Nematodiasis and larval migrans in Kiwi (*Apteryx* spp.)

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Nicki van Zyl

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Abstract

The aim of this thesis is to investigate the inter-relationships between host and parasite specificity and aberrant larval migration, using the kiwi family (*Apteryx spp*) and their nematodes as the study system. Kiwi are endemic to New Zealand, and have been estimated to have declined by 90% since human settlement in New Zealand. The effect of disease, mainly parasitism on kiwi populations have been a minor issue compared to predation, however with intensive conservation management the effect of parasites on kiwi are likely to increase. Very little is known about parasites in kiwi and how they impact the birds, especially the aberrant larval migrans syndromes.

I determined the prevalence of nematodes in all kiwi species from 1991-2012 (n=642) that were necropsied at Massey University, using the National Wildlife Pathology Database (Huia), with a specific focus on larval migrans. Brown kiwi (*Apteryx mantelli*) were found to have the highest prevalence of nematodiasis, and were also the only species to show evidence of neural larval migrans. Visceral larval migrans were found in brown, rowi (*A. rowi*) and little spotted kiwi (*A. owenii*). With the brown kiwi showing the highest incidence of larval migrans, I focused on this species for further investigation. It has previously been proposed that the larval migrans could be caused by nematodes from the *Toxocara* spp, since wild kiwi can share habitats with wild cats and dogs, which are the normal host of this nematode). However, with specific PCR analysis conducted on archived tissue, I concluded that neither *Toxocara cati* or *T. canis* was present in the tissues. I also investigated the cause of cutaneous larval migrans (CLM) in rowi. Uniquely, CLM has not been previously recorded in any avian host. A generic nematode PCR analysis followed by DNA sequencing were used in this study to identify the nematode involved in CLM as being closely related to a nematode from the *Trichostrongylus* species.

My results suggest that introduced animal hosts are having an indirect effect on our native bird populations, but to what extent is still unknown. The parasites transmitted between the introduced species and our native species are causing adverse effects to our native populations' health, in the case of larval migrans; but how often are these introduced parasites infecting our native population and could our native species cope with this competition? From my study it can be presumed that our native species are struggling to adapt to new parasites, and competition between introduced and native species could be contributing to the decline in our species.

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Preface

This thesis has been written and organised as self-contained chapters that will act as submissions to peer-reviewed scientific journals. Because of this, individual chapters will contain unavoidable repetition. This thesis is the original work of the author, unless stated otherwise in the references, methods and acknowledgments.

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Chapter 1. General Introduction

1.1 Ecology and current status of kiwi

Kiwi (*Apteryx* spp) are endemic to New Zealand (Sales, 2005). They are nocturnal birds that live mostly in native forests around New Zealand, however they can also be found in pasture, low land tussock and even sand dunes (Sales, 2005). Kiwi probe the forest floor for invertebrates and plant material with their highly specialised long bills (Cunningham et al., 2007).

Kiwi have existed in their current form since at least 65 million years ago (Holzapfel et al., 2008). The decline in the abundance and distribution of kiwi have gone largely unnoticed until two decades ago (McLennan & Potter, 1992; Sales, 2005). All five species, however, have been declining significantly in range and abundance ever since there has been human settlement in New Zealand (McLennan et al., 1996). For example, kiwi abundance has declined by at least 90% in the North Island forests. It is suggested that former forest densities consisted of 40-100 adults km^2, whereas present densities are about four adult kiwi per km^2 (McLennan et al, 1996). This has led to all kiwi species having the status of being 'threatened' and some are 'critically endangered' (Miskelly et al., 2008; Sales, 2005). The main causes of decline in these populations are predation due to a variety of introduced mammalian predators as kiwi have evolved in the absence of mammalian predators. However, today they co-exist with many obligate or facultative carnivores that have been introduced by Europeans and Polynesians (Sales, 2005). Some of their carnivorous predators include cats, dogs, ferrets, pigs, possums, stoats and weasels (Sales, 2005). The effect of disease and, in particular, parasitism on kiwi populations has been minor in comparison to the rates of predation, but as the intensive management of remnant populations increases, the effect of parasites on the success of conservation efforts are likely to be increasing (Holzapfel et al., 2008).

1.1.1Taxonomy

Kiwi were first described in the early 19th century, and since then they have been divided into as many as ten different species. This division of species was primarily based on morphological features such as colouration and feathers (Potts, 1872). However, with the development of technology and the ability to carry out genetic testing, this has prompted a change in how we recognise different species of kiwi (Burbidge et al., 2003). There are now five formally described species of kiwi (Burbidge et al., 2003; Sales, 2005); the brown kiwi (*Apteryx mantelli*), rowi (*A. rowi*), tokoeka (*A. australis)*, great spotted kiwi (*A. haastii)* and the little spotted kiwi (*A. owenii*) (Holzapfel et al., 2008). The brown kiwi is found throughout the North Island; Rowi at Okarito; Tokoeka in Fiordland, Haast Ranges and on Stewart Island/Rakiura; Great spotted kiwi in the northern South Island; and the Little spotted kiwi which is on several offshore island and at the Karori Wildlife Sanctuary in Wellington (Burbidge et al., 2003; Holzapfel et al., 2008). The distribution of kiwi around New Zealand is shown in figure 1.1 (Holzapfel et al., 2008).

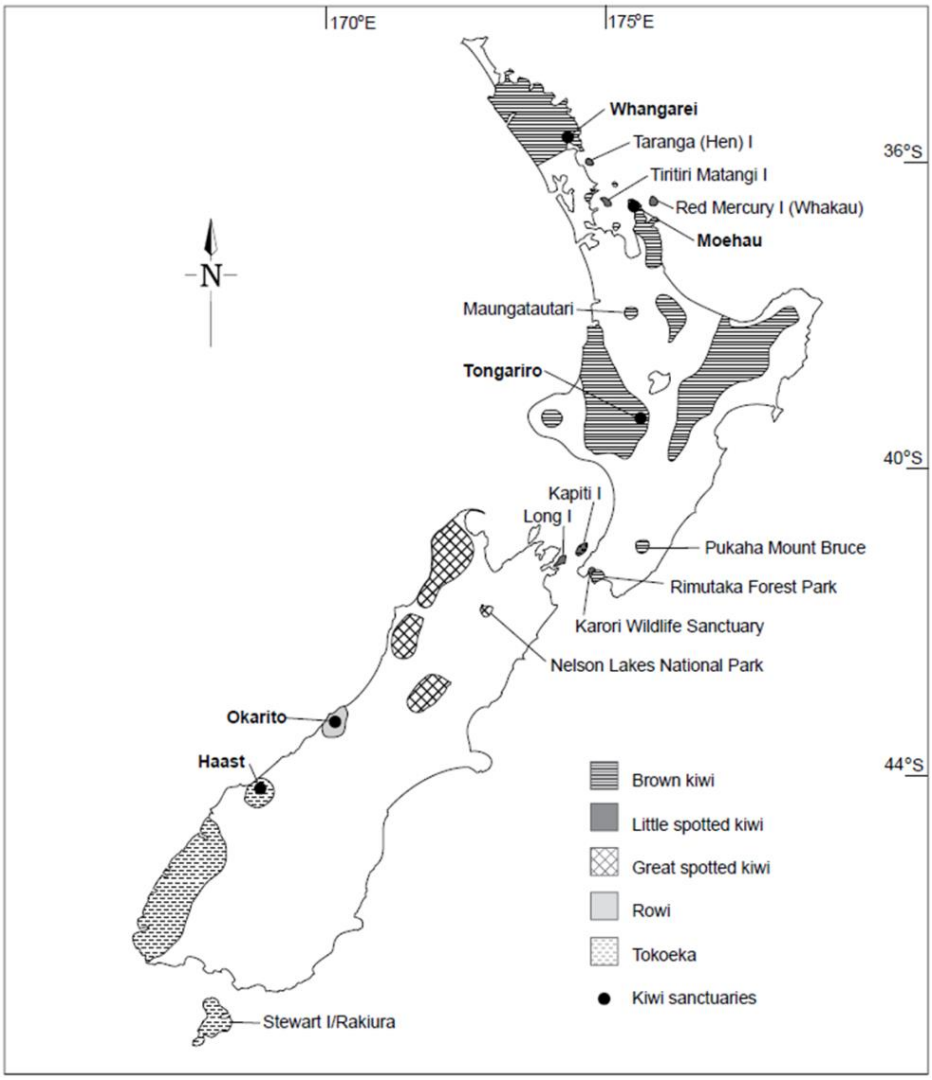

Figure 1.1 *The distribution of kiwi in New Zealand. The locations of five kiwi sanctuaries are also noted (Holzapfel et al., 2008).*

Within the brown kiwi species, four genetically and geographically distinct forms can be identified: Northland, Coromandel, Western and Eastern forms; and in tokoeka there are the Haast, northern Fiordland, southern Fiordland, and Stewart Island forms (Burbidge et al., 2003; Holzapfel et al., 2008). The other species do not show a clear genetic variation among geographic lines, although it has been shown that a North Island form of the little spotted kiwi did exist prior to its extinction in the late 1800s (Holzapfel et al., 2008).

1.2 Conservation management

The distribution and numbers of all five species of kiwi have declined substantially since the occupation of Europeans in New Zealand (Colbourne et al., 2005). Less than 5% of kiwi chicks survive into adulthood in unmanaged areas (McLennan et al., 1996). The most critical factor affecting survival and security of kiwi populations on the mainland of New Zealand is predation by stoats and in some places cats (especially to kiwi that are less than six months old) (Colbourne et al., 2005).

The general aim of kiwi conservation has until recently been to prevent the extinction of as many species as possible (Hunter Jr & Gibbs, 2009; Soulé, 1985). While this goal is desirable, it is becoming clear that more and more species will need to be managed intensively and therefore more pragmatic triage decisions need to be based around this. For instance, only the Eastern brown kiwi is now being bred in captivity. Kiwi are a national icon to New Zealanders and this has led to intensive management to help preserve this species.

Several experimental management projects have been carried out to protect kiwi populations. These generally aim to increase the productivity and survival of kiwi through trapping and/or poisoning the main predators (Colbourne et al., 2005). Another way of increasing productivity and survival is through captive management of kiwi. This can include captive rearing programmes which aim to improve the chances of recruitment of young kiwi into wild populations (Morgan et al., 2013).

Another approach to captive management is to remove eggs and/or chicks from predation risk in the wild; this approach is dubbed as Operation Nest Egg (ONE) (Colbourne et al., 2005). The eggs that are removed from the wild are artificially incubated and the resultant juvenile kiwis are reared in captivity and/or in a predator free environment “crèche” (Colbourne et al., 2005). The kiwi are reared until they reach a body weight of 800-1000g at which they are deemed large enough to protect themselves against stoats. This is usually more than six months old, after which they will then be released back into the wild (Colbourne et al., 2005). Releasing captive-reared animals back into the wild is a widely-used

conservation management technique world-wide. Kiwi have been held in captivity for many decades, but the captive population have been barely self-sustaining (Colbourne et al., 2005).

1.2.1 Effect of parasites on kiwi conservation

Captive management is a key management tool for kiwi conservation, with many kiwi kept in captivity as part of the ONE programme, on display for advocacy purposes or in off display breeding pairs (Barlow, 2011). However, intensive rearing of young kiwi increases the likelihood of the exposure to pathogens, in particular parasites, that are usually associated with high–density livestock situations (Morgan et al., 2013). For example, the conservation management practice of putting young kiwi together in outdoor crèches that are used repeatedly over many years has led to severe disease due to the protozoal parasite, coccidiosis (Morgan et al 2013). Similarly, a cluster of avian malaria cases was recently seen in a captive rearing facility for kiwi (Banda et al., 2013).

Alternatively, isolation of hosts from novel immunological challenges while in captivity may lead to increases in host prevalence when individuals are released back into the wild (Cunningham, 1996). It may be more beneficial for the animal to have limited exposure to these parasites especially if they are present in reservoirs in its natural habitat (Morgan, 2013). When pathogens are eliminated by veterinary treatment or through small population processes, the host can lose herd immunity thus creating a catastrophic effect if a disease breaks out in that animal (Lyles & Dobson, 1993).

Immunocompetence is determined not only by the host's genetic background, but also by its history of disease exposure. This includes inherited maternal antibodies, nutrition, environmental stress, behaviour patterns and many other factors (Lyles & Dobson, 1993).

1.3 Host and parasite specificity

Host specificity is one of the most fundamental properties of parasitic organisms. Host specificity is inversely proportional to the number of host species that can be used by a parasite at any given stage of its life cycle (Poulin & Keeney, 2008). The number of hosts a parasite can infect is determined by physiological and ecological factors, but is ultimately determined by the evolutionary and biogeographical history of the parasite and its hosts (Poulin & Keeney, 2008). It also links to the ability of the parasite to colonise new host species when the opportunity presents itself. Thus, there is a strong negative correlation between host specificity and parasites and their likelihood of 'jumping' to a novel host species and initiating an emerging infectious disease (Poulin & Keeney, 2008).

Parasites that are highly host specific and only occur in one host, have their fate very closely linked to that of the host species. This is in contrast to parasites that are generalists: those that can persist through host extinction by exploiting other alternative hosts (Poulin & Keeney, 2008). Parasites may be expected to become adapted to their local hosts, because parasites are often more numerous and have shorter generation times than their hosts. Therefore parasites should evolve faster than their host as they need to increase their fitness at the expense of the host's (Lajeunesse & Forbes, 2002). Thus, one aspect of local adaptation can be defined as the non-invasibility of a parasite population by competing foreign parasites (Kaltz & Shykoff, 1998). However, in co-evolutionary host-parasite systems each of the species will need to adapt in an ever-changing environment (Gandon, 2002).

Specialist parasites are less successful than generalists in invading new environments. The former are less likely to encounter new hosts which they can infect therefore reducing their potential impact on native fauna (Tompkins & Poulin, 2006). The impact of exotic infectious agents are also more severe when native species related to introduced hosts are present in the new environment (Tompkins & Poulin, 2006). This may be due to relatedness being able to facilitate host switching, yet the new hosts lack adaptive defences against the parasite of the

traditional host and therefore can suffer serious pathogenic effects (Tompkins & Poulin, 2006).

However, many parasites persist in multihost systems, making the identification of infection reservoirs crucial for devising effective interventions. Failing to identify such infection can lead to ineffective or counter-productive control measures with costly implications (Viana et al., 2014). A reservoir can consist of multiple connected populations of the same or different species. Assessing the ability of reservoirs to assist with pathogen infection can be difficult. Therefore, assessing more than one line of evidence of what comprises a reservoir is important in order to devise the best intervention (Viana et al., 2014).

1.3.1 Host adaption versus parasite adaptation

Following the introduction to its host, the success of parasite colonisation is controlled by three processes. Firstly, the parasite must be present on or in individuals of the host founder population. It may simply fail because the parasites could be aggregated across the host individuals in the founding population and the host may not be infected with the parasite(MacLeod et al., 2010). Secondly, the parasite's host must persist and establish in the new region, if the host fails to establish, so will the parasite. Finally, if the host persists, so must the parasite and not fail for other reasons (MacLeod et al., 2010).

MacLeod et al (2010) have proposed three mechanisms which may explain how a parasite species fails, despite the host successfully establishing. Firstly, the parasite may require another host species as part of its lifecycle, and if this is absent in the new area the parasite cannot persist. Secondly, parasite transmission amongst host individuals at the new locality may be insufficient to offset the parasite losses when the hosts dies. Insufficient transmission can include small founding populations, high mortality rates and low levels of social interaction between the hosts. Proposed parasite factors that contribute to insufficient transmission include low vagility and high host-specificity. Thirdly, parasite persistence is likely to be

influenced by the number of parasites that are introduced and their distribution among hosts (MacLeod et al., 2010).

The invasive success of a particular parasite within a host species is often partly contributed to the release of regulatory effects of parasites on their native range. Such a release may allow introduced hosts that lose their parasites to attain unnaturally high densities and become 'pests' in the new location. Also, many parasite species can themselves cause harm outside their native range, and understanding how parasites succeed in colonizing new regions is key to mitigating their spread and impact (MacLeod et al., 2010). Despite this, we have very little understanding of the mechanisms and processes that are important for determining the success of parasite colonization in new regions following host introduction (MacLeod et al., 2010).

1.4 Changes to Ecosystems

Parasites can have a strong influence on host populations, non- host populations, and ecological processes (Blakeslee et al., 2012). However, parasites are often studied over limited portions of their distributions. This makes understanding of demographic patterns throughout geographical range very minimal. Understanding these large-scale patterns is very important given the global movements of species through human-transport mechanisms (Blakeslee et al., 2012).

There has been empirical evidence that demonstrates how human-mediated introductions can strongly limit the abundance and diversity of parasites. The process of introduction filters out some parasite species, while it enables others to invade with their hosts, also known as 'parasite escape' (Blakeslee et al., 2012).

1.4.1 Phylogeography

Phylogeography is the study of the historical processes that may be responsible for the contemporary geographic distributions of individuals. Phylogeographical data may be useful in understanding the history and colonisation of hosts and their parasites in New Zealand. Theoretically, this kind of study could indicate possible source populations of parasites; the timing of the invasions; or to test whether multiple introductions or invasions have occurred (Criscione et al., 2005).

New Zealand has a rich exotic avifauna that comprises of more than 30 species from 14 families, resulting largely from the efforts of Acclimatisation Societies. These societies were founded in the Victorian period (mid to late 1800's) with the goal of 'enriching' the recipient country's fauna and flora. New Zealand has therefore had abundant opportunities for co-invasion by avian parasites (Ewen et al., 2012)

However, there has been little phylogeographical studies done on parasites. Given that parasites are more often than not closely tied to their host, it may be expected that parasites and their host share similar phylogeographical patterns (Criscione et al., 2005). Even if parasites were introduced with their hosts, they may still be lost. This can be due to stochastic effects, or the new host population initially may be of insufficient size or density (Tompkins & Poulin, 2006).

1.4.2 Parasite diversity and island ecosystems

Parasites are increasingly being spread by human activities and subsequently establishing exotic populations in locations beyond the limits of their native geographical ranges (Ewen et al., 2012). These exotic parasites may cause a major disease threat to not only other animals but humans as well, especially if they can jump between hosts at new localities to infect naïve native hosts.

If pathogen loss positively affects host traits such as reproduction and survival, then the likelihood that an exotic host population can become established may be

increased; a process also known as the 'Enemy Release Hypothesis' (Ewen et al., 2012). Conversely, an exotic host may benefit from the co-introduction of its parasites, if those parasites subsequently infect and cause population declines in the native species that would otherwise be competition to the exotic hosts; this is known as Novel Weapon Hypothesis (Ewen et al., 2012).

