts (*Himantopus novaezelandiae*). She also reported that greater masses of faecal material, as provided by pooled samples, are much more sensitive than small samples from single birds for detecting trematode infection, but pooled samples will not provide information on prevalence. Robertson (2009) used sedimentation methods for trematode diagnosis and observed a low egg recovery rate. The amount of faecal material from black stilts recovered by Robertson (2009) was certainly larger than the small amounts of around 0.2-0.5g in passerines collected for this study, and therefore this would reduce the likelyhood of detecting small numbers of eggs. Another possibility

could be that the trematodes found in saddleback were introduced with European birds, and the saddleback is a less than optimal host for these parasites, which might reduce the egg output. .

Another consideration is the low number of trematode species found, North Island saddleback being a rare species that has undergone several extreme population bottlenecks, could have been depleted of its parasites. As mentioned by Rozsa (1992), a major decrease in numbers of the host population, as happened with both saddleback sub-species in New Zealand, will also endanger the parasites inhabiting the ecosystem “host”, and these parasite species might have disappeared before being studied.

Generally, helminths do not seem to affect the examined birds severely in the wild, but might become a problem if the birds are held in captivity or quarantine for long time periods, as exemplified by the death of a saddleback from a captive facility at Orana Park (Christchurch) that died from intestinal rupture associated with a massive infestation with different helminths. Nematodes of the genus *Porrocaecum* also caused deaths in saddleback on Tiritiri Matangi (Alley et al. 2007).

A song thrush was also found which showed a gallbladder infestation with trematodes similar in size and appearance to the parasites found in saddlebacks. This finding raises the question as to whether these helminths were introduced to New Zealand together with passerines from Europe. Trematodes are generally not host specific, and can exist in a range of hosts, even spanning classes of animals like mammals and birds, so it is possible that trematodes from introduced birds also infect native and endemic species. For example, *Brachylaima cribbi*, an introduced trematode in Australia, uses many different bird species, ranging from chickens and emus to passerines, as end host (Butcher and Grove 2005), but can also be found in humans (Butcher and Grove, 2001). Similar ranges in end hosts in various birds can be seen in trematodes of the genus *Lutztrema* (formerly *Brachylecithum*) (Carney, 1970). Trematode infection of the gallbladder was found on Mokoia as well as in Orana Park/ Christchurch, both areas

inhabited by song thrushes, and in a song thrush in the Manawatu, so it is possible that this trematode species is widespread in New Zealand and distributed by thrushes. Clearly a first step is to establish whether or not the trematodes from the two hosts are the same species. The possibility that helminths of introduced birds may infect native fauna has been encountered before in New Zealand, with nematodes of the genus Porrocaecum which are known inhabitants of the gastrointestinal tract of starlings (*Sturnus vulgaris*) and blackbirds infecting saddlebacks.

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

A major concern when translocating rare birds is optimising their chances of survival. This is entirely understandable given the small numbers of birds usually available and the large investment of time and money made in raising them. It is usual, therefore, to carry out screening health tests including checking for parasites and treating those found infected. This raised a number of questions about the effectiveness of this practice in terms of both the sensitivity of diagnostic procedures and the effectiveness of any drug treatments It cannot be assumed that drugs effective in one host-parasite system will work equally well in another. This is illustrated by the study of the translocation of 40 North Island saddleback from Mokoia Island to a New Zealand mainland island, Bushy Park, near Wanganui, in which it was found that anti-coccidial treatment did not affect the shedding of oocysts and the survival of the birds post release (Thorne 2007).