1.5 Life cycle of parasitic nematodes

While there is considerable variation in some details of the life cycle of parasitic nematodes, there are some general features in common which are outlined here. In the Nematoda, the sexes are separate and the males are generally smaller than the females, which lay eggs or larvae (Taylor et al., 2007). During development, a nematode will moult at intervals, shedding its cuticle. The complete life cycle of all nematodes consist of the egg and four larval stages (L1, L2, L3, L4) before becoming an adult, which is the fifth stage (Lee, 2002; Taylor et al., 2007).

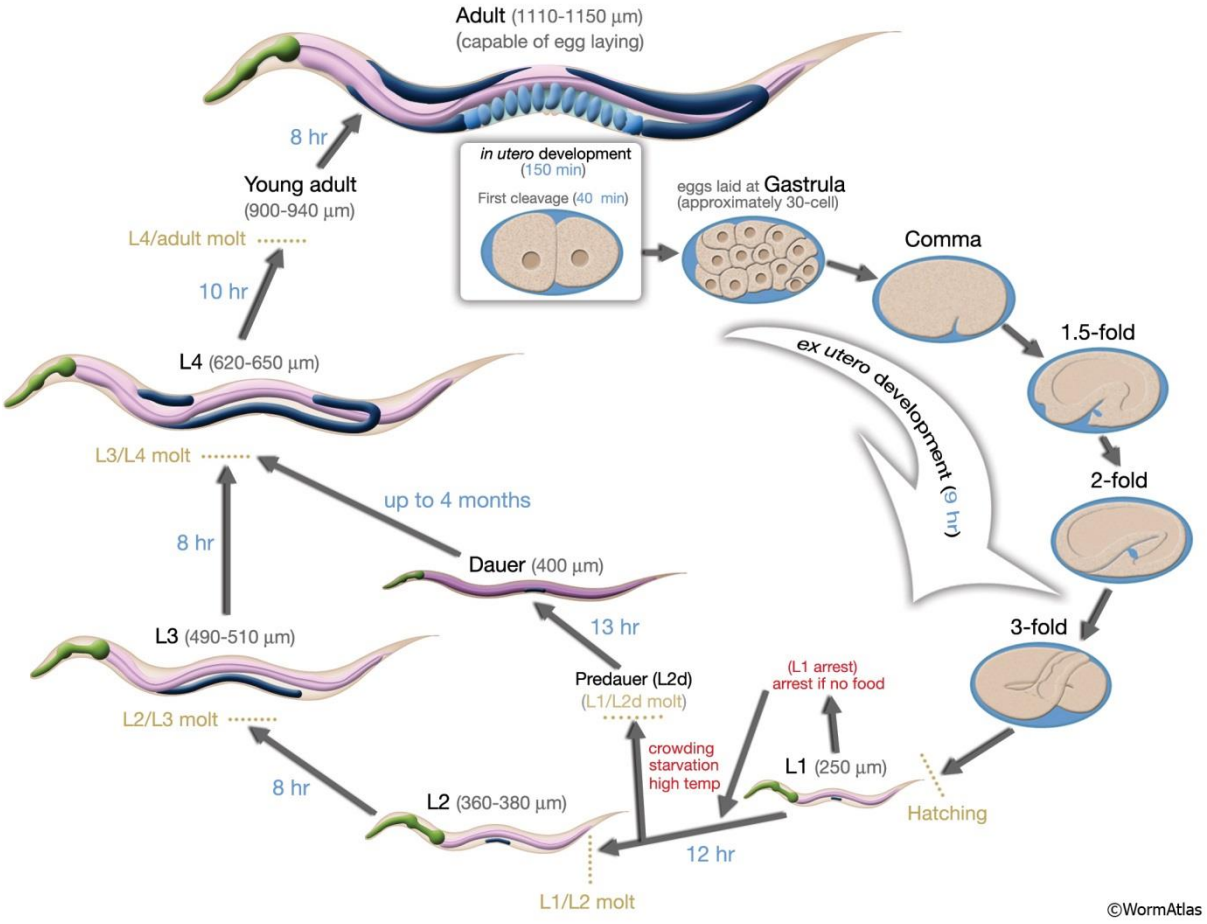

Figure 1.2. *Caenorhabditis. elegans* life cycle as an example of a Nematode life cycle. Taken from (Hall et al., 2012).

A feature of the basic life cycle of a nematode is that intermediate transfer of infection from one final host to another will rarely occur. Some development will take place normally in the faecal matter or in a different species of animal, the intermediate host, before infection of the primary host can take place (Taylor et al., 2007).

In the common form of the direct life cycle, the free-living larvae undergo two moults after hatching and the infection is by ingestion of the free L3. There are however, some important exceptions. Infections can sometimes be due to ingestion of the egg which contain the larvae or through infection in mammals, or through larval penetration of the skin (Taylor et al., 2007). Larval penetration of the skin has not yet been recorded in birds. In indirect life cycles, the first two moults

usually take place in an intermediate host and infection of the final host is either by ingestion of the intermediate host or by inoculation of the L3, when the intermediate host feeds, such as a blood-sucking arthropod (Taylor et al., 2007).

In general, parasitic nematodes enter the host when they are their third stage larvae (L3), which is therefore referred to as the infective larval stage, and will enter the host either orally or percutaneously. Oral infection takes place when the host either eats or drinks the infected food that contains eggs or larvae. The larvae that is swallowed or those that are hatched from eggs in the alimentary tract may then immediately penetrate the epithelial layer to reach the mucosal tissue. Some species of nematode may stay in the gut or start extensive migration through host tissues which is known as aberrant larval migration (Maruyama & Nawa, 2002). Percutaneous entry will be through the skin, and the parasite will travel to the gut or carry out an extensive migration as well.

After the infection occurs, two further moults take place to produce the L5 stage or immature adult parasite. Once maturation of these stages occurs, copulation and oviposition takes place completing the life cycle. In gastrointestinal parasites, the lifecycle may entirely take place in the gut lumen or with limited movement into the mucosa (Taylor et al., 2007).

1.6 Larval migrans - aberrant

If an infective larvae enters a host in which it is not able to complete its life cycle, instead of migrating to its appropriate final destination, it may migrate randomly through the host's tissues causing damage to the host cells and organs in its path (Maruyama & Nawa, 2002). This is known as aberrant larval migration. An example of this is shown in Figure 1.3.

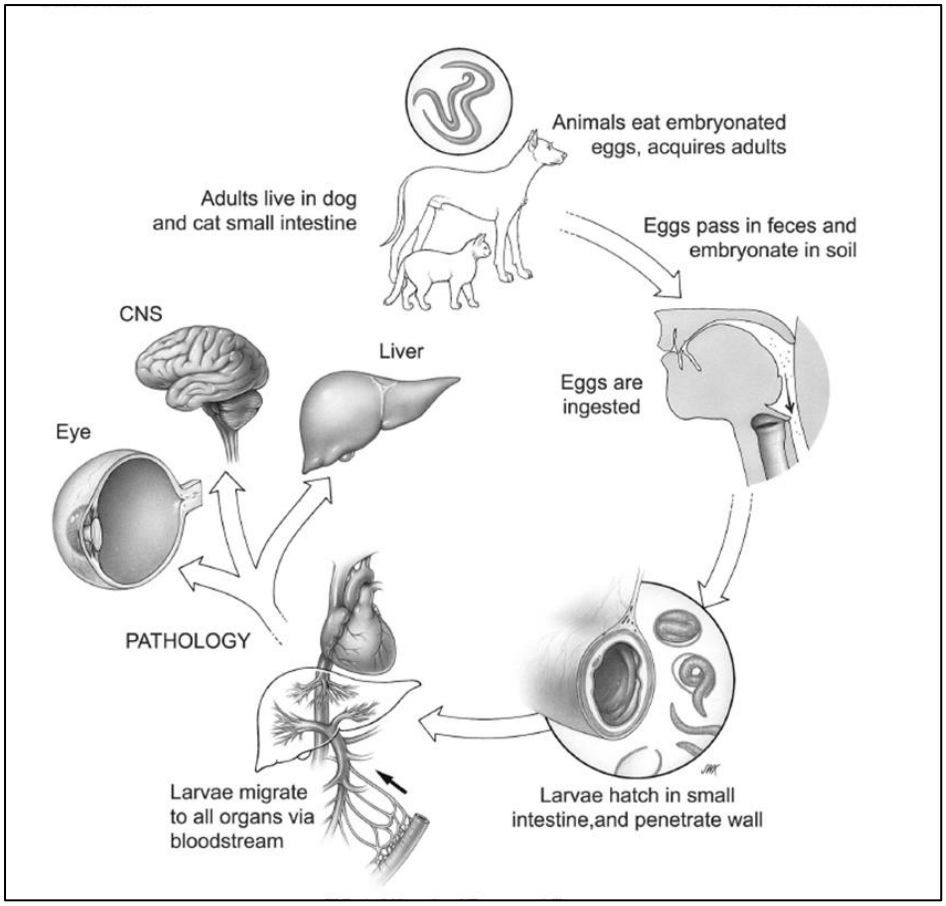

Figure 1.3. An example of aberrant larval migrans in humans through ingesting eggs of *Toxocara canis* or *T. cati*. Taken from (Despommier, 2003).

In aberrant hosts, the completion of the life cycle of the nematode differs. The infective larvae that are ingested will hatch, but the juvenile stage of the worm is unable to develop into a mature adult worm. Therefore, the worm is left to wander the body for days, months or several years, often causing extreme damage to any tissues or organs that it enters (Despommier, 2003). Alternatively, *Toxocara* eggs for example, if found in an aberrant host such as mice or humans, can remain in an arrested state. The host ingests the eggs, and the emerging larvae migrate to muscle and neurological tissues, where they can remain in the arrested state. Here they will not grow, reproduce or differentiate. In mice, the dormant tissue larvae can re-enter the transmission cycle by predation by a canid carnivore. In humans, however, migrating larvae can invade visceral tissue causing eosinophilia and non-pathogenic malaise, or move into the eye with some ocular larva migrans (Maizels

et al., 2006). There are also reported cases of central nervous system invasion, where larvae migrate into the brain (Maizels et al., 2006).

1.6.1 Visceral larval migrans

Visceral larval migrans (VLM) occurs when parasitic larvae migrate through the internal organs of the host that does not include the intestines or stomach (see Figure 4) (Iowa State University, 2014). The clinical signs vary with the number of parasite and the tissue(s) that is invaded in the animal.

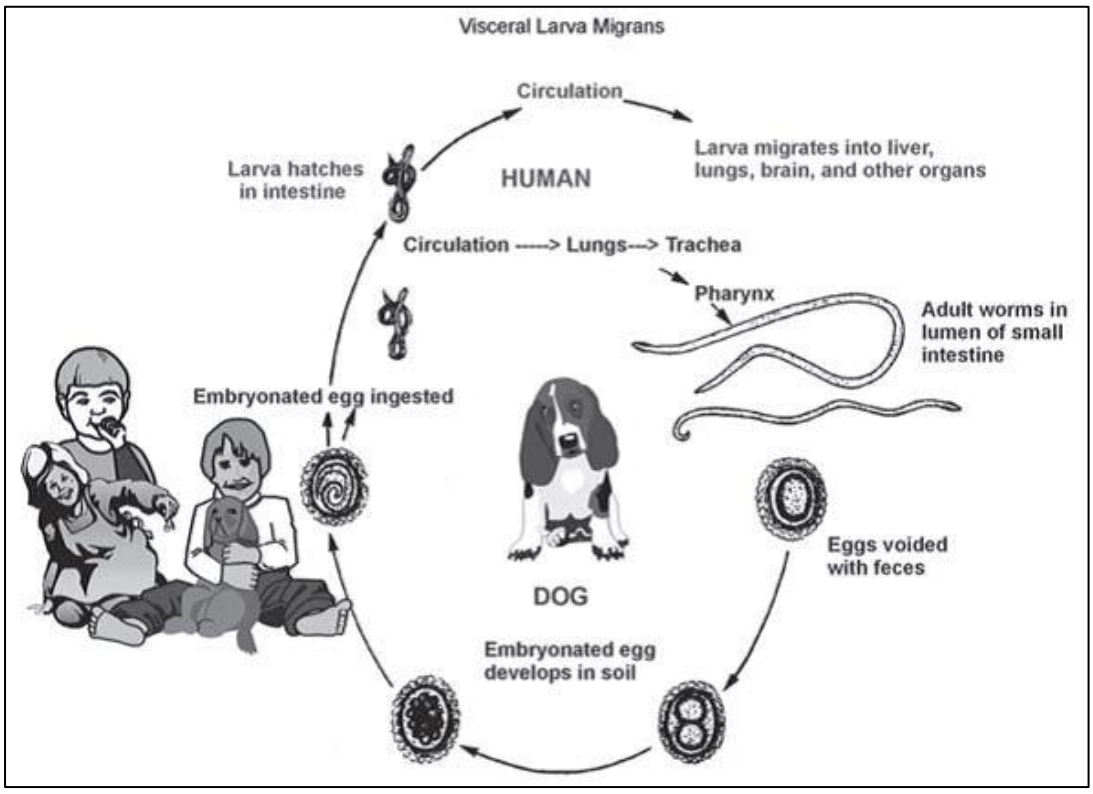

Figure 1.4. The lifecycle of *Toxocara canis* when ingested by humans. This figure is taken from Satoskar et al. (2009).

Toxocariasis are the most important causes of visceral larval migrans in humans. Most infections of *Toxocara* are asymptomatic, but some can cause death. The lifecycle is initiated by the ingestion of embryonated eggs in humans. After the

larvae hatch, the third stage larvae wander through the body, invading all organs (Figure 1.4). Unlike *Ascaris*, *Toxocara* larvae do not break out of the capillaries in the alveolar spaces in the lung; instead they pass through and eventually reach the systemic circulation. The degree of damage they cause is proportional to the number of organs and tissues that have been invaded. Ultimately the larvae will die and provoke the formation of granulomas (Katz et al., 1988).

Clinical signs in humans can include skin lesions, lymphadenopathy, pruritus, anaemia, decreased appetite, nausea, vomiting, headaches, pneumonia, as well as behavioural and sleep disturbances. Myocarditis, respiratory failure or seizures may complicate the infection. Other less frequent complications reported with toxocariasis can include; multiple ecchymoses with eosinophilia, pyogenic liver abscess, urticaria, Henoch-Schönlein purpura, nephrotic syndrome, secondary thrombocytosis and eosinophilic arthritis (Satoskar et al., 2009).

Ascaris suum is another parasite that has been known to migrate in aberrant hosts. The transfer of this parasite is very similar to that of the *Toxocara* species. The effect this parasite has on an aberrant host is also similar to that of *Toxocara*. VLM with the *A. suum* parasites have been identified in humans.

1.6.2. Neural larval migrans

Neural larval migrans (NLM) occur when a parasitic larvae travels through the organs of an aberrant host and into the brain or spinal cord (Sorvillo et al., 2002). This is often fatal to the animal if the cause is not found in time and treated.

The most commonly reported NLM parasite currently, apart from *Toxocara*, has been *Baylisascaris procyonis* which is a roundworm that infects racoons. *Baylisacaris procyonis* has a widespread distribution with up to 90% of juvenile raccoons having this roundworm (Sorvillo et al., 2002). As with other ascarids, eggs are excreted into the faeces and onto soil, and must develop externally to become

infectious. When raccoons ingest infective eggs, *B. procyonis* larvae hatch and enter the wall of the small intestine and subsequently develop into adult worms in the small bowel (Sorvillo et al., 2002). However, when other animals ingest these eggs from the soil, the larvae hatch and extraintestinal migration occurs.

More than 90 species of wild and domestic animals have been identified as infected with this parasite larva. Outbreaks of fatal central nervous system disease caused by *B. procyonis* have occurred in zoos and on farms (Sorvillo et al., 2002). There have been cases with infection in humans, more often than not being fatal or severe sequelae (Sorvillo et al., 2002). Most of these cases have been reported in children, as the mode of transmission is faecal-oral.

In 2007, a Moluccan Cockatoo was found with a single *Baylisascaris* nematode in its midbrain upon histological examination (Wolf et al., 2007). The larval migration of this parasite caused encephalitis which caused the clinical signs the bird displayed. The bird also had multifocal granulomas in the left ventricle of the heart (Wolf et al., 2007).Prognosis for the return to normal central nervous system function is regarded to be too poor for birds who have encephalitis due to cerebrospinal nematodiasis. Therefore, an emphasis needs to be placed in preventing this disease (Wolf et al., 2007). However, it is worth noting that *Baylisascaris* has not been recognised in New Zealand and is currently considered an exotic disease.

1.7 Classification of nematodes

Parasite identification used to be based mainly on morphological characteristics, but over the last decade or so, molecular tools have been developed. Selected DNA sequences can be used to identify parasites and achieve a higher level of discrimination among morphologically similar species (Poulin & Keeney, 2008).

For effective diagnosis, treatment and control of parasitic diseases, it is essential that parasite isolates can be accurately identified (McManus & Bowles, 1996). With many parasite groups, precise identification is often more problematical than mere

recognition of samples that have been previously characterised in groups. This is because variations in biological features with practical implications are now being detected with rapidly growing numbers of parasite species, implying a high cross over in morphological features (McManus & Bowles, 1996). Knowledge of taxonomic relationships and phylogeny of parasite species and their variants are becoming more important, as it provides a much needed framework for understanding parasitism and further experimental studies (McManus & Bowles, 1996).

There are a growing number of sophisticated and sensitive methods for discriminating parasite isolates. Some of these techniques are not always broadly accessible or field-based, or intended for clinical diagnostic work; their purpose is for detection and characterisation of variants (McManus & Bowles, 1996). Precise knowledge of the identity of a parasite, and an understanding of the range of variation in a particular parasite group, is crucial in the development of vaccines and new diagnostic methods (McManus & Bowles, 1996).

1.7.1 Morphological

In the past, nematode identification was previously based mainly on morphological characteristics of the eggs, larvae and adult parasites, and this is still considered the mainstay for both identification and taxonomy of nematodes (McManus & Bowles, 1996). However, precise identification is often problematical due to variations in biological features with practical implications now being detected with rapidly growing numbers of parasite species and identification based soley on morphological techniques can be unreliable (McManus & Bowles, 1996).

1.7.1.1 Identification of eggs

There are many characteristics that are used to identify the species of parasite eggs. Some of these characteristics include; size, shape, stage of development, thickness

of the egg shells, colour, and the presence of characteristics like lids (opercula), spines, plugs, hooklets or mammillated outer coats (World Health Organization, 1991).

Occasionally atypical or distorted eggs will be seen. In these cases it will be necessary to look for more typical forms in order to make a reliable diagnosis (World Health Organization, 1991).

1.7.1.2 Identification of larvae

Firstly the larvae must be stained and suspended on a slide, usually stained with iodine solution. The larvae normally examined is already at the L3 stage. Here it is important to differentiate the caudal and cranial extremities for the L3 stage of most parasitic nematodes. Other features may also be important such as the shape and length of the oesophagus (Van Wyk et al., 2004).

Identifying species of parasites are often very difficult to do, owing to their limited morphological characteristics. In addition to this the primary samples of parasites may be from unidentifiable egg or larval stages from a particular host (Criscione et al., 2005). Therefore genetic identification of the species is by far the most commonly used application for molecular techniques in parasitology (Criscione et al., 2005). Morphological methods can also be limited when one has to look at nematode sections in tissue samples; a situation where molecular tools have a clear advantage.

DNA-based identification and discovery of parasite species also has implications of our understanding of the biodiversity on a global scale. The limited morphological characters of many parasitic helminths have most likely resulted in a gross underestimation of the true number of the species in biodiversity surveys (Criscione et al., 2005).

1.7.1.3 Identification of adult nematodes

The general external morphology of a parasite provides the most immediate source of comparative features, while the study of the internal morphology (including the genitalia, musculature, organs and glands) provides useful characters for identification (McManus & Bowles, 1996). This is a relatively inexpensive method and can be used in the field, however it does require expertise to identify between species. A range of morphological features can be limited in many organisms, and morphologically indistinguishable sibling species are also common among parasites.

1.7.2 Molecular identification of nematodes

DNA sequencing is often the most direct and often used approach for characterisation of distinct species (McManus & Bowles, 1996). DNA-based procedures are extremely sensitive in detecting variation, since even silent nucleotide positions can be compared (McManus & Bowles, 1996). Selected DNA sequences can be used to identify parasites and achieve a higher level of discrimination among morphologically similar species (Poulin & Keeney, 2008).

1.7.2.1 Overview of genomic studies

Over evolutionary time, all regions of the genome accumulate mutations. Introns and non-coding regions are unlikely to be highly constrained by function and will generally evolve more rapidly than coding regions. It is important to note that some nucleotides positions can be indefinitely conserved in the genome (for example active sites of enzymes). Spontaneous mutations can arise, but preservation of function at certain nucleotide positions is vital to the survival of the organism and therefore those mutations are not fixed at the genome (McManus & Bowles, 1996). Hence, different genes and various segments within a single genome will evolve at different rates (McManus & Bowles, 1996).

The nuclear genome is made up from a number of linear chromosomes and contains mostly non-coding sequences. Non-coding DNA evolves more rapidly,

however it is mostly uncharacterised and therefore not readily accessible for PCR-based methods (McManus & Bowles, 1996).

1.7.2.2 Polymerase chain reaction

Polymerase chain reaction (PCR) provides a highly sensitive method for DNA amplification and identification (Powers & Harris, 1993). A particular fragment of DNA is specifically selected from a complex genome and enzymatically amplified *in vitro*. Double-stranded genomic DNA is denatured by heating, and then the temperature is lowered to allow two specific primers to anneal to their complementary sequences on opposite strands of the DNA. Template-directed DNA synthesis proceeds from the primed sites in both directions when catalysed by a polymerase. The double-stranded products result and the procedure is repeated approximately 30 times (McManus & Bowles, 1996).

1.7.2.3 Ribosomal DNA genes

The genes encoding the rRNA subunits include regions with varying rates of evolution, from highly conserved (the small subunit gene) to the highly variable (the intergenic spacer regions (IGS)). Although multiple copies of rDNA exist in the genome, they evolve in a concerted fashion rather than independently, so that the rDNA sequences have a tendency to be homogeneous within an individual (McManus & Bowles, 1996).

Internal transcribed spacer (ITS) refers to the piece of non-functional RNA that is situated between the structural ribosomal RNAs (rRNA). It's situated between 18S and 28S or rDNA genes (refer to figure 5) (Powers et al., 1997) . Genes encoding rRNA and spacer occur in tandem repeats and are thousands of copies long, each of these separated by regions of non-transcribed DNA also known as IGS or NTS (non-transcribed spacer). Sequence comparison for the ITS region is widely used in taxonomy and molecular phylogeny because it is easy to amplify in small amounts

of DNA, and it contains a high degree of variation between closely related species (Powers et al., 1997). Due to this, the ITS region has received the most attention by nematologists, and it is currently the preferred region for taxonomic identification.

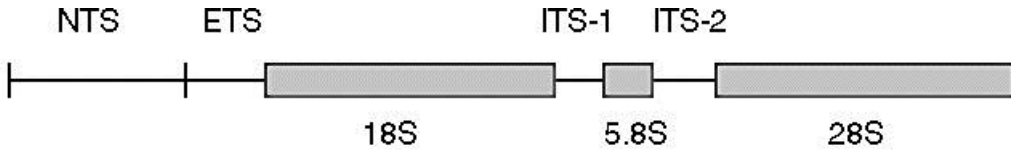

Figure 1.5. Diagram of the ribosomal DNA gene family. The regions coding for 5.8S, 18S and 28S are shown with bars. NTS= non-transcribed spacer, ETS= external transcribed spacer, ITS= internal transcribed spacer regions. Sourced from: (Nebraska-Lincoln, 2013).

This is the region where primers are used to start DNA synthesis for nematode identification. Many primers have been developed for nematodes within this region. Table 1 shows a variety of primers developed for nematodes of interest in this study.

Table 1.1 Primers developed for nematodes of interest in this study

Reference	Parasite Species	Normal host species	Abnormal host species	Region	Primer set	ID of where they are sitting (flanking region)
(Jacobs et al., 1997)	Toxocara cati	Cats	Humans, kiwi, laboratory animals	ITS-2	Tcat1 (F) / NC-2 (R)	
(Fogt-Wyrwas et al., 2007)		Dogs		ITS-2	Tcan1 (F) / NC-2 (R)	
	Toxocara canis					
	Toxocara canis	Dogs	Humans	ITS -2	YY1 (F) / NC2 (R)	
(Li et al., 2007)	Toxocara cati	Cats	Laboratory animals and the Potential for human infection (zoonotic)	ITS-1 and 5.8S (partial) And complete ITS-2	JW4(F) / NCS (R)	
(Traversa et al., 2008)	*A. abstrusus*	Cats and other felids	Cockatoos and frogmouths	ITS-2	Aabfor (F) /Aabrev (R)	5.8S and 28S
(Dangoudoubiyam et al., 2009)	*B.procyonis*	Racoon	100 species of birds and mammals including humans	Cox2	BpF (F) / BpR (R)	25 (146bp)
					18SF (F) / 18SR (R)	18S

Reference	Species	Normal host species	Abnormal host species	Region	Primer set	ID of where they are sitting (flanking region)
(Zhu et al., 1999)	*Ascaris*	Humans, pigs		ITS-1 and ITS-2	NC5 (F) / NC2 (R)	5.8S
(Pecson et al., 2006)	*Ascaris lumbricoides and A. suum*	Humans, pigs		ITS-1		18S, 5.8S and 28S
(Ishiwata et al., 2004)	*Ascaris – toxocara canis and ascaris suum*	Dog and pig	Humans, laboratory animals	ITS-1 and 2	F2662 / R3124 (ITS1) F3207 / R3720 (ITS2)	
(Matoba et al., 2006)	*Toxocara, toxoscaris* and *Baylisascaris*	Cats, dogs, racoon	Humans and other mammals, birds	ITS-2	LC1F / HC2R	
(Gatcombe et al., 2010)	*B. procynois*	Racoon	Humans and other mammals, birds	Cox2	SrF1 / JVBPR	12S rRna

1.8 Known nematodes of kiwi

According to Weekes (1982) and McKenna (2010), 17 species of helminth and protozoan parasites have been identified in kiwi, mostly in the brown kiwi and little spotted kiwi (refer to Table 1.2). Of these 17 parasite species, there are five species of nematodes that have been found in the brown kiwi, but none have been identified in the other species of kiwi. However, there have been no studies done on the effects these parasites have on kiwi.

Table 1.2. Internal parasites previously identified in kiwi (*Apteryx* spp) (McKenna, 2010).

Brown Kiwi	**Nematoda**	*Ascaris apterycis*
		Cyrnea apterycis
		Heterakis gracilicauda
		Porrocaecum spp
		Toxocara cati
	Cestoda	*Anomotaenia minuta*
		Davainea spp
		Paricterotaenia apterygis
		Raillietina spp
		Taenia apterycis
	Trematoda	*Lyperosomum megacotylosum*
	Acanthocephala	*Echinorhynchus spp*
	Protozoa	*Babesia kiwiensis*
		Eimeria spp
		Hepatozoon kiwi
		Plasmodium spp
Little Spotted Kiwi	**Protozoa**	*Toxoplasma gondii*

From the nematodes found to infect kiwi (Table 2), I have selected those which have previously been found in aberrant hosts: *Heterakis gracilicauda, Porrocaecum spp and Toxocara cati .* Although, no work has been done to identify the parasites causing larval migrans in kiwi, it has been suggested that the larval migrans could be caused by *Toxocara* because of the histopathological and epidemiological features only (M.Alley, personal communication, November 23, 2012).

1.8.1 Reported cases of larval migrans in kiwi

The first recorded incidence of larval migrans in kiwi was in 2002. A young male kiwi was found dead on the east coast of the North Island, with injuries consistent of that of a dog attack. Histology of the brain revealed a large parasitic granuloma, containing several cross sections of nematode larvae. The left ventricular myocardium and gizzard also contained parasitic granulomas. The parasite causing the larval migrans has not yet been identified. It was suggested that the lesions in the heart and brain had significant effect on the animal's fitness, which could have predisposed it to predation (Alley & Gartrell, 2003).

Another report of visceral and neural larval migrans in kiwi was in 2004. A three month old kiwi chick was found dead in Northland. Upon necropsy at Massey University, lesions in the liver showed parasitic granulomas with similar lesions being found in the lung also. Upon closer examination in the brain, a parasite granuloma was also found in the cerebellar granular layer. This was suggestive of a migrating tract cause by nematodes, although no identification was made of the parasite (Alley et al., 2004).

The latest recorded case was in 2006, where post-mortem revealed microscopically severe lesions in the colon, gizzard and brain. Large numbers of nematodes were present in the mucosal surface of the colon, and were identified as *Heterakis* species. The gizzard also had a large nematode embedded in the mucosa. The brain contained small focal areas of inflammatory necrosis within the neuropil of the midbrain, that showed a type of lesion that has been seen previously in the brains of kiwi affected with neural larval migrans. This nematode has not yet been identified (Alley & Gartrell, 2006).

All these incidences highlight the importance of nematode burdens in kiwi and illustrate the fact that free-living birds may sometimes be vulnerable to life threatening nematode infections. It also highlights the need to identify these nematodes for future treatment and prevention of these infections.

1.9 Thesis aims and organisation

The overall aim of this thesis is to investigate the inter-relationships between host and parasite specificity and aberrant larval migration using the kiwi family as the affected host system and nematodes as the parasites.

In Chapter Two, I investigate the prevalence of nematodiasis in all kiwi species from 1991-2012 that were necropsied at Massey University (n=699). Archived records from the National Wildlife Pathology Database (Huia) were examined for any mention of nematodes in tissues. The information gathered from the database was then quantified and analysed to compare species, sex, age and habitat with nematodes found the intestine, ventricular; neural and visceral larval migrans.

In Chapter Three, I focus on the molecular techniques used to identify the nematode species involved in extra-intestinal nematodiasis, in particular looking at *Toxocara cati* and *T. canis* species. I extracted DNA from formalin fixed tissue and ran PCRs with the extracted DNA.

In Chapter Four, I investigate and identify the cutaneous larval migrans recently found in the Rowi species. Using molecular techniques, I extracted DNA from skin biopsies preserved in wax blocks and carried out PCR analysis and sequencing. With the sequencing results, I was able to BLAST the sequence to match the DNA of the parasite and identify which parasite was causing the cutaneous migrans.

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Chapter Two

Presence and prevalence of helminths in kiwi

Chapter 2. Presence and prevalence of helminths in kiwi

2.0 Abstract

The relationship between host and parasites are ancient and on-going, and like many other animals in the world, our native kiwi in New Zealand, are host to many parasites including gastrointestinal helminths such as cestodes, nematodes and trematodes. Parasites can cause a variety of problems in their host at high levels of infection or through larval migration in aberrant hosts. Therefore, this study focused on finding any correlation with age, sex, habitat and species of kiwi with the prevalence of nematodiasis of different tissues at the time of post mortem through the years of 1991 to 2012 (n=699). Our results showed brown kiwi have the highest prevalence of nematodiasis and were interestingly also the only species found to have neural larval migrans; visceral larval migrans were found in brown, rowi and little spotted kiwi. It was also found wild adult kiwi have a higher percentage of intestinal and ventricular nematodiasis as well as visceral larval migrans. No significant correlation was found between prevalence of nematodiasis in intestinal, ventricular or visceral larval migrans when compared to sex or species of kiwi. Interestingly, we found kiwi kept in crèches to have a higher prevalence to visceral larval migrans. No significant risk factors were identified for neural larval migrans in kiwi.

Keywords: kiwi, parasites, nematdoes, larval migrans, prevalence

2.1 Introduction

Kiwi (Order Struthioniformes, Family Apterygidae) are endemic to the three major islands of New Zealand (Sales, 2005). Recent molecular work has revealed that kiwi are close relatives to elephant birds (Aepyornithidae) that went extinct on Madagascar (Mitchell et al., 2014). Thus it is known that kiwi have existed in their current form for at least 65 million years (Holzapfel et al., 2008; McLennan & Potter, 1992; Sales, 2005). The decline in the abundance and distribution of kiwi has gone largely unnoticed until two decades ago (Mitchell et al., 2014). They are classified as 'threatened' with extinction, mainly due to the low recruitment rate of young kiwi into the wild populations (Basse et al., 1999; Miskelly et al., 2008).

The main causes of decline in these populations are predation due to a variety of introduced mammalian predators as kiwis have evolved in the absence of mammalian predators. However, today they co-exist with many obligate or facultative carnivores that have been introduced by Europeans and Polynesians (Sales, 2005). Some of their carnivorous predators include cats (*Felis catus*), dogs (*Canis lupus*), ferrets (*Mustela furo*), pigs (*Sus domesticus*), possums (*Trichosurus vulpecula*), stoats (*Mustela erminea*) and weasels (*Mustela nivalis vulgaris*) (Sales, 2005). The effect of disease, particularly parasitism on kiwi populations, has been a minor issue in comparison to the rates of predation. However, as the intensive management of remnant populations increases, the effect of parasites on the success of conservation efforts will likely increase.

Parasites are increasingly cited as a major threat to wildlife conservation, being implicated in the decline and/or extinction of many high-profile species worldwide (Tompkins & Poulin, 2006). The majority of living organisms are parasitic and it is apparent that they are a major selective force that influences almost every conceivable aspect of the host's ecology (Holmstad et al., 2005). Parasites, by definition, take nutrients from their hosts and can inflict pathophysiological damage. Consequently this can have a negative effect on the hosts' fitness as it reduces its somatic growth, reproduction and survival (Holmstad et al., 2005; Torchin & Mitchell, 2004).

There are documented examples of the impact of helminth parasites on native New Zealand passerines. These includes the nematodes of the ascarid family, genus *Porrocaecum*, which has caused death in saddlebacks (*Philesturnus carunculatus*); and *Acanthocephala* species that infect the pied stilt (Schoener et al., 2012). However, little is published about the effects helminths have on kiwi (*Apteryx spp*). According to McKenna (2010) about 17 different kinds of helminth and protozoan parasites have been identified in kiwi, mostly in brown (*Apteryx mantelli)* and little spotted kiwi (*Apteryx owenii*). Out of these 17 helminths and protozoan parasites, five species of nematodes found in the brown kiwi, and currently no nematodes have been identified in the other species of kiwi. Also, the prevalence and epidemiology of disease caused by helminths in kiwi in New Zealand is largely undescribed.

One group of parasites investigated in this study are gastrointestinal helminths, which are endoparasitic worms that belong to three groups; trematodes, cestodes and nematodes. At low levels, these parasites cause a constant demand on their hosts, to maintain its immune defences and to continually repair damage inflicted by the parasites. As a result, the behaviour and activity of the host may be altered, at times significantly depending on the parasite load. As a consequence these infections may hinder the animals ability to cope with physiological and environmental stresses, such as nutritional demands, weather exposure, predation pressures and habitat loss (Hatcher et al., 2006; Kristan & Hammond, 2000; McCallum & Dobson, 1995, 2002; Robertson, 2009).

Another less common detrimental effect of parasites is larval migrans, defined as the prolonged migration of a larval parasite in the skin or internal organs of an abnormal host (Beaver, 1969). In kiwi, both neural larval migrans (NLM) and visceral larval migrans (VLM) have been recognised. Visceral larval migrans is a syndrome of inflammatory damage incited by nematode larvae that move through various body organs (Barron & Saunders, 1966), and neural larval migrans is the inflammatory damage caused by traveling nematode larvae in the brain and spinal cord. Larval migrans is caused by nematodes in aberrant hosts. The nematode cannot fulfil its

life cycle in the aberrant host and therefore travels in unexpected tissues and may cause damage to the accidental host (Maruyama & Nawa, 2002).

The main aim of this study was to document the presence and prevalence of nematodiasis and associated syndromes of larval migrans in kiwi. I also wanted to assess if there was any correlation between sex, age, habitat and kiwi species with the prevalence of nematodiasis in different tissues.

2.2 Materials and methods

2.2.1 Huia and Post Mortem database review

Archived records of kiwi necropsied at the Wildbase pathology laboratory between 1991 and 2012 (n=699) are held on Huia, the National Wildlife Pathology Database, and the post-mortem database of the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North. These were searched to identify cases of neural, visceral and intestinal nematodiasis. The full necropsy report of each case was reviewed, including the absence and presence of any nematodes in all body tissues as well as the primary cause of death as determined by the pathologist. In some cases a wide range of tissues including the brain, gizzard, kidney, intestines, lung, spleen, heart and liver were examined histopathologically, usually with haematoxylin and eosin (H&E) stains and the results discussed. It should be noted, however, that not all necropsy reports recorded findings from all the tissues examined (Morgan et al., 2013).

The databases also included details about the individual animals' age at the time of death, sex, location and habitat, gross findings and other concomitant diagnoses. In keeping with previous publications: chicks were classified as less than 25 days old (McLennan, 1988); juveniles as aged between 26 days and 18 months old; and adults as those that were greater than 18 months old (Basse et al., 1999).