Although the effects of treatment on gastrointestinal parasites and their passerine hosts were not examined in this study, the results mentioned above raise questions as to whether these treatment regimes are a necessary cost of translocations and whether their reduction or extinction might be preferable. Together with the questionable success of these treatment regimes, there is also the issue of whether the extermination of the gastro-intestinal parasites of these birds is desirable. Hudson et al. (2006)

have proposed that a healthy ecosystem is one that is rich in parasites. According to these authors, parasite-mediated effects on the ecosystem could be important. For example parasites influence a range of ecosystem functions, have effect on food webs, host population dynamics, change interspecific competition and also seem to be important drivers of biodiversity. As Rozsa (1992) mentioned, parasites are not just to be regarded as undesirable agents, but as species with their own evolutionary value. This is exemplified by host species that have been saved without their specific parasites and therefore cannot maintain their intraspecific genetic diversity. In addition, Rozsa (1992) and Perez et al. (2006) both point out that the rare status of an endangered host can threaten the existence of the parasites inhabiting that host. In this context, indiscriminately treating all endangered native birds and exterminating their specific parasite fauna will diminish the biodiversity in New Zealand as well as threaten the survival of the host species in the long run due to diminishing the genetic diversity in the hosts. That the majority of parasitic infections in wild passerines do not lead to overt disease is another consideration. Achieving the most appropriate balance between these competing considerations is undoubtedly difficult and warrants careful weighing of the pros and cons.

5.4 Further Research

Further studies are necessary in order to gain a better picture of the diversity of gastrointestinal parasites of passerines in New Zealand and whether these parasites significantly affect the health, survival, or reproductive ability of their hosts.

In the light of our results, additional critical questions need to be addressed. Firstly, what is the life cycle of these parasites, in particular what are the reservoir species? Secondly, what is the prevalence and the shedding rhythm of the passerine coccidia found in New Zealand avifauna? Thirdly, are the gastro-intestinal parasites found native or introduced, and in the case of the helminths, which families, genera and species are present?

5.4.1 Potential life cycle Investigations

To investigate further the life cycle of coccidia in hihi, the examination of hihi (and other species) blood by molecular methods would be a fruitful line of inquiry. This would determine if it is possible to find extra intestinal stages in the blood of highly affected birds showing symptoms of disease, for example weight loss and/or a high faecal count over 10000 eggs/g. If such stages can be detected, this may facilitate the diagnosis of coccidiosis in birds that are not or not yet shedding oocysts in the faeces. (Dolnik et al. 2009) demonstrated that at least some haplotypes of *Isospora* spp. found in their research had extra intestinal stages in the blood, as described in canaries by (Box 1975). Some of these parasites did not complete their life cycle at once, so oocysts showed up in the faeces at a later stage.

In the case of the trematodes found in tui and saddleback, nothing is known so far about the life cycle of these worms, raising interesting questions as to how the birds become infected. Trematodes need at least 2 hosts to complete their life cycle. The first obligate host is always a mollusc, and in case of passerines, the bird is the final host in which sexual reproduction of the parasite occurs. Further research could help to identify the first host or hosts of these parasites, most likely molluscs, insects or other invertebrates.

Further work also needs be done on the range of vertebrate hosts of these worms. There is a striking similarity between the adult trematodes found in saddleback (order passeriformes) and trematodes found by Robertson (2009) in her work on gastrointestinal parasites in black stilt (*Himantopus novaezelandiae* of the order charadriformes), birds belonging to two different orders of birds (Ian Scott, pers. comm. IVABS/ Parasitology). Archived formalin fixed material from black stilt used in this research is still available at IVABS, which could be used for another study comparing recently found passerine trematodes with the ones from black stilts.

5.4.2 Potential shedding rhythm experiments

Studies in a variety of passerines have shown that coccidian oocysts are shed in a diurnal cycle, with faecal counts highest in the late afternoon (Hudmann, Ketterson et al. 2000, Brown, Ball et al. 2001; Misof 2004; Lopey, Figuerola et al. 2007; Cassey 2008). Unfortunately the time of day when the faecal samples were collected in this study was not recorded, something that could have a considerable effect to the faecal oocyst counts, as noted by (Brawner 1999). Collecting samples at all times of the day means that the results from this study must be viewed with caution (Lopez, Figuerola et al. 2007) in terms of both detecting and quantifying coccidia. Although it is as yet not known if coccidia in passerines native to New Zealand follow the same diurnal cycle, there is no obvious reason to expect the contrary. Therefore, one possible field of future study would be to determine if saddleback and other native New Zealand passerines also show diurnal shedding of coccidial oocysts.

5.4.3 Potential phylogenetic relationships

The molecular findings presented in this study have to be seen as preliminary because there is a lack of sequence information available with which new sequences can be compared. Consequently, it is difficult to make biological sense of some of the phylogenetic findings in this study. For example coccidia from tui showed a 94-99% similarity to *Eimeria* species in bats and rodents, a closer relationship than to species known in passerine species from North America and Asia (Schrenzel, Maalouf et al. 2005). So far, only few coccidia of wild animal species, birds and mammals alike, have been sequenced, therefore the significance of this finding awaits further work in this field. No work has been done on the genetics of passerine coccidia in Australia and New Zealand before, and a comparison between coccidia of Australia and New Zealand as well as the comparison between the already obtained sequences and coccidia from New Zealand native bats might explain the unique relationships between these species. Considering the

geological and geographical relationship between New Zealand and Australia, there is clearly considerable scope for comparative studies of coccidia from passerines in the two countries. An additional and interesting challenge in New Zealand is the number of introduced passerines that occur here and comparison of their coccidial parasites with those in their place of origin.

There is much to be done in New Zealand, for example to establish the identity of coccidia species found in silvereye and fantail, as PCR and sequencing have failed to yield any results so far. The examination of further saddleback faecal samples to get an overview of the diversity of coccidia species infecting these birds would also be valuable.

In Fantail, a PCR followed by sequencing may be carried out to investigate the presence of *Caryospora,* as described in (Barta 1997; Barta, Martin et al. 2001).

In the hihi, further molecular examinations are required on extraintestinal material, for example liver tissue containing coccidian parasites. So far, the attempt at sequencing has not produced conclusive results, and specifically designed primers for these parasites will have to be developed. If this can be achieved it will help in identifying the life cycle of coccidia in hihi and passerine birds in general as well as helping with diagnosis, treatment and care, especially because extra intestinal stages of coccidia cannot be found by only examining faecal samples, because only sexual products (oocysts) are shed.

The helminths found in this research still need to be identified, both morphologically and with the help of molecular methods. The trematodes found in this study are likely to be digeneans of the family dicrocoeliadae. Members of the genus *Lutztrema* (formerly *Brachylecithum*) and *Oswaldoia* have been recorded in the biliary system of a variety of passerines (Binder 1970; Carney 1970) and it is possible that the trematodes recorded in this study belong to one or the other of these genera.

This further research will increase the knowledge of diversity, prevalence, life cycle, pathology and potential threats of gastrointestinal parasites in passerine birds in New Zealand. In time it will help with the diagnosis, treatment and general management of passerine species in captivity, in the wild and in translocation programmes.

Last but not least it needs to be recognized that parasites are part of the biodiversity in New Zealand's native fauna, a part that is hardly known and understood and also at risk of disappearing before anything is known about them.

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Appendix

Appendix 1

Appendix 1, Table 12: Descriptive statistcs of two types of coccidia oocysts found in hihi (Twentyman, 2001)

	Type A length	Type A width	Type A length/ width ratio	Type B length	Type B width	Type B length/width ratio
N	26	26	27	21	21	21
Mean	20.03	18.71	1.07	24.46	19.63	1.25
SD	1.72	1.86	0.05	2.08	1.54	0.09
SE Mean	0.34	0.37	9.96E-03	0.45	0.34	0.02
Minimum	16.4	15.36	1	18.8	15.44	1.11
Maximum	22.96	22.08	1.16	28.4	21.84	1.44

Appendix 2

Avian Necropsy Technique

Maurice Alley
Institute of Veterinary, Animal and Biomedical Sciences, Massey University

Field necropsies have the advantage of providing some early information on the nature of a mortality (e.g. is it trauma or a toxic or infectious disease). They will also allow the collection and rapid preservation of samples before further decomposition occurs. Ideally however, the aim of the investigator should be to deliver whole birds (that have been cooled and have not decomposed), to the laboratory to be examined by a trained avian pathologist.