2.2.2 Statistical Analysis

Statistical analysis were performed using SAS version 9.3 (SAS, 2011). Univariate analysis was performed using the Fisher's exact test to determine the effect of each independent variable on the prevalence of nematodiasis. The dependant variables were neural larval migrans, visceral larval migrans, ventricular nematodiasis and intestinal nematodiasis. The independent variables were age (chick, juvenile, adult); sex; habitat (captive, wild, crèche); species (brown kiwi, *Apteryx mantelli*, great spotted (GSK), *A. haastii*, little spotted (LSK), *A. owenii*, rowi, *A. rowi*, tokoeka, *A. australis*).

ArcGIS (ESRI, 2010) was used to create a map of the location of the post mortem database cases. Only the brown kiwi species was used to show their geographic distribution around New Zealand. A second map was created to show the distribution of cases with nematodiasis as well as those cases that were not infected with nematodes.

2.3 Results

2.3.1 Presence and prevalence of nematodiasis by species

Data was collected from the post mortem data base and placed in a spreadsheet. All species of kiwi were used for the analysis to compare nematodiasis in different tissues examined (Table 2.1). Brown kiwi were the most highly represented kiwi species in the cases examined (n=551) with nematodiasis being commonly identified in the intestine (16.6%) and ventriculus (26.3%), while evidence of larval migrans was less commonly found in the viscera (4.5%) and brain (3.8%). Post mortem records from other kiwi species were less common and therefore, the prevalence of nematode infections in various tissues carries a greater degree of uncertainty. As a result of this, further analysis of the data set was focussed solely on brown kiwi. From this data, the tissues that were actually involved in the neural

and visceral larval migrans were identified (Figure 2.1). The liver and lung were the tissues mostly affected by larval migrans (Figure 2.3).

Table 2.1 The presence and prevalence of nematodes in kiwi cases at Massey University (1991 to 2012). The denominator represents the number of tissues examined and the numerator is the number of positive cases of nematodiasis. The percentage equates to the prevalence of nematodiasis in kiwi and their respective tissues. Sample sizes within each species vary due to incomplete examination of some tissues due to decomposition, especially of the intestinal tract and brain or limitations imposed by the extent of the post mortem analysis.

	BK	GSK	LSK	Tokoeka	Rowi
Intestinal	36/217 (16.6%)	1/20 (5.0%)	1/8 (12.5%)	0/20 (0%)	2/12 (16.7%)
Ventriculus	60/228 (26.3%)	0/36 (0%)	2/9 (22.22%)	0/20 (0%)	2/7 (28.6%)
VLM	15/333 (4.5%)	0/33 (0%)	1/12 (8.3%)	0/32 (0%)	1/16 (6.25%)
NLM	6/158 (3.8%)	0/19 (0%)	0/8 (0%)	0/12 (0%)	0/10 (0%)

BK, brown kiwi (Apteryx mantelli); GSK, great spotted kiwi (Apteryx haastii); LSK, little spotted kiwi (Apteryx owenii); Tokoeka (Apteryx australis); Rowi (Apteryx rowi); VLM, visceral larval migrans; NLM, neural larval migrans.

2.3.2 Geographic distribution of nematodiasis in brown kiwi

The geographic distribution of kiwi sent for post mortem examination at Massey University, is shown in figure 2.1.The map shows that there was a greater number of cases from the North Island of New Zealand, and very few from the South Island which reflects the wild and captive distribution of brown kiwi. Brown kiwi from the South Island of New Zealand were exclusively from captive institutes and were from

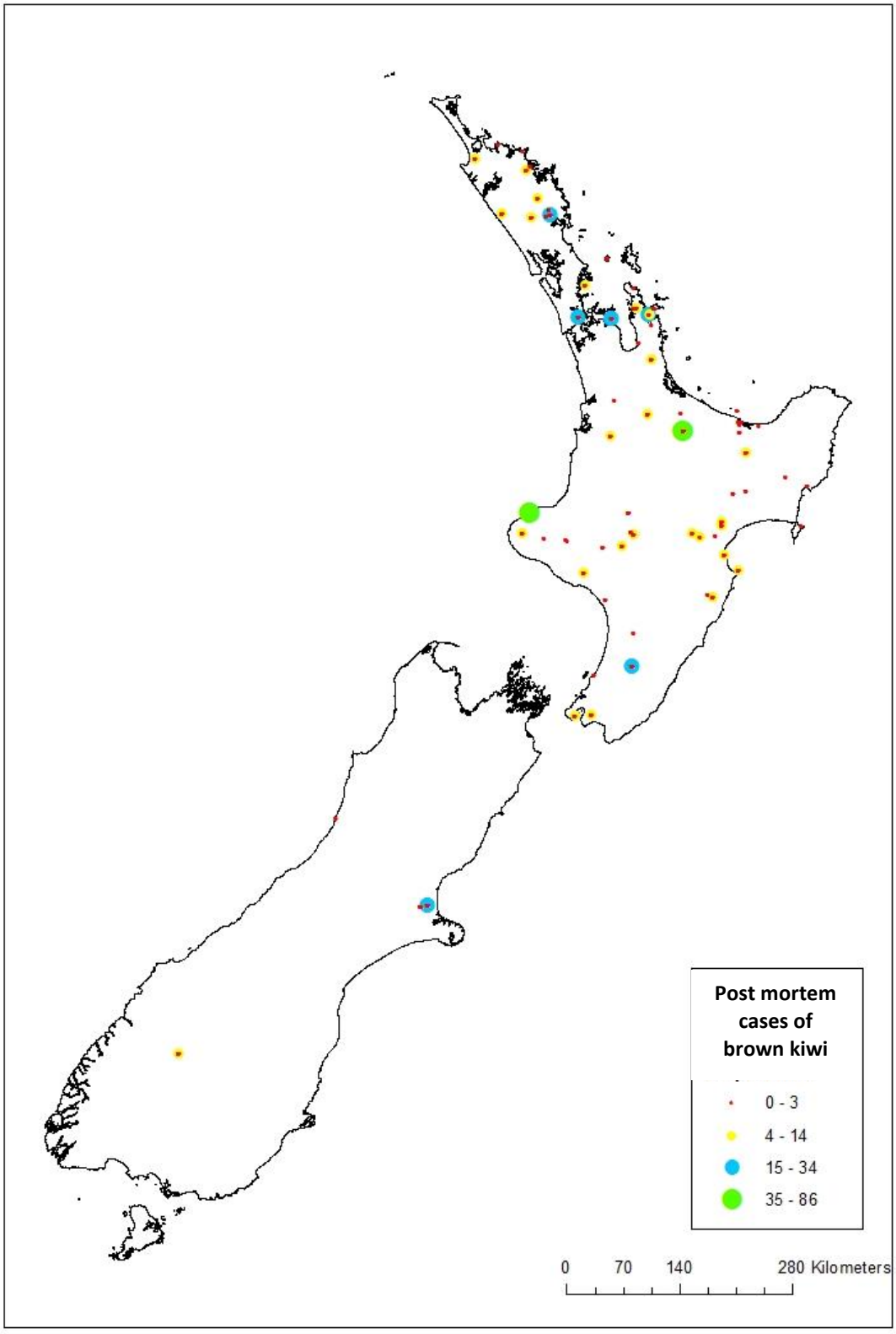

Figure 2.1 Geographical distribution of brown kiwi (*Apteryx mantelli*) (n=551) submitted for post mortem examination from 1991-2012. This includes wild and captive birds from the North Island and captive birds only from the South Island.

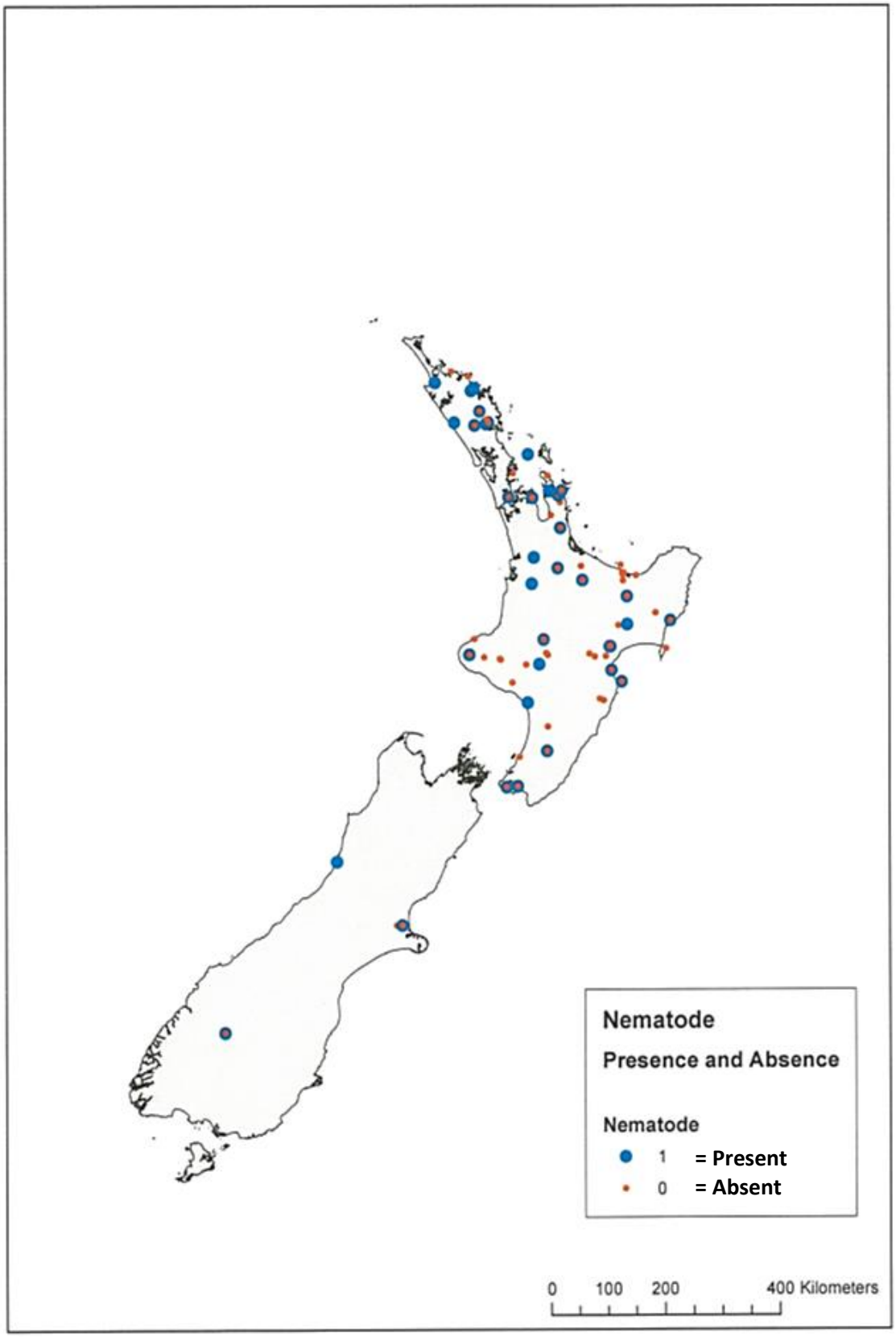

Figure 2.2 Geographic distribution of brown kiwi (*Apteryx* mantelli) (n=551) with the presence and absence of nematodiasis identified at post mortem examination from 1991 to 2012.

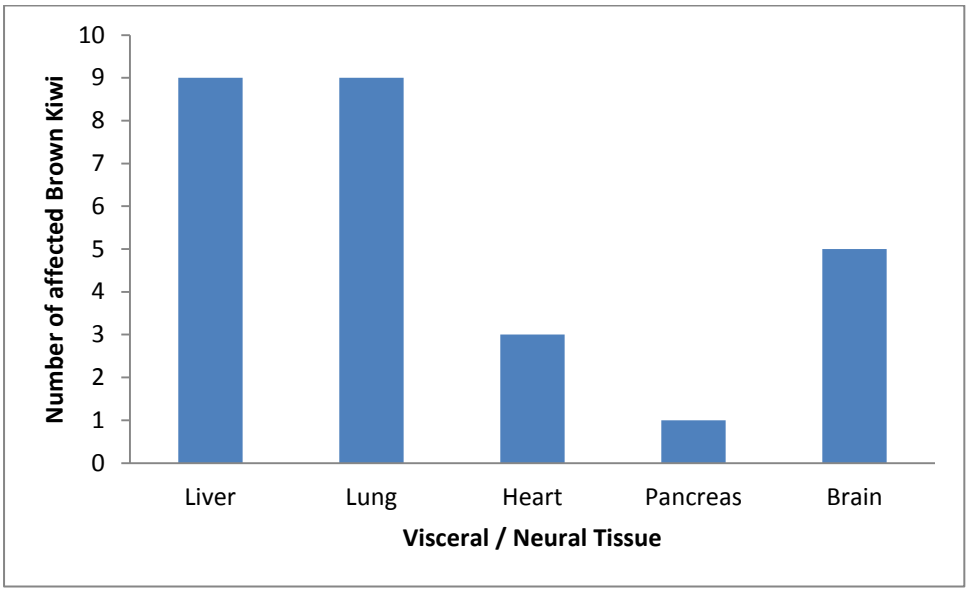

Figure 2.3. Frequency of visceral and neural larval migrans identified in tissues of brown kiwi (*Apteryx mantelli*) (N=551) submitted for post mortem examination at Massey University from 1991-2012.

three main areas; Greymouth, Christchurch and Queenstown. This map was also the basis to further identify the geographic distribution for cases of nematodiasis in brown kiwi in New Zealand (Figure 2.2).

2.3.1 Univariate logistic regression analysis

Of the 699 cases (including all kiwi species) that were examined in this study, 137 were chicks, 295 were juveniles and 248 adults (Table 2.2) (4 eggs and 15 birds were of unknown age). Adults in general had a significantly higher prevalence of nematodiasis , with 25% of adults infected with intestinal nematodiasis compared to 14.6% in juveniles and 2.7% in chicks. Adults also had the highest prevalence of neural (5.1%) and visceral larval migrans (5.6%).Visceral larval migrans was also more likely to occur in adults (Fisher's Exact test; p=0.025). Chicks were also recorded to have the lowest prevalence (under 6%) of nematodiasis, with no chicks infected with neural larval migrans.

Table 2.2.The associations of post mortem cases of nematodiasis in all five kiwi species (*Apteryx spp.*) by site of infection with respect to age, sex, habitat and host species (N = 462). The numerator denotes the number of positive cases, and the denominator denotes the number of relevant tissues available within each group.

Variables		Ventricular Nematodiasis		Intestinal nematodiasis		Neural Larval Migrans		Visceral Larval Migrans	
Age (n=303)	**Chick**	4/68	5.9%	2/75	2.7%	0/49[1]	0.0%	2/94	2.0%
	Juvenile	33/108	30.6%	15/103	14.6%	4/88	4.5%	7/173	4.0%
	Adult	24/61	39.3%	18/72	25.0%	3/59	5.1%	8/142	5.6%
	Chi square p value	<0.001*		0.0029*		Not valid		Not valid	
	Fisher's Exact Test P-value	<0.001*		<0.001*		0.2977		0.0254*	
Sex (n=226)	**Male**	24/89	27.0%	17/81	21.0%	3/75	4.0%	6/148	4.1%
	Female	26/84	48.1%	13/98	13.3%	3/71	4.0%	8/157	5.1%
	Chi square P- value	0.6678		0.2463		Not valid		0.6782	
	Fisher's Exact Test P-value	0.1163		0.0807		0.3182		0.1984	
Habitat (n=224)	**Captive**	13/83	15.7%	9/105	8.6%	1/66	1.5%	3/140	2.1%
	Crèche	1/9	11.1%	0/6[1]	0.0%	0/7[1]	0.0%	1/11	9.1%
	Wild	36/79	45.6%	21/67	31.3%	5/70	7.1%	10/151	6.6%
	Chi square P-value	0.0056		0.0372		Not valid		Not valid	
	Fisher's Exact Test P-value	<0.001*		0.0194*		0.2060		0.0164*	
Kiwi Species (n=462)	**Brown**	48/124	38.7%	26/138	18.8%	6/101	5.9%	12/232	5.2%
	Great Spotted	0/23[1]	0.0%	1/15	6.7%	0/16[1]	0.0%	0/26[1]	0.0%
	Little Spotted	2/7	28.6%	2/6	33.3%	0/8[1]	0.0%	1/11	9.1%
	Rowi	0/5[1]	0.0%	1/8	12.5%	0/9[1]	0.0%	1/11	9.1%
	Tokoeka	0/12[1]	0.0%	0/11[1]	0.0%	0/9[1]	0.0%	0/22[1]	0.0%
	Chi square P- value	Not valid		Not valid		Not valid		Not valid	
	Fisher's Exact Test P-value	0.2419		0.0298				0.1250	

[1] Variable not used in analysis due to lack of data; *Significant difference

Of the 699 cases examined, 226 were female and 239 were male (234 were unknown). A higher percentage of female kiwi were shown to have ventricular nematodiasis (48.1%) than males (27%). Males had a higher percentage of intestinal nematodiasis (21%) than females (13.3%). However, none of these differences were statistically significant and the differences probably reflect the distribution of the sexes that were received for necropsy. There was also no difference in the prevalence of neural larval migrans between males and females, and a 1% difference between the sexes with visceral larval migrans, with the females being higher. However, none of these differences were statistically significant.

Habitat was classified into three categories, crèche, wild and captive. Of the 699 cases necropsied, 422 were wild, 28 were crèche and 244 were captive (with 5 unknown). Kiwi populations in the wild had a higher prevalence of nematodiasis when compared to the other habitats. Kiwi that were in crèches had the lowest prevalence of nematodiasis; with no recorded cases of neural and intestinal nematodiasis. However, there was a significantly higher prevalence ($p=0.0164$) of visceral larval migrans in kiwi from crèche sites, compared to the prevalence in wild birds (6.6%). However it should be noted that birds from crèches are all young birds and this result is likely to be confounded. Therefore, a more valid analysis is to compare crèche birds to birds of the same age cohort from the wild or captivity, as seen in Appendix I. However no statistical significance was found for this analysis.

All species of kiwi were represented in the overall data set (699); although the data was heavily biased to brown kiwi ($n = 551$), with lesser numbers of the other species represented; great spotted ($n = 42$); little spotted ($n=12$); rowi ($n=40$); and tokoeka ($n=54$). No other species apart from the brown had any neural larval migrans. All species apart from tokoeka were diagnosed with intestinal nematodiasis, with little spotted kiwi having the greatest prevalence of intestinal nematodes (33.3%). Brown kiwi have the highest prevalence of ventricular nematodiasis (38.7%) compared to little spotted (28.6%), and the other three species of kiwi had no recorded diagnoses of ventricular nematodiasis.

2.3.2 Multiple Logistic Regression Analysis

Due to the lack of data, analysis of more than one independent variable was not possible with many cases (Appendix I). However, where statistical analysis was appropriate no multiple variable interactions were shown to be associated with any form of nematodiasis in kiwi.