History

Before beginning the examination it is important to obtain a good history. This should include morbidity and mortality rates (when applicable), clinical signs of the affected birds as well as the nutrition and location of dead and affected individuals.
If the bird is alive, the clinical signs should be carefully observed and recorded as these may assist in making a diagnosis.
Blood samples in serum and EDTA tubes can be collected from the wing, jugular or leg veins.

The method of euthanasia will depend on the circumstances and available materials. Intravenous barbiturate or inhaled gas (anaesthetic, CO or CO_2) can be used. It should be remembered that if the bird is killed by cervical dislocation or a blow to the head, this will produce traumatised tissue in these areas which may mask any underlying pre-existing lesions.

External Examination

Identify the individual and record the leg bands, age, species etc.
Weigh and record the weight of the intact bird.
Record the body condition score.
Examine the plumage for evidence of loss of waterproofing, wounds and abrasions, scales, nodules and external parasites.
All body orifices should be examined for discharges, ulcers etc and may be swabbed at this stage if required.
Palpate the limbs and neck noting the presence of any swellings or evidence of trauma .
Lay the bird on its back and smooth the feathers of the ventral neck, thorax, abdomen and legs with detergent (or soapy water) to allow a clean dissection of the internal organs.

Internal Examination

Using forceps and scissors, incise the skin and abdominal wall immediately beneath the sternum and extend the incision transversely across the abdomen taking care to avoid cutting the internal organs. Extend this incision caudally down the midline to the cloaca and examine the abdominal airsacs and ventral surface of the viscera.
Remove the skin from the pectoral muscles and extend this skin incision cranially along the ventral neck to the lower beak.
Return to the abdomen and with a strong pair of scissors or bone forceps (depending on the size of the bird) cut through the ribs at the costochondral junctions and then through the coracoid bones on both sides. Reflect the sternal musculature away from you to expose the heart, thoracic airsacs, liver and gizzard. The thickness of the pectoral muscles and amount of subcutaneous and abdominal fat are good indicators of the nutritional state of the bird (wide seasonal variations are expected in migrating birds). The abdominal and thoracic airsacs should be clear. If any abnormalities (cloudiness, exudate, plaques or nodules) are noted, consider collecting samples for microbiology and histology.
Examine the pericardial sac and thyroid glands located near the thoracic inlet and remove remove and examine the heart.
Using a pair of forceps, grasp the gizzard and pull it together with the liver, caudally and upwards. Cut through the proventriculus cranially and carefully remove the entire gastrointestinal tract from the body leaving behind the genital organs, adrenals and kidneys. In small birds the bursa of Fabricus and cloaca can also be removed attached to the colon.

Inspect the liver and free it from the gizzard and intestine then locate and examine the spleen and pancreas.
The gastrointestinal tract can now be straightened and each part can be opened starting with the proventriculus and gizzard. The nature of the contents should be noted and smears or mucosal scrapings may be taken and examined for the presence of parasites.
The bursa of Fabricus, adrenal glands, urogenital tract and kidneys can now all be examined *in situ* and removed if necessary for further examination.
Extend the ventral neck incision forward into the oral cavity by cutting through one side of the beak and mandible. The oral cavity, tongue and oesphagus can now be examined and mucosal smears taken for parasite examination.
The respiratory system can be examined next beginning with the nasal cavity and infra-orbital sinuses. Cut down the full length of the trachea with scissors then free each lung from the thoracic wall and examine dorsal and ventral surfaces.
Carefully remove the skin from the cranium, neck and dorsal surface of the thorax and lumbar regions noting the presence of any penetrating wounds, haemorrhage or contusions.
The method of examining the brain will depend on the size of the bird. In large or medium sized birds, the dome of the skull can be removed with a fine saw or strong scissors beginning and ending with foramen magnum. In smaller birds, a midline incision can be made through the entire head using a sharp knife or scalpel working from the ventral surface of the hard palate.
When examination of the spinal cord is required it is usually best to fix the vertebral column, freed of muscle in formalin for subsequent removal or decalcification.
Check all the bones and joints of the wings and legs by palpation and open at least 2 major joints to examine their articular surfaces.