2.4 Discussion

All species of kiwi were infected with nematodes at post mortem examination, apart from tokoeka (Table 2.1). Brown kiwi had the highest prevalence of nematodiasis at the time of necropsy and this species was also the only species identified as infected with neural larval migrans (NLM), with 6 out of 158 brains examined being positive for larval migrans. Interestingly, three kiwi species were positive for visceral larval migrans (VLM); brown, little spotted and rowi.

2.4.1.1 Intestinal nematodiasis

Adult kiwi and kiwi in the wild had the highest prevalence of intestinal nematodiasis (25% and 31.3% respectively) (Table 2.2). There are no significant results that link intestinal nematodiasis with the sex or species of kiwi. One might conclude the adult kiwi in the wild are more likely to pick up intestinal nematodes than are younger kiwi and those kept in captivity or crèches. Alternatively, kiwi in captivity and crèches are regularly treated with anthelmintics. Kiwi in the wild may encounter faecal droppings from many animals including other kiwi, therefore transmission of nematodes, especially intestinal nematodes would be more common.

2.4.1.2 Ventricular nematodiasis

Ventricular nematodiasis shows the same pattern as that of intestinal nematodiasis. Both adult kiwi and kiwi in the wild showed a high prevalence to ventricular nematodiasis. There is no significant relationship between prevalence of ventricular nematodiasis and sex or species of kiwi (Table 2.3). Ventricular and intestinal nematodiasis show the same pattern, as both of these categories are in adjacent parts of the digestive system of an animal. However, the species of nematodes that inhabit these tissues may differ significantly. We did not differentiate the species of nematodes in this study but future research should examine this.

2.4.1.3 Visceral larval migrans

Adult kiwi have a significantly higher prevalence of VLM than chicks and juvenile kiwi. Interestingly, we found that kiwi kept in crèches have a higher prevalence to VLM compared to wild and captive kiwi. There was no significant relationship between prevalence of VLM and sex or species of kiwi. However, there were a greater number of tissues sampled from kiwis in the wild and in captivity. Therefore, any interpretation of these results should consider this sample bias. The diagnosis of both VLM and NLM depends on the identification of parasitic granulomas (measuring approximately 100-500µm) within tissues. These are usually randomly distributed and not all contain readily identifiable parasitic remnants. For this reason, prevalence of infection in this study is likely to be underestimated in this study.

2.4.1.4 Neural larval migrans

There were no significant risk factors identified for NLM in kiwi. However, the number of brains necropsied is low compared to other tissues.

2.4.2 Likely impacts on kiwi

2.4.2.1 Intestinal nematodiasis

Intestinal nematodiasis is common in every kiwi and many of the parasites found in this study could be highly host adapted gut parasites. Nematodes can cause a range of direct health impacts on birds from complete tolerance of low numbers of host adapted species, through a spectrum of loss of fitness and nutrients, and in some cases serious disease and death if nematodes are found in high numbers or are poorly host adapted (Poinar & Boucot, 2006; Schoener et al., 2012).

When birds are kept in captive conditions or in close contact with each other, helminths could have a greater negative effect on the birds' condition, through increased exposure to infective ova and a greater number of parasites occurring in the animal (Schoener et al., 2012). However, my results were contrary to this and may reflect the likelihood that captive kiwis are often treated with anthelminthics, or are perhaps kept in more hygienic conditions, and therefore have a lower parasite load than kiwi in the wild.

2.4.2.2 Ventricular nematodiasis

Ventricular nematodes could also be host-adapted parasites of the kiwi, and therefore be causing minimal harm to the host. However, as mentioned before, if the parasite load becomes greater the host may exhibit clinical signs. If the nematodes found in the ventriculus are not host-adapted parasites, they could cause harm to the kiwi by travelling through other tissues. There is confusion as to where and when the ventricular nematodes attached to the gastrointestinal mucosa become larval migrans. There is no accepted definition for this in the literature, and many parasitologists have different and sometimes conflicting ideas

about this disease. Thus, ventricular nematodiasis could have a greater impact on kiwis than is thought; particularly if they are not normal host adapted parasites.

Ventricular nematodes in ostriches of the genus *Libyostrongylus* often results in severe erosions of the koilin and bacterial and fungal ventriculitis (Andrade et al., 2011). The predisposing role of ventricular nematodes in kiwi in causing more severe ventricular disease by disrupting the koilin and mucosa of the ventriculus is worthy of further study.

2.4.2.3 Visceral larval migrans

Visceral larval migrans has the potential to have a large impact on the health of kiwi. The migrating nematodes cause significant damage to tissues and organs that they enter in the viscera and could lead to the hosts' death. In my results, VLM occurred relatively frequently in kiwi and interestingly was common in crèche habitats of kiwi. This is a major problem for intensive conservation management of kiwi. If parasites are causing larval migrans and are extensive enough in the kiwi to cause death, our kiwi population in crèche habitats could be placed at risk. Therefore, we need to identify what nematode species are causing larval migrans in kiwi, and what the primary reservoirs are for these nematodes.

2.4.2.4. Neural larval migrans

Neural larval migrans has a significant impact on individual birds but is of low frequency in the kiwi population. It is likely these are non-kiwi adapted nematodes, but the normal environmental reservoirs of these parasites is currently unknown. Neural larval migrans may occur in association with VLM, as the nematode(s) travel through the tissues and organs to enter the blood stream and eventually reach the brain. This would be critical for any animal, and the kiwi would be no different, as without treatment the kiwi will die. If neurological symptoms are present in kiwi, and NLM is the cause, it may be too late as a large amount of damage could already

have occurred in the brain and other organs. NLM along with VLM could cause secondary infections and/or behavioural changes in kiwi which may lead to death of the bird. Some nematode species are more likely to result in NLM so identifying the species of nematodes and its reservoirs in the New Zealand environment will be important for managing this problem.

2.4.3 Limitations and bias

2.4.3.1 Geographical and host species bias

In all four categories where nematodiasis was investigated (intestinal, ventricular, VLM and NLM), sample sizes of tissues examined varied from >150 in the brown kiwi to a range of 8-36 in other species. This sampling bias is due to the relative abundance of the brown kiwi species around the North Island and in captive institutes as well as the ease of access to the pathology services at Massey University in Palmerston North.

In Figure 2.1 (which only focuses on brown kiwi) we can see that there is a large population of brown kiwi in the North Island and in Christchurch, which have been sent for necropsy to Massey University. The spatial distribution of the sample sources in the North Island are clustered around the Wairarapa, Taranaki, Whangarei, Auckland and Coromandel regions. Many of these regions correlate to where kiwi sanctuaries are around New Zealand. The Whangarei region has a Whangarei Kiwi Sanctuary (Anonymous, 2012). The Auckland region has Auckland Zoo which houses kiwi. The Coromandel has Moehau Kiwi Sanctuary, which covers about 16,000 hectares at the northern tip of Coromandel Peninsula. The Taranaki region has a Taranaki Kiwi Trust, which operates a trapping program to help catch the predators of the brown kiwi. The last region in the North Island that has a high number of brown kiwi sent to Massey University is the Wairarapa region. A wildlife restoration centre, Pukaha Mount Bruce is located here which houses brown kiwi. Thus, most of these regions in the North Island will have combined help from the communities and the Department of Conservation (DOC) to help with trapping and

keeping the population free from pests. This will include sending ill kiwi for treatment or dead kiwi to determine a cause of death to Massey University.

Rowi and little spotted kiwi had the least number of tissues examined. This would be due to the low population numbers of these species. Rowi is classified as 'threatened: nationally critical', as they have a very small population size of around 200 birds (Anonymous, 2014). This species relies heavily on the Operation Nest Egg (ONE) scheme that DOC implemented in the mid 1990's. The little spotted kiwi also has a low population, estimated to be 1,500. However, this species is located mainly on offshore islands such as Kapiti Island, Long Island, Tiritiri Matangi, Hens Island, Red Mercury Island and the Karori Sanctuary (which is situated in Zealandia in Wellington) (Anonymous, 2012). Therefore, retrieving dead kiwis from the islands for necropsy is logistically difficult, as rangers may not find the kiwi before they begin to decompose. This would make necropsy diagnosis difficult, or near impossible if the carcass is decomposing or scavenged and all organs have been removed.

Great spotted kiwi still have a large population size around 16,000 (Anonymous, 2012) and these are found around northwest Nelson region, Paporoa Range and near Arthur's Pass. They have also been transferred to Lake Rotoiti mainland Island. Due to this species living at higher altitudes it is thought that they are protected against some predators due to the harsher conditions that go with high altitude. The difficulties in retrieving kiwi from these environments is a cause of bias and sending them long distances to Massey University is expensive and time consuming.

Lastly the tokoeka species is the most numerous kiwi species when the four regions (Haast Range, Stewart Island, Northern Fiordland and Southern Fiordland) are combined (Holzapfel et al., 2008). However, the Haast tokoeka have a low population size of about 300, which makes it more vulnerable that the other forms of tokoeka. The tokoeka species also occupies rather remote areas and the same problem occurs in sending birds for necropsy as with the other remote species. This in return creates a bias in our data where as little as 32 (Table 2.1) tissues where sampled from this species, compared to the brown kiwi (N=551).

2.4.3.2 Identification

There is a serious gap in our knowledge in the identification of nematodes after necropsy, as identification solely relies on histology and/or morphology and at this stage does not include molecular work. This causes limitations as there are very few morphological characteristics that identify nematode species apart, especially if a whole parasite is not available. It may also be difficult to identify the parasite because it not known what larvae stage is present at the time of necropsy. DNA-based identification is an extremely sensitive method for identifying; parasites allowing even silent nucleotide positions to be compared (McManus & Bowles, 1996). However, doing molecular work can be expensive and very time consuming. In an ideal world, including molecular methods would allow more accurate diagnosis to be available in necropsy reports.

2.4.3.3 Post mortem study

One of the limitations in our study include that samples were only examined from dead birds. This meant that there was variation in numbers of birds submitted from each species of kiwi; and the number of tissues examined post mortem varied. The difference in the number of tissues examined at post mortem reflects that varying states of preservation in which birds were submitted for examination, or may include constraints placed on the extent of post mortem examination for cultural reasons.

2.4.3.4 Routine treatment of captive kiwi for nematodes

In most cases where chicks are kept in captivity they are surrounded by a sterile environment which would make infection of nematodes near impossible. This is reflected in the low number of nematodes present in chicks that were kept in captivity compared to those chicks which were found in the wild. It could be

assumed that kiwi kept in captivity would also be routinely treated for parasites and other diseases. Thus, our study would bias nematode infection toward wild kiwi.

2.4.4 Future research

Parasites are increasingly cited as a major threat to wildlife conservation, being implicated in the decline and/or extinction of many high-profile species worldwide (Tompkins & Poulin, 2006). Parasites may be expected to become adapted to their local hosts, because parasites are often more numerous and have shorter generation times than that of their host (Lajeunesse & Forbes, 2002). This research has identified some gaps in the taxonomic identification of nematodes in kiwi, their environmental reservoirs and the host-pathogen dynamics that occur between kiwi, introduced birds and their nematodes. Because this research is the first step into looking into these patterns there are many paths that still need to be explored.

Identification of the nematodes through molecular methods, where whole nematodes are not available is an important area to explore in future work. This is especially important in the neural and visceral larval migrans cases. These aberrant larval migran parasites would need to be identified through molecular work to encourage specific treatment of these parasites as well as encourage better management plans for kiwi to prevent future infection. Molecular work is the best option to correctly identify the parasite, especially if a whole parasite cannot be extracted.

Having the nematode identified through molecular work would further the investigation of the life cycle of that nematode. How it causes the migration through tissues and organs, and at what stage this migration occurs. With this knowledge it could further open up the possibilities of other parasites going through the same cycle and being a potential threat to our native and endangered birds. It could potentially also lead to the investigation of host interaction of the 'normal' host of that parasite. This would lead to advances in better management plans where appropriate, as well as increased surveillance in kiwi species. This is

particularly important for the rowi species, as they are the currently the species most critically at risk. Having increased surveillance in this population on their crèche islands could help prevent parasites and potentially even aberrant larval migration of unknown nematodes.

2.4.5 Management implications

There are important management implications from the findings in this study. We have seen that visceral larval migrans is common in kiwi from crèche habitats. Therefore, a better management plan would need to be advised for conservation managers of these areas. This would include routine nest box clean outs and regular relocation, if the parasite is from animals that share the same roosts. Potentially looking at more pest control or looking at ways to prevent nest box sharing to avoid parasite transmission. This is particularly important for rowi, as a big part of their breeding programme involves the juveniles going to offshore crèches on predator free island until they reach maturity. If VLM becomes a major factor on crèche islands, it has the potential to put the already declining population at further risk.

There is also the potential to do faecal collections in the wild to monitor parasite load in kiwi. The correlation between faecal egg counts and the associated total worm burdens and pathogenic effects of nematodes in kiwi needs validation. The use of anthelmintics in the intensive management of kiwi in captivity and in conservation programmes such as ONE also needs investigation.

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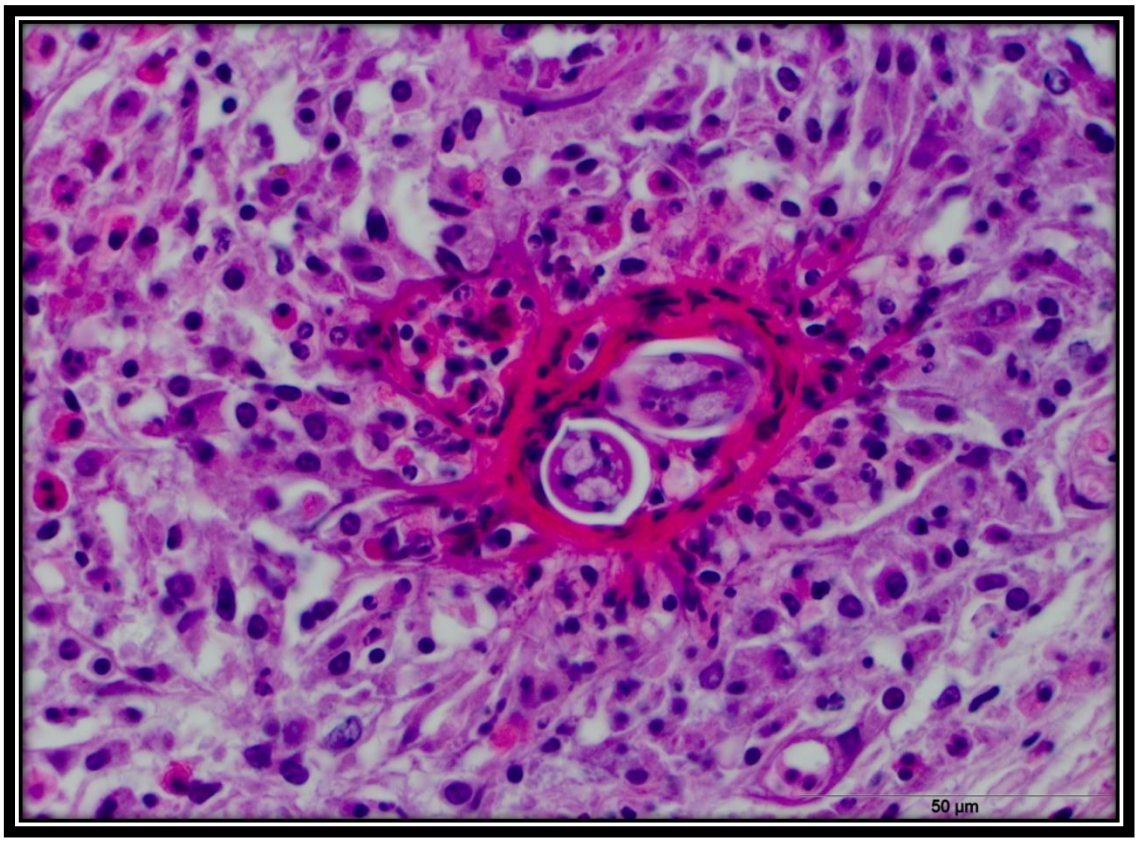


Chapter Three

The use of molecular techniques to attempt to identify the cause of neural and visceral larval migrans in Brown kiwi (*Apteryx mantelli*)

Chapter 3. The use of molecular techniques to attempt to identify the cause of neural and visceral larval migrans in Brown kiwi (*Apteryx mantelli*)

3.0 Abstract

Larval migrans occurs when a parasite, normally nematodes, cannot complete their life cycle within an aberrant host. This causes the nematode to travel through the organs of the aberrant host, which can cause tissue damage and inflammation. Larval migrans have been noted in the Brown kiwi species (*Apteryx mantelli*), and has been proposed to be due to either *Toxocara cati* or *T. canis* species due to the presence of a lateral alae on the nematode larvae in tissue sections. This led to this study which used molecular work on these infected archived tissues using specific *Toxocara* primers. The results showed no evidence of infection with *T.cati* or *T. canis*. This paper then discusses other nematodes that could possibly be the cause of these larval migrans in brown kiwi.

Keywords: Brown kiwi, aberrant hosts, nematodes, larval migrans, *Toxocara*, PCR

3.1 Introduction

The brown kiwi (*Apteryx mantelli*) is the most widespread, best known and most abundant kiwi species, having a total population size of about 25,000 (Anonymous, 2012; Castro, 2011; McLennan & Potter, 1992). Within this species four genetically and geographically distinct forms have been identified: Northland, Coromandel, western and eastern brown kiwi (Burbidge et al., 2003; Department of Conservation, 2011). However, the overall population numbers are still declining, and the species has been classified as 'threatened: nationally vulnerable' by the Department of Conservation (Anonymous, 2012; Sales, 2005).

Although the population decline is mainly due to predators such as stoats (*Mustela erminea*), possums (*Trichosurus vulpecula*), ferrets (*Mustela furo*), pigs (*Sus domesticus*), cats (*Felis catus*) and dogs (*Canis lupus*) (Basse et al., 1999; McLennan & Potter, 1992; Sales, 2005) it is important to study all factors that could influence the decline of this species. One cause of illness and mortality in brown kiwi are parasites and heavy parasite burdens can make kiwi prone to predation or accidental trauma (Alley & Gartrell, 2006). On a total biomass, the majority of living organisms are parasitic, and it is apparent that they are a major selective force that influence almost every conceivable aspect of their hosts' biology (Holmstad et al., 2005).

The kiwi family have 17 recognised different kinds of helminth and protozoan parasites (McKenna, 2010). Of these, 5 have been identified as nematodes and have been recovered from brown kiwi. Most of these nematodes have been identified only from the gastrointestinal tract (Clark & McKenzie, 1982; McKenna, 2010; Weekes, 1982). However, in the pathological reports of larval migrans in brown kiwi completed to date, the species of nematode involved has not been identified (Alley & Gartrell, 2006; Alley et al., 2004). Larva migrans is defined as the prolonged migration of a larval parasite, usually that of a nematode, in the skin or internal organs of an abnormal host (Beaver, 1969). It has previously been proposed that the nematode species causing this larval migrans in the brown kiwi are either *Toxocara cati* or *T. canis* based on the presence of lateral alae visible on

nematode larvae in some histopathological sections (M.Alley *pers. Comm*). However, no whole larvae could be extracted from tissues at post mortem examination and morphological identification of the parasite was not possible.

Both *T. canis* and *T. cati* are ascarid nematodes, in the family Toxocaridae. Their definitive hosts are the domestic dog and cat, respectively, where they live as adults within the lumen of the small intestine (Despommier, 2003). Infection can occur when a host ingests viable embryonated eggs from contaminated sources (e.g. soil) or paratenic hosts (e.g. earthworms); or via intrauterine or mammary transmissiom (Despommier, 2003; Iowa State University, 2014; Lim, 2008; Pietsch et al., 2002). Both *T. cati* and *T. canis* have been recognised as causing zoonotic disease. The impacts these migrating species have on humans have sometimes been very damaging and occasionally lethal (Fisher, 2003). They are associated mainly with two syndromes; ocular larval migrans (OLM) and visceral larval migrans (VLM) (Fisher, 2003).

Larval migrans occurs when nematodes cannot complete their lifecycle within an aberrant host (eg. humans and kiwi). The infective larvae will hatch after their ingestion as eggs, but the juvenile stages fail to develop to mature adult worms (Despommier, 2003). Instead these juvenile stages wander throughout the body of the host for months or up to several years, causing damage to tissues they migrate through (Despommier, 2003; Lim, 2008; Yu et al., 2012).

The specific aim of this chapter was to use molecular techniques to determine whether the migrating larvae identified in the tissues of brown kiwi were from the *Toxocara* species.

3.2 Methods:

3.2.1 Huia database

Archived records from "Huia", the National Wildlife Pathology Database, of the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North, were searched for all kiwi necropsied at our laboratory between 1991 and 2012 (n=699) (Morgan et al., 2013). Cases with visceral and neural larval migrans were identified and the histological slides were extracted from storage. These contained the affected tissues fixed in 10% neutral buffered formalin and embedded in paraffin blocks.

3.2.2 DNA extraction from Toxocara cati and T.canis

Fresh *Toxocara* nematodes were sourced from a wild cat and a domestic dog that came for post mortem at Massey University, Palmerston North to utilise as positive controls. DNA was extracted using a Qiagen DNeasy kit, following the Quick-Start protocol (Qiagen, CA, USA).

3.2.3 Histopathology

The archived tissue samples were prepared for histological analysis by the Histology lab in IVABS at Massey University, Palmerston North, New Zealand. The samples were fixed at 10% neutral buffered formalin, processed routinely, cut at 4 µm, and stained with haematoxylin and eosin. New sections were cut from the blocks, if slides from cases were not found.

3.2.4 Isolation of genomic DNA and PCR analysis

DNA was extracted from 10 µm sections of tissue samples that had been formalin fixed,and paraffin embedded. The sections that were used for molecular analysis were from a sandwich cut between two slides that showed histological evidence of nematode larvae. This was composed of a 4 µm section, then a 10 µm section followed by another 4 µm section, with the 4 µm sections used for histology and the 10 µm for DNA extraction. DNA was extracted using a Qiagen DNeasy kit, following the Pretreatment for Paraffin-Embedded Tissues protocol (Qiagen, CA, USA). The internal transcribed spacer 2 (ITS-2) from within the ribosomal DNA (rDNA) genes was amplified using primer sets described by Jacobs et al. (1997). *T. canis* was amplified using the species-specific forward primer T.can (5'-AGTATGATGGGCGCGCCAAT-3') and genus-specific reverse primer NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3'), and *T. cati* was amplified using species-specific forward primer T.cat (5'GGAGAAGTAAGATCGTGGCACGCGT-3') with reverse primer NC2. All samples were amplified using both primer sets.

For PCR amplification the conditions were as follows: 1U Platinum Taq (Invitrogen, CA, USA), 1 X PCR buffer (200mM Tris HCL pH8.4, 500mM KCl), 1.5mM $MgCl_2$, 200µM dNTPs each, 1µM of each primer, 50µg of template DNA in a total reaction volume of 25µL. The reaction was carried out under the following conditions in a SensoQuest Labcycler (Germany); an initial 5 minute denaturation at 94°C, was followed by 30 cycles of, 94°C, 55°C, and 72°C, with each step lasting for 30 sec. A final extension at 72°C for 7min concluded the amplification reaction (Fogt-Wyrwas et al., 2007). A negative control (water) and two positive controls (*T. cati* and *T. canis* DNA) were used for quality control standards. An aliquot of 10µL of the PCR product was then separated on a 1% agarose gel that contained 0.2mgml^{-1} of ethidium bromide for staining. The gels were run at 100V for one hour and were visualised with a UV transilluminator. A 100bp molecular ladder (Promega Madison W1, USA) was concurrently run with the PCR products to provide size estimation.

3.3. Results

Eighteen cases of kiwi with visceral and/or neural larval migrans with accessible archived tissues were identified out of the 699 cases in the databases. Many of these cases contained more than one affected organ, and a total of 29 slides containing affected tissues were available.

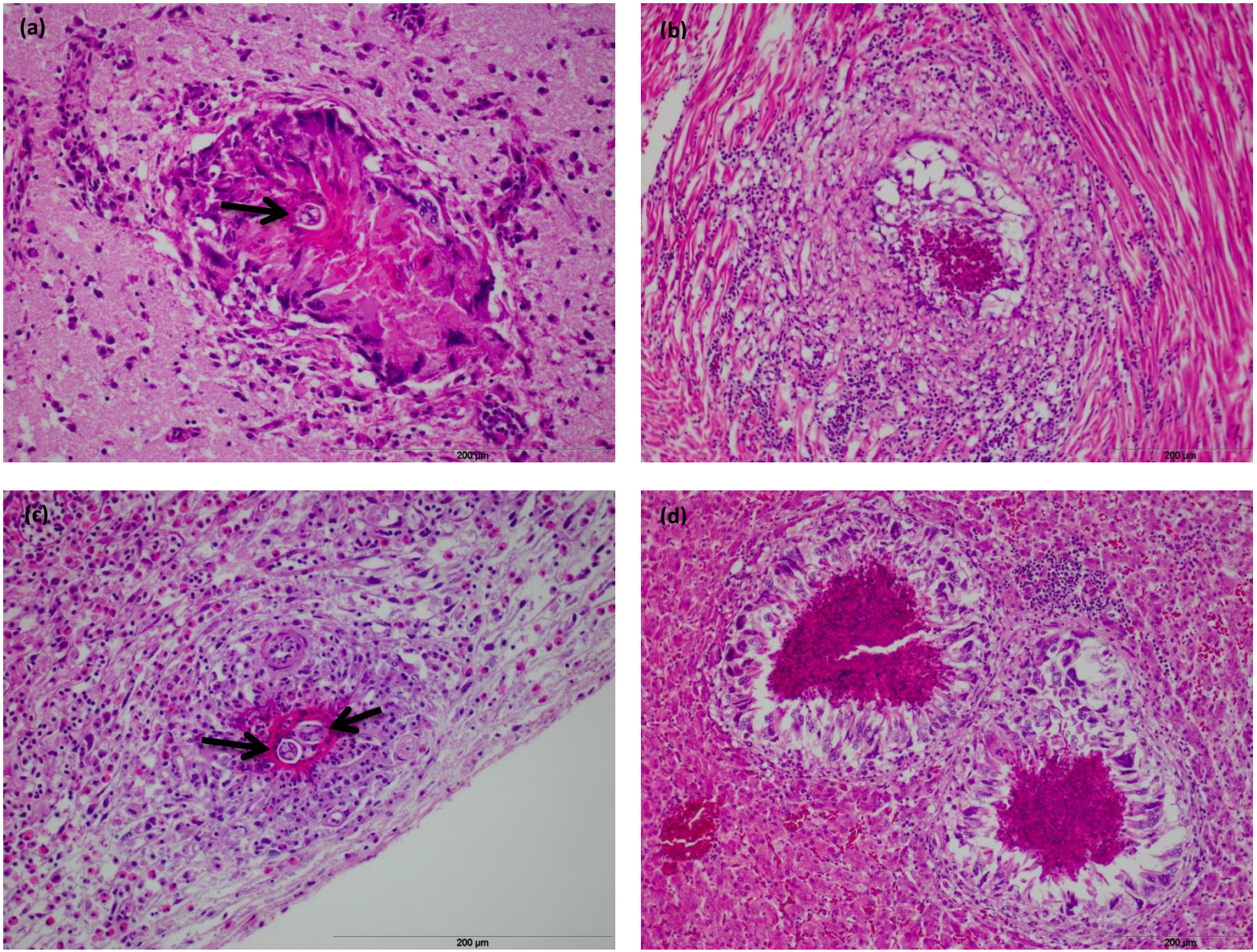
3.3.1 Histopathology

Of the 18 kiwi affected with larval migrans, 15 cases were of visceral larval migrans and 6 were cases of neural larval migrans. However, some kiwi had more than one organ affected and some also had both forms of larval migrans, (Table 3.1). In the total of 29 slides examined histologically, 3 tissues showed cross sections of nematodes, and 13 slides showed granulomas suspicious of larval migrans, as shown in Figure 3.1.

Table 3.1. The distribution of larval migrans lesions in 18 Post mortem cases in brown kiwi (*Apteryx mantelli*).

	Liver	Lung	Heart	Brain
Parasitic granulomas	11	6	2	6
Parasitic granulomas with larvae	1	2	0	3
Total cases[1]	12	8	2	9

[1]Note there is an overlap of cases where kiwi had more than one organ affected


(a)
200 µm
(b)
200 µm
(c)
200 µm
(d)
200 µm

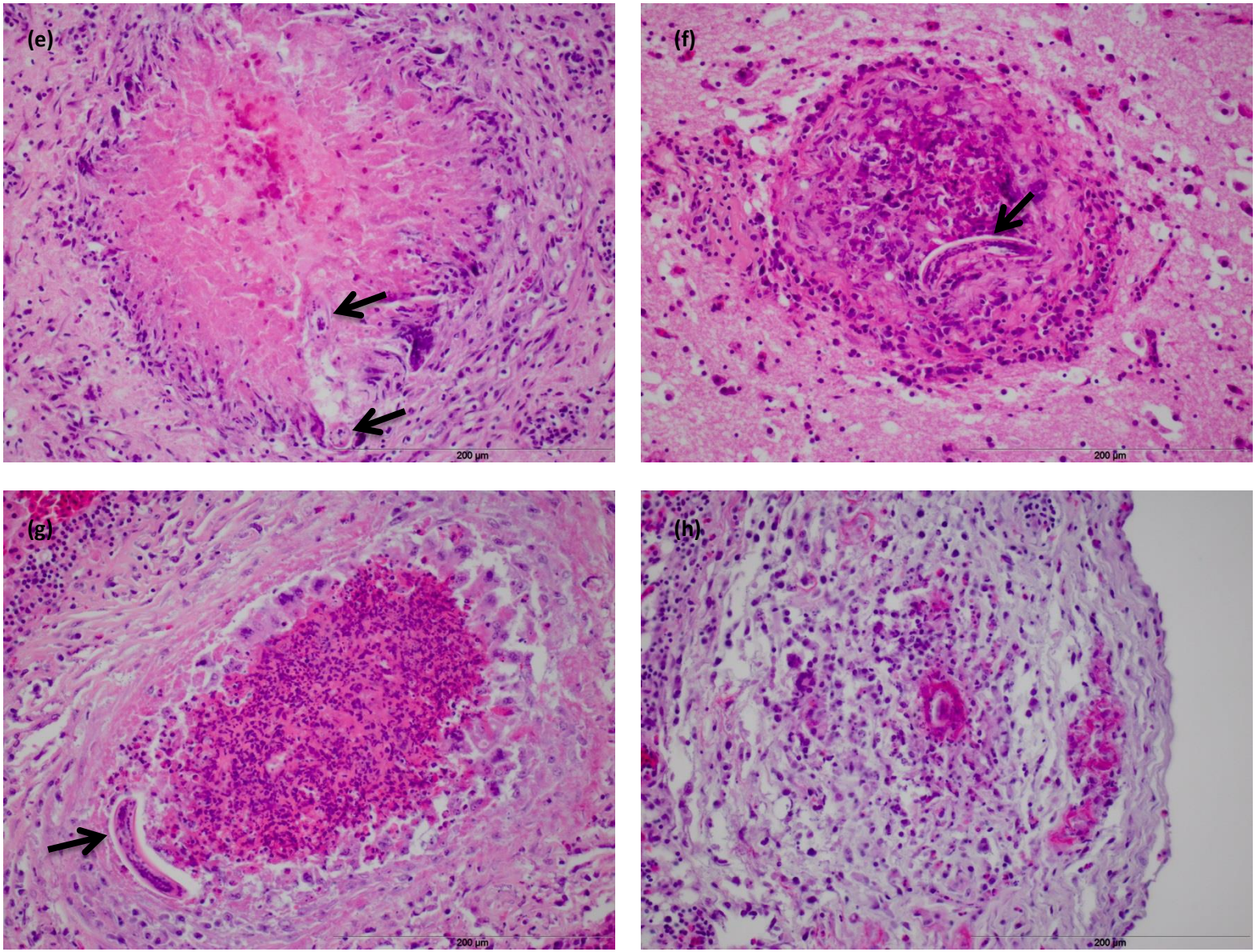

(e)
200 µm
(f)
200 µm
(g)
200 µm
(h)
200 µm

Figure 3.1. Photomicrograph of parasite granulomas in (a): the brain of a juvenile brown kiwi (*Apteryx mantelli*; Case 47257). A cross-section of a nematode larva is evident in the granuloma. Hematoxylin and eosin (HE) stain. Scale bar= 200µm. (b) the myocardium of the same kiwi. HE. Scale bar= 200µm (c): the lung of the same kiwi, containing a parasite granuloma with cross-sections of nematode larvae. Diffuse mixed inflammatory response can be seen. HE. Scale bar= 200µm. (d): the liver of the same kiwi showing two large granulomas. HE. Scale bar=200µm. (e): the brain of an adult brown kiwi (Case 37499). With a cross-section of a nematode larva, as well as part of a nematode larva evident in the granuloma. HE. Scale bar= 200µm. (f): the brain of a juvenile brown kiwi (Case 38174) showing a nematode larvae within the granuloma. HE. Scale bar= 200µm. (g): the liver of the same kiwi showing a nematode larva within the granuloma. HE. Scale bar= 200µm. (h): the lung of the same kiwi. HE. Scale bar= 200µm.

Typically granulomas were composed of central cores of eosinophilic and pyknotic granular debris (with and without the presence of larval nematode/s) bordered by a rim of epithelioid macrophages and multinucleated giant cells; although eosinophils and heterophils were not a prominent feature. When granulomas were present in the brain, there was often cuffing of surrounding blood vessels with small numbers of lymphocytes and plasma cells. In some instances nematode larvae were surrounded by necrotic debris and small numbers of heterophils and eosinophils. There was also minimal macrophage or multinucleated giant cell component which would suggest an initial inflammatory response to a more recent arrival of the nematode larvae, before granuloma formation has taken place. When larval nematodes were present, they were either in cross or longitudinal sections. Larvae were approximately 20-30 microns in diameter and approximately 150-200 microns in length. Cuticle thickness and internal structures were difficult to discern, but there was one transverse section in which the larva had paired lateral alae. When more than one transverse section was present it was difficult to tell whether these represented one coiled larva or two separate larvae.

3.3.2 Amplification

No successful amplification of PCR product occurred in any of the 28 samples (Figure 3.2) despite successful amplification of the positive controls (*T .cati* and *T. canis*) at approximately 320bp. The PCR was repeated 2 times for all samples with the same result.

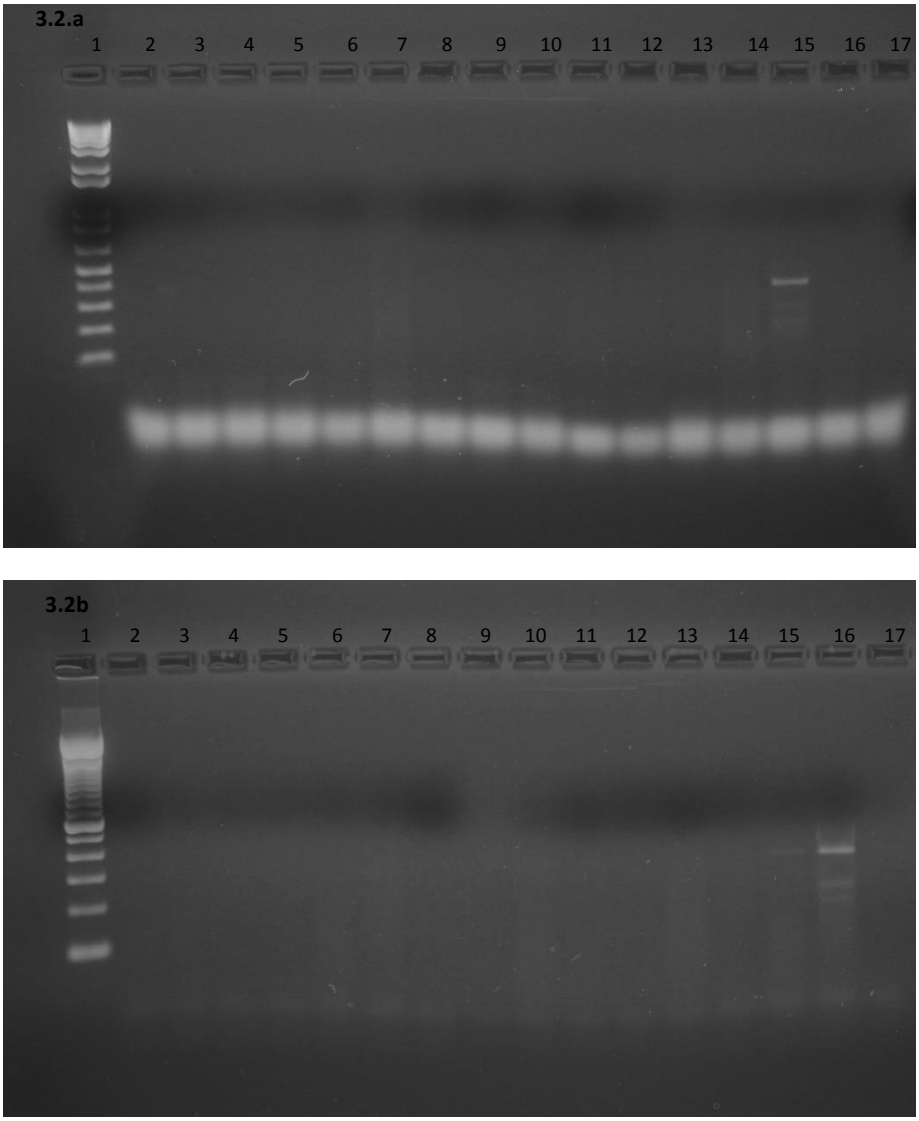


Figure 3.2. PCR amplification product showing positive results for controls only. **3.2a** The lanes 1= Ladder, 2-14= different kiwi DNA, 15=*T. cati*, 16=*T. canis*, 17= water. **3.2b** The lanes 1= Ladder, 2-14= different kiwi DNA, 15=*T. cati*, 16=*T. canis*, 17= water.