Collection of Specimens for the Laboratory

Label all specimens for laboratory examination carefully and preserve them according to the requirements of the laboratory involved. It is easier to discard excess specimens than be faced with the embarrassment of having inadequate samples to reach a diagnosis.

Microbiology:- Samples of fresh tissue or microbiological swabs in transport media should be kept at 4C and dispatched in a cooled chilli bin as soon as possible after collection. Always place each tissue in a separate container for transportation.

Histopathology:- Samples of all visible lesions together with < 5mm slices of liver, spleen, lung, heart, kidney, muscle, and in the case of smaller birds, the entire gastrointestinal tract including the bursa should be fixed in 10% formol saline. The brain can be left whole and submitted *in situ* provided the cranium has been opened. Try to use 10x as much formalin as tissue for fixation although reduced amounts can be used during transportation. Plastic bottles inside sealed plastic bags can be used for holding tissues. Tissues are of little or no use for histopathology once they have been frozen.

Parasitology:- Blood parasites are best identified in air dried blood smears made from a live or freshly dead birds. Worms, flukes and mites can be sent to the laboratory fresh, in saline, or preserved in 70% alcohol or 5% formalin.

Toxicology:- The samples required for chemical analysis will vary according to the toxin suspected. In general, samples of liver, gastrointestinal contents, kidney, muscle, body fat, brain, serum and whole blood are adequate for most toxins. Generous tissue samples should be placed in clean, separate containers before freezing.

DNA analysis:- This can be undertaken on small samples of fresh or frozen tissue. If preservation is required, a slice of muscle can be placed in 70% alcohol. Never fix tissues for DNA analysis in formalin.

Examinations Requiring Limited Equipment

Wing fat analysis:- The last reserves of fat to be utilised by starving birds are those reserves present in the bone marrow of peripheral bones such as the ulna bones of the wings. Following predation or when the carcass has been extensively scavenged, the wing bones may still be left intact. Analysis for fat by the method of Moore and Battley (Notornis 2003,50:133) can at this stage still provide useful information on the nutritional state of the bird**.**

Cytology:- Smears made from lesions or from skin and mucosal scrapings may provide information on the presence of parasites when examined under a microscope. Quick and simple stains such as Difqik can be used to identify cell types and bacteria.

Appendix 3

Avian Body Condition Scores used at Necropsy

(M.R.Alley, National Wildlife Health Centre, Massey University, Palmerston North New Zealand)

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Score	Necropsy Findings
1	Severely emaciated. No body fat reserves (including bone marrow), severe atrophy of skeletal muscles (severe concavity of pectoral muscle*) and atrophy of internal organs eg. GI tract and liver.
2	Emaciated. No body fat reserves (including bone marrow), atrophy of skeletal muscles (concavity of pectoral muscles
3	Very thin. Very little body fat reserves (eg..in thoracic inlet and bone marrow). Mild concavity of pectoral muscles
4	Thin. Small fat reserves in thoracic inlet and omentum. Pectoral muscle flat.
5	Average. Moderate fat reserves in thoracic inlet, omentum and subcutaneous tissues. Pectoral muscle slightly rounded.
6	Good. Good fat reserves in thoracic inlet, omentum and subcutaneous tissues Pectoral muscles firm and rounded.
7	Very Good.. Very good fat reserves in all depots. Pectoral muscles, firm and convex.with a sternal depression palpable
8	Obese. Excessive fat reserves in all depots including subcutaneous issues of thorax and flanks.
9	Severely obese. Excessive fat reserves in all depots including subcutaneous issues of thorax and flanks Fatty liver.