3.4 Discussion

In order to identify the nematode sections in the visceral organs and sections of brain, DNA was isolated from fresh *T. cati* and *T. canis* and from archival formalin-fixed, paraffin-embedded sections of the viscera and brain. Although ITS-2 sequences consistent with *Toxocara* spp. were successfully amplified from control nematodes in all combinations, the amplification of the same sequences from the formalin-fixed, paraffin embedded tissues was not successful (Tišljar et al., 2007). However, several other studies have been able to successfully amplify rDNA from archived samples stored in this manner, including successful amplification of rDNA from cutaneous larval migrans in the rowi kiwi species (Chapter 4). Dermal samples from rowi kiwi were formalin fixed and embedded in paraffin wax using the same techniques as in the current study, and amplification of rDNA was successful, although different primers were used. This suggests that the negative findings in the current study were not due to difficulties in extracting DNA but rather indicates the absence of *Toxocara* in the tissues examined.

The negative findings obtained may, never the less, be due to technical difficulties as it is often difficult to amplify long DNA fragments from formalin-fixed and paraffin-embedded tissues. Formic acid formed in formalin solution, the fixation time in formalin, and the period of storage of the tissue blocks may all affect the quality of DNA (Sato et al., 2001). Because DNA is not well preserved or can be degraded in fixed tissue it can be difficult to obtain consistent results. It has been shown that DNA extracted from specimens fixed in formalin for longer than 24 hours showed a marked decline in the efficiency of DNA amplification (Sato et al., 2001). However, the method used for DNA extraction in the current study consisted of many steps, including deparaffinization in xylene, protein digestion, phenolchloroform purification, and ethanol precipitation, and this has the advantage of obtaining a high DNA purity (Sato et al., 2001).

While these results do not support the hypothesis that *Toxocara* nematodes are the causative agents of the larval migrans in kiwi, there are many other possible causative organisms that need to be specifically investigated. Three other possible species of nematodes that could be the cause of the larval migrans of kiwi include *Angiostrongylus cantonesis*, *Ascaris suum* and *Baylisascaris procyonis*. Neither *A. cantonesis* or *B. procyonis* have been identified in New Zealand but the primary host of *Angiostrongylus cantonesis,* rats, are very common introduced pests in the New Zealand environment. *A. suum* is a common parasite found in most pigs worldwide including New Zealand. *Baylisascaris* is a more remote possibility given that its primary host, the racoon, is not found in New Zealand, but as a common cause of neural larval migrans elsewhere, features of this nematode will also be discussed.

3.4.1 Angiostrongylus cantonesis

Angiostrongylus is a genus of parasitic nematode that generally inhabits the lungs of rodent hosts. With the invasive spread of rodents throughout the world, the corresponding spread of parasites and diseases carried by these vectors into newly colonised environments may impact humans and native fauna (Stokes et al., 2007). This rat lungworm, is one parasite that causes zoonotic disease (neuro-angiostrongyliasis) in non-target human, bird and mammalian hosts globally (Stokes et al., 2007).

Adult worms reside in the pulmonary arteries and the right ventricle of the definite host. While many species of rats carry patent infections, the Norway rat (*Rattus norvgicus*) and the black rat (*Rattus rattus*) are considered to be the most important definitive hosts and vectors for this parasite (Ma et al., 2013). Eggs released by a mated female *A. cantonesis* will embolise in pulmonary capillaries and the larvae will migrate up the trachea to be swallowed and shed in the faeces of the rat. Intermediate hosts including various species of molluscs are infected by ingesting the first stage larvae in the rat faeces (Ma et al., 2013).

Larvae of the *A. cantonesis* have an obligatory migration through neural tissues of the definitive host as part of their development to the adult stage. Following ingestion by rats or other non-target species, which can include humans, dogs and other wildlife; the larvae will penetrate the intestinal wall and spread through the blood via the lymphatics to the brain and spinal cord. Neurological disease in non-target hosts is the result of both direct damage to neural tissues by migrating larvae and the damage caused by host inflammatory response (Ma et al., 2013).

This parasite has already been found in some wildlife in Australia, for example captive Bennett's wallabies (*Macropus rufogriseus*), captive bettongs (*Aepyprymnus rufescens*), captive tamarins (*Sanguinus spp.*), wild black and grey headed flying foxes (*Pteropus poliocephalus*), a captive yellow-tailed black cockatoo (*Calyptornchus funereus*) (Monks et al., 2005), tawny frogmouths (*Podargus strigoides*) and brushtail possums (*Trichosurus vulpecula*) (Ma et al., 2013; Stokes et al., 2007). Even though this parasite has not yet been identified in New Zealand, this does not exclude the possibility that it is present. The nematode could occur in our introduced rats as well as possums, and transmission from these pests to native wildlife could easily occur. Kiwi probe for their food in soil, and their diet consist of roughly 40-45% earthworms, 40-45% other invertebrates and 10-15% plant material (Colbourne & Powlesland, 1988; Sales, 2005). *A. cantonesis* have an intermediate host that consist of molluscs, however kiwi do not ingest molluscs as part of their diet, so immediate transfer cannot occur. However, rat and possum faeces could be ingested by kiwi through means of direct contact through probing in the soil or through ingestion of earthworms that could contain contaminated faeces.

3.4.2 Ascaris suum

Another possible cause of the larval migrans seen in kiwi could be the nematode *Ascaris suum* whose primary host is the pig. *Ascaris suum* eggs are shed in grass or in mud where they may encounter salt concentrations of a few mM, oxygen

tensions of 100-200mm Hg and a near neutral pH (Thompson & Geary, 2002). The eggs that are shed into this environment may survive desiccation for long periods and even cooling to temperatures that are below freezing level. Though the pre-parasitic moults occur by about 3 weeks after the egg is passed, a period of maturation is necessary, and the egg is not usually infective until a minimum of four weeks after it has been passed, even in its optimal temperature of 22-26^{o}C. The egg of *A. suum* is very resistant to temperature extremes, and is viable for more than four years (Satoskar et al., 2009; Taylor et al., 2007). The life cycle of this parasite is direct (Taylor et al., 2007).

When these eggs are ingested, the infective larvae will enter the stomach of the host (normally porcine). This is a highly acidic environment that includes an array of digestive enzymes, osmotic pressures from 200-500 mOsm and oxygen tension that is very close to zero (Thompson & Geary, 2002). From the stomach they pass into the intestine, where they will hatch (L3 larvae), and penetrate the intestinal mucosa and before travelling to the liver (Taylor et al., 2007; Thompson & Geary, 2002). The larvae then passes into the bloodstream to the aerobic environment of the lungs. The L3 larvae (or the infective larvae) will then migrate to the trachea, where they are swallowed back into the stomach by the host and where they will eventually return to the anaerobic environment of the small intestine. Here they will mature and reproduce (Thompson & Geary, 2002) and the young adult worms inhabit the lumen of the small intestine (Taylor et al., 2007).

If the eggs are ingested by an earthworm or dung beetle they will hatch, and the L3 travel the tissues of this paratenic hosts, where they will remain fully infective for a long period. The prepatent period is about 7 and 9 weeks (Taylor et al., 2007). This would be a key route for the infection of this parasite into kiwi, presumably an aberrant host. Kiwi consume large numbers of earthworms, and the earthworm could contain eggs of *A.suum*. The eggs would hatch in the duodenum or jejunum of the aberrant host and the emerging larvae will penetrate the intestinal wall and enter the circulatory system through which they reach the liver and lungs (Inatomi et al., 1999). Visceral larval migrans following ingestion of eggs from *A.suum* have been recorded in humans.

In the wild, kiwi often share their habitat with feral pigs, so that infection by this parasite is definitely possible. Ineson (1954) found that *A. suum* was present in 43% of samples from 62 feral pigs that he obtained during pig hunting trips.

3.4.3 Baylisascaris procyonis

The last possibility is the common intestinal roundworm of the racoon (*Procyon lotor*) *Baylisascaris procyonis* (Evans, 2002). This parasite has been a well-documented cause of VLM, OLM and NLM in many species including 90 species of domestic birds (Sorvillo et al., 2002) and mammals, including humans (Evans, 2002; Page et al., 1998). As with other ascarids, eggs are excreted into the soil, and must develop externally to become infectious. Once infectious, the eggs can remain viable in the environment for years, even during harsh weather conditions (Shafir et al., 2006) When raccoons ingest infective eggs, larvae hatch and enter the wall of the small intestine and subsequently develop into adult worms in the small bowel (Sorvillo et al., 2002). However, when aberrant hosts ingest these eggs, the larvae hatch, then penetrate the intestinal wall, enter the bloodstream, and are disseminated to various organs and tissues. The sites of larval migration will vary between hosts, although the larvae will often migrate to the central nervous system, which causes behavioural changes in those animals (Shafir et al., 2006). *Baylisascaris* has not been recognised in New Zealand, and therefore is considered as an exotic disease. Further, its primary host, the racoon has never been introduced to New Zealand. It is therefore unlikely that this could be the cause of VLM and NLM in kiwi.

In conclusion, the results of this investigation suggest that the causative organism of the larval migrans affecting kiwi is unlikely to be *Toxocara*. However, more molecular work will need to be undertaken on these and future samples to identify the parasite. It is recommended that priority be given to molecular analysis of the tissues for *Angiostrongylus cantonensis and Ascaris suum*. If these species are also

ruled out as causative agents of the larval migrans in kiwi, then consideration should be given to screening the tissue samples for *Baylisascaris procyonis.*

We should also consider that often a diagnosis of neurological disease in wildlife has been based on clinical presentation rather than a definitive diagnosis through gross and microscopic post mortem examination with ancillary tests (Ma et al., 2013). Future investigative work should include the archiving of whole organs where such a parasite has been found, recovering whole nematodes where possible for morphological identification and attempting to carry out molecular diagnostics on fresh tissue as soon as possible after post mortem examination

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Chapter Four

Identifying the nematodes causing cutaneous larval migrans in Rowi (*Apteryx rowi*) using molecular techniques

Chapter 4. Identifying the nematodes causing cutaneous larval migrans in Rowi (*Apteryx rowi*) using molecular techniques

4.0 Abstract

Rowi (*Apteryx rowi*) are a critically endangered species of kiwi with only 375 birds left. They are intensively managed with young kiwi being moved to predator free islands (crèches). On the crèche islands a number of chicks showed signs of feather loss from the ventral abdomen and a scurfy dermatitis around the abdominal area. Skin biopsies were taken from the birds, and after histological examination, it revealed that the dermatitis was caused by cutaneous larval migran (CLM). CLM has never been recorded in any avian hosts. Upon molecular investigation it was found that the nematodes involved in the CLM is closely related to the *Trichostrongylus axei* species. *T. axei* is a nematode most commonly found in ruminants, and should not be present on the crèche islands. This study highlights that intensive management could potentially lead to rowi being exposed to novel parasites on these crèche islands.

Keywords: Rowi, cutaneous larval migrans, *Trichostrongylus axei*

4.1 Introduction

Rowi (*Apteryx rowi*), also known as Okārito brown kiwi were recognized as a unique species of kiwi in 2003. Before this, it was thought that rowi were a variety of brown kiwi (Burbidge et al., 2003). Rowi are the rarest of the five kiwi species, with an estimated total of 375 birds in the population (Anonymous, 2014; Department of Conservation, 2011). This has led the Department of Conservation in New Zealand to classify the population as 'threatened: nationally critical', but they have not yet been listed on the IUCN Redlist (Miskelly et al., 2008). The only wild population is found in the protected Okārito forest near Franz Josef, on the West Coast of the South Island (Anonymous, 2014; Department of Conservation, 2011).

Rowi are intensively managed by the Department of Conservation. Despite the fact that over 3000 traps were used in the Okārito Forest from 2001 to 2006, stoats continue to kill a high percentage of wild rowi chicks (Anonymous, 2014). Therefore, a "BNZ Operation Nest Egg" programme (BNZONE) was implemented for this species. Eggs are removed from the burrows of the wild birds and transferred to hatching facilities at the West Coast Wildlife Centre in Franz Josef (Anonymous, 2014). The eggs are artificially incubated and hatched at the centre. Once the chicks have hatched they are moved to outside pens and then subsequently to a predator free crèche on Motuara Island in the Marlborough Sounds (Anonymous, 2014). Here the chicks live independently and are only monitored intermittently (Colbourne et al., 2005). Finally, they are returned to the wild population when the chicks are sufficiently grown to reduce the risk of predation mortality.

In July, 2013, a ranger from the Department of Conservation noticed that a number of chicks on the island were showing signs of feather loss from the ventral abdomen and a scurfy dermatitis around abdominal skin and vent margin. No similar condition has been previously seen in rowi of any age group, nor has it been reported in other kiwi species. Histological analysis of skin biopsies showed that the dermatitis was caused by cutaneous larval migrans (CLM) (unpublished).

Cutaneous larva migrans (CLM) is a distinctive self-limiting cutaneous eruption that results from the skin penetration by the larval forms of nematodes (Blackwell & Vega-Lopez, 2001; Heukelbach & Feldmeier, 2008). CLM are common in humans (Blackwell & Vega-Lopez, 2001; Brenner & Patel, 2003), and is mainly associated with the hookworm nematode of a cat or dog (Heukelbach & Feldmeier, 2008; Purdy et al., 2011). Humans are accidental hosts to these parasites and infection can occur after contact with soil or sand that have been contaminated with faeces by the infected animal (Blackwell & Vega-Lopez, 2001; Heukelbach & Feldmeier, 2008). However, similar parasitic cutaneous lesion may be caused by parasites such as the *Gnathostoma* spp., *Loa loa* (a filarial nematode), *Sacroptes scabiei*,(causing sarcoptic mange) and larvae of parasitic flies (Heukelbach & Feldmeier, 2008). CLM occurs world-wide, but infection is more prevalent in warmer climates (Blackwell & Vega-Lopez, 2001). However, to the author's knowledge, cutaneous larval migrans has never been reported in any avian host. This chapter describes the molecular identification of the species of nematode causing the cutaneous larval migrans in the rowi.

4.2 Materials and Methods

4.2.1 Tissue Biopsy

Biopsies of the affected skin and vent margins were taken using 5mm biopsy punches under general anaesthesia. The tissue biopsies were placed immediately into 10% buffered formalin.

4.2.2 Histological Analysis

The samples were also preserved for histological analysis by the Histology lab in IVABS at Massey University, Palmerston North, New Zealand. The samples were

fixed at 10% neutral buffered formalin, processed routinely, cut at 4μm, and stained with haematoxylin and eosin.

4.2.3 Isolation of genomic DNA and PCR analysis

DNA was extracted from 10μm sections of skin biopsy samples that were prepared for histological examination. The sections that were used for molecular analysis were from a sandwich cut, between two histologically positive nematode slides. This was composed of a 4 µm section, then a 10 µm section followed by another 4 µm section, with the 4 µm sections used for histology and the 10 µm for DNA extraction. DNA was extracted using a Qiagen DNeasy kit, following the manufactures instructions (Qiagen,CA, USA). Polymerase chain reaction (PCR) was performed to amplify the second internal transcribed spacer region (ITS-2) of the ribosomal DNA (rDNA) region. Amplification of the rDNA was performed using the NC1-NC2 primer constructs designed by Gasser et al. (1993). NC1: 5'-ACGTCTGGTTCAGGGTT-3' and NC2: 5'-TTAGTTTCTTTTCCTCCGCT-3' which sits within the 5.8S and 28S regions.

For PCR amplification the conditions were as follows; 1U Platinum Taq (Invitrogen, CA, USA), 1 X PCR buffer (200mM Tris HCl pH8.4, 500mM KCl), 1.5mM $MgCl_2$, 200µM dNTPs each, 1µM of each primer, 50 µg of template DNA in a total reaction volume of 25µL. The reaction were carried out under the following conditions in a SensoQuest labcycler (Germany); an initial 5 minute denaturation at 94°C was followed by 40 cycles of 94°C, 55°C, and 72°C, with each step for 30 seconds. A final extension at 72°C for 10 minutes concluded the amplification reaction. A negative control (water) and positive controls (a *Haemonchus contortus* nematode and a cloned *Haemonchus contortus* ITS-2 DNA that was prepared by a molecular biological technician at Massey University) were used for quality control.

An aliquot of 10µL of the PCR product was separated on a 1% agarose gel that contained 0.2mgml^{-1} of ethidium bromide for staining. The gels were run for 1 hour at a 100V and were visualised with a UV transilluminator. A 100bp molecular ladder

(Promega Madison WI, USA) was concurrently run with the PCR products to provide size estimation.

4.2.4 Sequencing of rDNA

Samples which contained a single PCR product of approximately 320bp in size were considered to be positive for the amplication of a nematode ITS-2 region. The remaining 15µL of the PCR product was cleaned using 70% ethanol and re-suspended in elution buffer (10mM Tris HCl, pH 8.0). The clean PCR product was sent to Massey Genome Service for Sanger sequencing in both directions, using the NC1 and NC2 primers.

Paired sequence data was aligned using Genious v 6.6.1 (www.genious.com). BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) of the paired sequences was performed against published sequence data in NCBI (National Center for Biotechnology Information). PCR reactions and sequencing of the 'rowi region' was performed twice to confirm results.

4.2.5 Phylogenetic anaylsis

A total of 18 published ITS-2 regions for nematodes were selected from species that included *Trichostrongylus* spp. and other nematodes that are known to be associated with cutaneous, neural or visceral larval migrans in fish, birds, mammals and humans. All sequenced data was obtained from NCBI. When necessary some sequences were trimmed to just the ITS-2 region using Geneious v. 6.6.1. Utilising the tree building function of Geneious v.6.6.1, a distance matrix was constructed using the algorithm of Tamura and Nei (1993), and phylogenetic tree construction was performed using the Neighbor-Joining method (Saitou & Nei, 1987).