*Not seen in flightless birds

M.R.Alley

Appendix 4

Individual measurements of coccidia oocysts and sporocysts

Appendix 4, table 13: Measurements of unsporulated oocysts from a North island robin in µm

oocyst length	oocyst width
17.5	17.5
17.5	17.5
18.75	18.75
17.5	17.5
20	20
17.5	17.5
15	15
16.25	16.25
17.5	17.5
17.5	17.5
17.5	17.5
17.5	17.5
20	20
17.5	17.5
17.5	17.5
20	17.5

Appendix 4, table 14: Measurements of oocysts from tui in µm

Bird	Condition	oocyst length	oocyst width	sc1 length	sc1width	sc2 length	sc2width
1	0	25	16				
1	0	30	20				
1	0	30	25				
1	0	30	25				
1	0	20	20				
1	0	30	25				
1	0	30	25				
1	0	25	25				
1	0	30	20				
1	0	25	25				
1	0	30	25				
1	0	30	25				
1	0	25	20				
1	0	30	20				
1	0	30	25				
1	0	30	25				
1	0	30	25				
1	0	30	25				
1	0	30	25				
1	0	30	25				
1	0	30	25				
1	0	30	25				
1	0	30	25				
1	0	25	25				
1	0	30	25				
1	0	30	25				
1	0	30	25				
1	1	25	25	15	12.5	15	12.5
1	1	30	25	15	15	15	15
1	1	30	25	20	15	20	15
1	1	30	25	20	10	20	10
1	1	30	25	25	10	25	10

Appendix 4, table 15: Measurements of oocysts and sporocysts (sc) from North Island saddleback in µm. Condition: 0 unsporulated, 1 sporulated.

Bird	Condition	oocyst length	oocyst width	sc1 length	sc1width	sc2 length	sc2width
1	1	25	25	15	10	20	12.5
1	1	25	25	15	10	15	10
1	1	25	25	15	10	15	10
1	0	25	15				
1	1	25	25	20	12.5	17.5	12.5
1	1	22.5	22.5	15	10	15	12.5
1	0	25	22.5				
1	1	25	22.5	17.5	10	15	10
1	1	27.5	25	17.5	10	15	10
1	1	22.5	22.5	15	10	17.5	10
1	1	25	20	12.5	10	15	10
1	1	25	20	15	7.5	12.5	10
2	0	20	17.5				
2	0	20	15				
2	0	20	20				
2	0	20	17.5				
2	0	20	20				
2	0	25	20				
2	0	20	20				
2	0	20	20				
2	0	20	20				
2	0	20	20				
2	0	17	15				
2	0	20	20				
2	0	20	20				
2	0	15	15				
2	0	17.5	15				
2	0	20	20				
2	0	20	15				
2	0	20	15				
2	0	20	17.5				
2	0	20	17.5				
3	1	17.5	15	7.5	7.5	7.5	7.5
3	0	17.5	15				
3	1	20	17.5	10	7.5	12.5	7.5
3	1	20	17.5	7.5	7.5	7.5	7.5
3	1	20	15	7.5		7.5	5
3	1	20	15	7.5		7.5	5
3	1	20	17.5	10	7.5	10	7.5
3	1	17.5	15	7.5	5	10	7.5
3	1	20	15	7.5	5	7.5	7.5
3	1	20	15	10	7.5	7.5	7.5
3	1	20	17.5	10	7.5	10	7.5
3	1	17.5	15	10	7.5	10	7.5

Appendix 4, table 16: Measurements of blackbird oocysts and sporocysts (sc) in µm. Condition: 0 unsporulated, 1 sporulated.

Condition	oocyst length	oocyst width	sc1 length	sc1width	sc2 length	sc2width
0	17.5	20				
0	20	20				
0	17.5	17.5				
0	20	17.5				
1	20	20	10	7.5	15	7.5
1	20	20	15	10	15	10
1	20	17.5	15	10	15	10
1	20	15	15	7.5	15	10
1	20	15	15	10	15	10
1	17.5	17.5	15	7.5	15	7.5
1	17.5	15	12.5	7.5	15	7.5
1	17.5	15	10	7.5	10	7.5
1	20	17.5	12.5	10	12.5	10
1	20	17.5	10	10	10	10
1	20	17.5	15	10	12.5	10
1	17.5	15	12.5	7.5	12.5	7.5
1	20	17.5	15	10	15	10
1	17.5	15	10	7.5	10	7.5

Appendix 4, table 17: Measurements of oocysts and sporocysts (sc) from a silvereye in µm. Condition: 0=unsporulated, 1=sporulated

Bird	Condition	oocyst diameter	sc1 length	sc1width	sc2 length	sc2width
1	0	27.5				
1	0	28.75				
1	0	27.5				
1	0	26.25				
1	0	26.25				
1	0	28.75				
1	0	27.5				
1	0	27.5				
1	0	27.5				
1	0	27.5				
1	0	25				
1	0	27.5				
1	0	25				
1	0	27.5				
2	1	30	17.5	12.5	20	10
2	1	28.75	20	10	18.75	12.5
2	1	27.5	18.75	12.5	17.5	10
2	1	28.75	17.5	12.5	15	11.25
2	1	30	20	11.25	17.5	10
2	1	25	15	10	15	10
2	1	27.5	20	12.5	17.5	12.5
2	1	27.5	20	12.5	17.5	12.5
2	1	28.75	17.5	12.5	17.5	11.25
2	1		17.5	12.5	20	12.5

Appendix 4, table 18: Measurements of oocysts and sporocysts (sc) from a fantail in µm.

Bird	Condition	oocyst length	oocyst width	sc1 length	sc1width
1	1	20	17.5	16.25	12.5
1	1	20	17.5	17.5	11.25
1	1	20	16.25	17.5	12.5
1	1	18.75	17.5	12.5	12.5
1	1	17.5	17.5	13.73	12.5
1	1	20	17.5	17.5	12.5
1	1	21.25	15	15	10
1	1	17.5	17.5	16.25	12.5
1	1	17.5	17.5	15	12.5
1	1	20	17.5	17.5	12.5
1	1	20	17.5	17.5	12.5
1	1	18.75	17.5	17.5	12.5
1	1	20	17.5	17.5	12.5
1	1	20	17.5	17.5	12.5
1	1	21.25	16.25	17.5	12.5
1	1	20	17.5	17.5	13.75
1	1	20	17.5	18.75	12.5
1	1	20	17.5	17.5	12.5
1	1	20	17.5	17.5	12.5
1	1	20	17.5	17.5	13.75
1	1	20	18.75	17.5	12.5

Appendix 5

Individual measurements of helminth eggs

Appendix 5, table 19: Measurements of Capillaria eggs of hihi in µm.

length	width
71.25	27.50
68.75	28.75
66.25	30.00
67.50	31.25
70.00	28.75
70.00	28.75
66.25	30.00

Appendix 5, table 20: Measurements of helminth eggs from tui in µm

Trematode		Cestode	
length	width	length	width
20	12.5	32.5	30
22.5	11.25	42.5	22.5
22.5	12.5	31.25	25
22.5	12.5	27.5	28.75
20	12.5	30	25
22.5	12.5	25	25
22.5	13.75	30	27.5
20	12.5	28.75	27.5
22.5	12.5	22.5	25
25	11.25	28.75	22.5
22.5	13.25	27.5	25
23.75	12.5	27.5	21.25
22.5	13.25	25	28.75
22.5	10	32.5	27.5
22.5	12.5		
20	12.5		
25	15		

Appendix 5, table 21: Measurements of helminth eggs from North Island Saddleback in µm

Trematode	
length	width
42.5	20
25	16.25
32.5	16.25
47.5	20
46.25	20
45	17.5
47.5	10
45	20
45	22.5
42.5	23.75
45	17.5
45	22.5
42.5	22.5
42.5	21.25
47.5	22.5
45	20
42.5	20
42.5	21.25
31.25	21.25

Cestode (Mokoia)/		Cestode (Orana Park)/	
length	width	length	width
45	37.5	75	42.5
45	32.5	70	37.5
50	40	65	37.5
40	32.5	70	35
40	32.5	75	37.5
40	37.5	40	37.5
45	37.5	70	40
45	40	50	40
37.5	35	42.5	37.5
40	37.5	75	37.5
40	32.5	77.5	37.5
40	37.5	75	37.5
55	42.5	75	35
45	37.5	50	37.5
37.5	30	67.5	35
57.5	35	87.5	40
		62.5	32.5
		80	37.5
		40	37.5
		62.5	33.75

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