4.3 Results

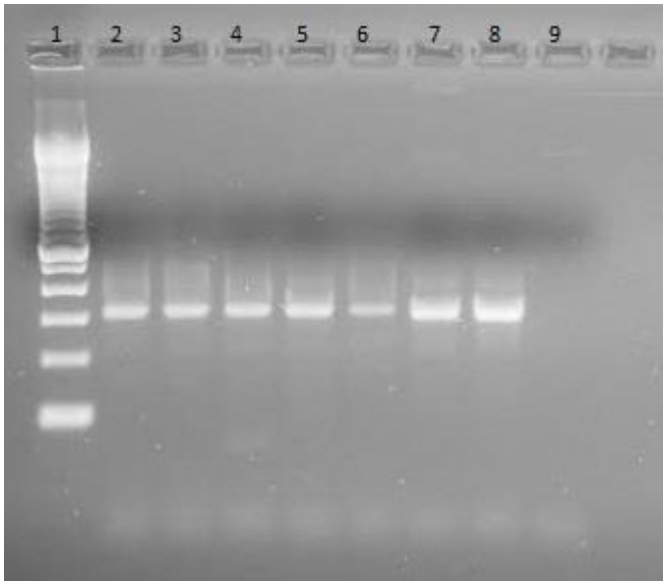

Figure 4.1. PCR product from amplification of tissue samples from rowi kiwi with cutaneous larval migrans using nematode specific primers NC1 and NC2, separated and visualized on a 1% agarose gel. Lane 1 is the ladder, lanes 2-6 contain rowi kiwi samples, lane 7 is a positive control using a cloned nematode (*Haemonchus contortus)*, lane 8 is a positive control using a nematode (*H. contortus*), and lane 9 contains a negative water control.

4.3.1 Amplification

Successful amplification of a single appropriately sized PCR product (approximately 320bp) occurred in all five rowi samples (Figure 4.1).

4.3.2 DNA Sequencing

A paired sequence was aligned and trimmed to remove any ambiguous sequences before BLAST analysis. The submitted sequence that had the highest expected value (e-value) for the sample analysed was a series of four *Trichostrongylus axei* field isolates rDNA (KC998724, KC998725, KC998726 and KC998727), each with e-vaules

of $3e^{-130}$. Additional *Trichostrongylus* species (*T. vitrinus*, *T. colubriformis* and *T. retortaeformis*) also showed a high alignment to the rowi samples. Each had an e-value less than $1e^{-100}$, which provides high confidence in the similarity of these samples to the *Trichostrongylus* genus.

4.3.3 Phylogenetic Analysis

The phylogenetic tree produced from the analysis of the ITS-2 sequences of the 18 putatively related nematodes and the rowi sequence demonstrates the association of the rowi sample with the *Trichostrongylus* genus. The rowi sample is shown to cluster with all examined *Trichostrongylus* species (Figure 4.2). The calculated Tamura and Nei (1993) genetic distance matrix for all 19 examined sequences can be found in Appendix II.

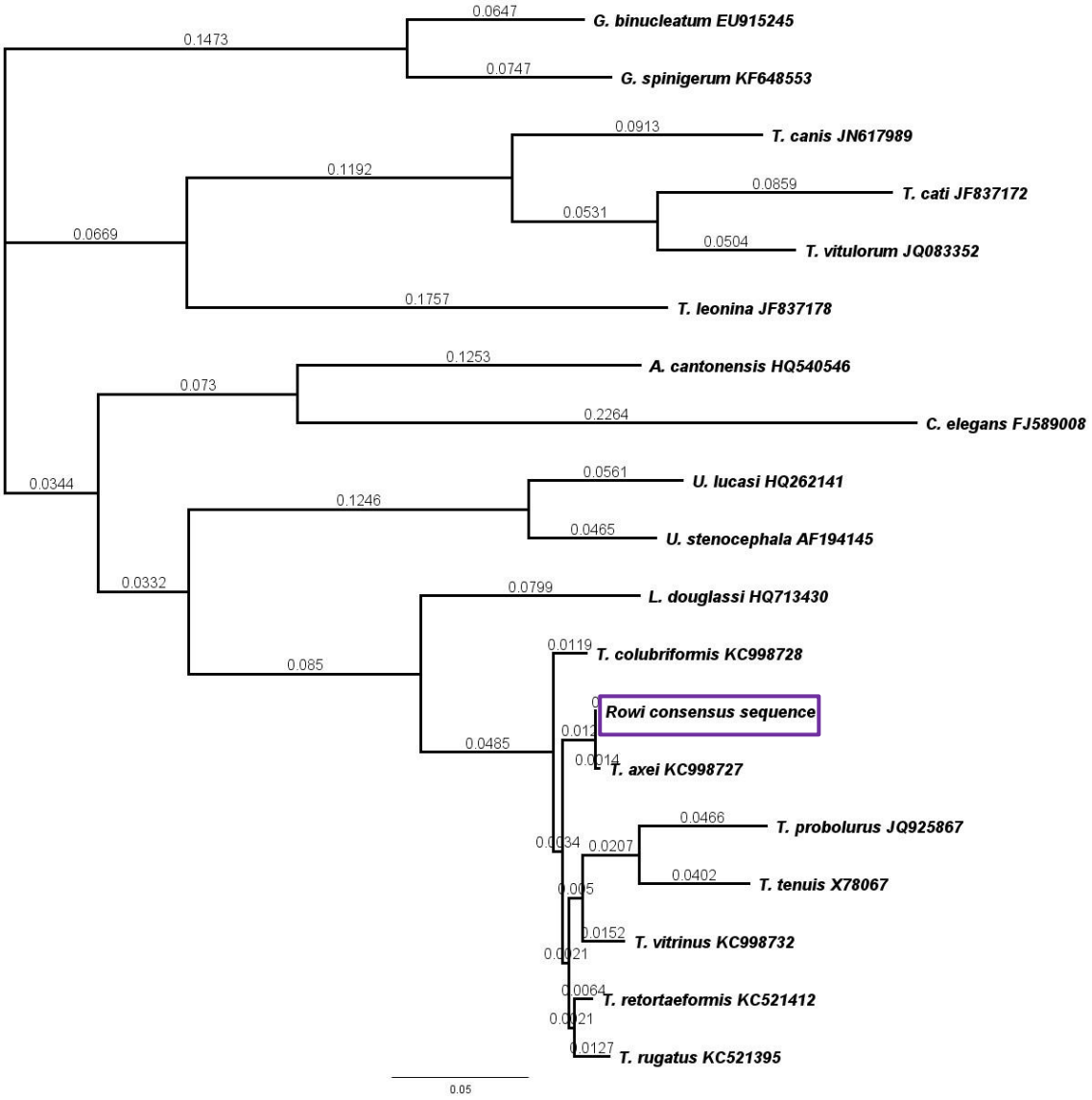

Figure 4.2. The phylogenetic tree created with the Neighbor-Joining method comparing eighteen nematode species with published ITS-2 regions to the material extracted from the rowi (*Apteryx rowi*) skin samples ("rowi consensus sequence"). Included species are; *Angiostrongylus cantonensis* (HQ540546), *C. elegans* (FJ589008), *Gnathostoma binucleatum* (EU915245), *G. spinigerum* (KF648553), *Libyostrongylus douglassi* (HQ713430), *Toxocara canis* (JN617989), *T. cati* (JF837172), *T. leonina* (JF837178), *T. vitulorum* (JQ083352), *Trichostrongylus axei* (KC998727), *T. colubriformis* (KC998728), *T. probolurus* (JQ925867), *T. retortaeformis* (KC521412), *T. rugatus* (KC521395), *T. tenuis* (X78067), *T. vitrinus* (KC998732), *Uncinaria lucasi* (HQ262141) and *U. stenocephala* (AF194145).

4.4 Discussion

The results obtained in this study suggest that the cutaneous larval migran infecting rowi kiwi is most similar to *Trichostrongylus axei*. The molecular sequence data used to create a phylogenetic tree (Figure 4.2) shows a visual association with other nematode species. The nematode species that were chosen for the phylogenetic tree were from the *Trichostrongylus* genus and a range of species that are known to cause larval migrans in any form. The phylogenetic tree shows that the consensus sequence from the present study clearly aligns within the *Trichostrongylus* genus, and the greatest association with the rowi consensus sequence is shared with that of *T.axei*.

Members of the *Trichostrongylus* genus are small, fine, reddish worms that infect a number of hosts including cattle (*Bos primigenius*), horse (*Equus ferus caballus*) , sheep (*Ovis aries*), pig (*Sus domesticus*), rodents (*Rodentia*), lagomorphs (Lagomorpha), birds (*Aves*), man (*Homo sapien*) and several other species of wild ruminants (Callinan, 1978; Drudge et al., 1955; Tompkins, 2008). They are also found in ground dwelling birds specifically, including red grouse (*Lagopus lagopus scotica*) (Saunders et al., 1999), partidges (*Perdix perdix*) (Purdy et al., 2012), capercailie (*Tetrao urogallus cantabricus*) (Millán et al., 2008), poultry (Sherwin et al., 2013), and snow geese (*Chen caerulescens caerulescens*) (Shutler et al., 2012). There are currently two species of *Trichostrongylus* that have been described in birds, *T. tenuis* and *T. pergracilis* (Tompkins, 2008).

Trichostronglyus have not been recorded to cause cutaneous larval migrans in any host species, but their larval forms have shown migration on plants (to the tips of plants) to increase their chance of being ingested by their host (Saunders et al., 1999; Tompkins, 2008). In this case, the likelihood of larvae coming in contact with the skin of the kiwi was increased by the communal roost boxes used on the island which had not been moved or cleaned for several years. Also young rowi often sleep on their abdomens with their legs stretched out behind them (T. Makan *pers. comm.*). However, the primary host of the nematode causing CLM in the rowi has not been determined. The normal host of *T. axei* is ruminants, and these are not

present on the predator free crèche on Motuara Island. The possible primary hosts may include seabirds such as little penguins *Eudyptula minor* that occasionally use the rowi burrows, or marine mammals such as New Zealand fur seals (*Arctocephalus forsteri)* which occasionally haul out on the island (T. Makan pers. comm.).

Examination of the second internal transcribed spacer (ITS-2) of the nematode ribosomal DNA was used in the present study. Previous studies have shown that congeneric species of parasitic helminths differ in the sequence of the ITS-2 region of their rDNA (Hoste et al., 1995). It has been shown that rDNA genes and their associated spacer regions do provide suitable targets for developing diagnostic markers for species identification of *Trichostrongylus* (Hoste et al., 1995). The results showed that there were consistent sequence differences between the five *Trichostrongylus* species, however the level of interspecific differences in nucleotide sequence was low (Hoste et al., 1995). Bott et al. (2009) have also shown that specific and semi-quantitative amplification from used genomic DNA of a number of nematode species, including *Trichostrongylus* spp*.,* can be achieved by using a specific forward oligonucleotide primer located in the second internal transcribed spacer (ITS-2) of nuclearrDNA together with a conserved reverse primer (NC2) in the large subunit of rDNA (18S-rDNA).

Sequence results of the present study may suggest that, due to the lack of interspecific variation between the various *Trichostrongylus* species, it is not possible to exclude other members of the *Trichostrongylus* species. However, the BLAST analysis produced a very low e-value, but this may still not be enough to confidently conclude that the cutaneous larval migran is indeed *T*. *axei*. Molecular work would need to be furthered to completely rule out other species of *Trichostrongylus*. One method which could provide more conclusive information is DNA metabarcoding, a next generation sequencing approach. This method sequences every target copy of interest (both host and nematode), and show each variant. However, due to time and cost restraints, metabarcoding was not feasible in the present study.

As a result of this research, other rowi on Motuara Island were treated with anthelmintics. Wide-scale application of anthelmintics can be useful for management of endangered species that are infected with *Trichostrongylus*, particularly since anthelmintic resistance is not known for this genus of parasites in birds (Tompkins, 2008).

Results from the present study conclude that the rowi consensus sequence produced is most similar to *T. axei*. There is only one parasitic nematode that has its full genomic information available publically and that is *H. contortus*. Even though the *T. axei*, *T. vitrinus* and *T. columbriformis* are agriculturally and economically important (Parkins & Holmes, 1989; Vlassoff & McKenna, 1994), only two genes (rDNA and cytochrome oxidase I (COX1)) are present and publically accessible on NCBI. Therefore, with the limited data available, the result was the most similar to *T.axei*. To have an accurate result we need to do more metabarcoding of general samples of nematodes. It is recommended the rDNA of all newly described parasites is determined and entered into the NCBI database, and thus the collaboration of parasitologists would also be needed.

In conclusion, the diagnosis of the CLM being caused by a nematode that is most likely within the *Trichostrongylus* species has enabled other rowi on the island to be treated with anthelmintics and changes made to the management of the roostboxes to prevent similar infections in the future. The strategic use of anthelmintics can be beneficial for the management of endangered species that are infected with *Trichostrongylus*, particularly since anthelmintic resistance is not known within this genus of parasites in birds (Tompkins, 2008).

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Chapter Five

General Discussion

Chapter 5. General Discussion

Understanding the parasite communities of our native fauna is of increasing concern, particularly with human intervention and the management of wildlife populations becoming more important in ensuring the survival of species (Adams, 2003). Parasites are increasingly being cited as a major threat to wildlife, being implicated in the decline and/or extinction of many species worldwide (Tompkins & Poulin, 2006). The overall aim of this study was to investigate the inter-relationships between host and parasite specificity and aberrant larval migration using the kiwi family as the affected host system and nematodes as the parasites. All three parts of this study highlight the importance of parasites, specifically aberrant nematodes and their effect on kiwi.

Even though the main cause of decline in kiwi numbers is predation through a variety of introduced mammalian predators (Sales, 2005), the effect of disease through parasitism is of increasing concern due to the intensification of conservation management. This may in turn limit the success of these conservation efforts.

I began this study with an epidemiological investigation of nematodiasis in New Zealand kiwi. The detection of a high rate of infection of nematodes, specifically visceral larval migrans (4.5%) and neural larval migrans (3.8%) in brown kiwi from different geographical areas in New Zealand suggests a previously unrecognised issue of host response to aberrant parasite interactions. This raises the possibility that non-host adapted species of nematodes are spilling over from introduced species into kiwi and causing disease. Interestingly, adult kiwi appear to be more susceptible to VLM than younger kiwi, while kiwi kept in crèches show a higher prevalence compared to those in the wild. This is contradictory as only juvenile kiwi are kept in crèche habitats. A common pattern observed in host-parasite assemblages is a higher intensity of parasites in juveniles compared to that of adults (Sol et al., 2003). Sol et al. (2003) studied the effects *Haemoproteus columbae* (a

red blood cell parasite) have on pigeons, and have suggested that immunity plays a large role in parasite infection in younger birds. Juvenile birds suffer higher parasite intensities than older birds, as they have not yet acquired a complete immune response against the parasite (Sol et al., 2003). Therefore, one would expect to have a higher parasitism load in juvenile kiwi compared to adults, as adults would have gained the correct immune responses to combat parasites. However, VLM and NLM are caused by aberrant parasites and thus kiwi would have limited immunity to these effects regardless of age, species or sex.

However, within my epidemiological study, the gastrointestinal tract, specifically the ventricular organs were not further studied due to the amount of time available. Identifying what parasites are present in these tissues would be crucial in future studies, as there is a potential that these parasites could be causing larval migrans in kiwi also. Of particular concern would be the pathogenic nematode *Libyostrongylus douglassi*, which is found under the mucosal lining of the proventriculus (McKenna, 2005) or the ventriculus of ostriches (Andrade et al., 2011). This parasite infection is mainly found in ostriches in South Africa, South America, Zimbabwe, Australia and some parts of Europe; and has also been identified in New Zealand (McKenna, 2005). Although this parasite is generally considered to infect only ostriches, the potential for cross transmission to other birds has not been investigated (Barton & Seward, 1993; Hoberg et al., 1995). Nonetheless, there has been one report of an emu with *L. douglassi* infection in Sweden by Jansson and Christensson (2000) however, the frequency of cross-contamination is unknown. There have also been no reports of this parasite causing any form of larval migrans (Tišljar et al., 2007) any host. Ostriches and kiwi are also unlikely to share habitats, nonetheless, we cannot disregard that transmission is possible, and could potentially infect our native birds.

With the broad overview of the prevalence of nematodiasis in kiwi in mind, I investigated a hypothesis that an introduced parasite of the *Toxocara spp.* might be responsible for neural and visceral larval migrans in kiwi. For this investigation I used a PCR molecular diagnostic method that is highly specific for the *Toxocara spp.* nematodes. DNA was extracted from preserved tissue and tested for the presence

of *Toxocara cati* and *Toxocara canis*. Molecular work undertaken on both neural and visceral tissues that showed histological evidence of NLM and VLM detected no evidence of *T. cati* or *T. canis.* In retrospect, this approach may not have been the most efficient way to identify the parasites. It may have been more beneficial to start the investigation with more general primers and then focus on a few specific primers; or look at using the general primers and then doing multiple DNA sequencing with the results. These more systematic but longer procedures could have provided a more definitive result, instead of targeting a single specific parasite species.

Therefore, less specific molecular methods were used to investigate the nematodes responsible for a syndrome of cutaneous larval migrans in an endangered kiwi species, the rowi. There have been no previous reports of CLM in birds, but this form of LM is common in humans (Blackwell & Vega-Lopez, 2001; Brenner & Patel, 2003). The aim was to identify this nematode through DNA sequencing. The results suggested that the nematode is closest to *Trichostrongylus axei*, a parasite that infects a variety of hosts such as cattle, horses, sheep, pigs, rodents, birds and man (Callinan, 1978; Drudge et al., 1955; Tompkins, 2008). However, due to lack of interspecific variation among the *Trichostrongylus* spp., our result may not provide adequate evidence to exclude other members of this species. This is a controversial result as *Trichostrongylus* spp. have not previously been implicated in larval migrans. Therefore, more molecular work needs to be completed to definitively conclude that *T. axei* is indeed responsible for the CLM in the rowi. One avenue that would be worth exploring for this nematode would be metabarcoding. For this technique to work we would need to design generic degenerative primers which would enable us to amplify any nematode ITS-2 sequence, which would also contain product from both the host and nematode. By then performing metabarcoding on the PCR product we could hopefully identify the nematode. Another avenue to explore would be entire genome analysis with next generation sequencing; however one would preferably need a pure sample to begin with. Both whole genome sequencing and metabarcoding requires extensive knowledge of the

host genome. An additional and recommended step to continue this research would be to concurrently sequence the whole genome of the rowi.

5.1 Implications

Biological invasions of introduced animal hosts, most likely avain hosts, along with their parasites has potentially led to adverse effects on our native species. However, very little is known about parasites of our native birds or what effect they have; as well as what effects invasive species parasites are having on our birds. Most studies on exotic parasites address the likelihood that invading hosts carry their parasites with them during invasion; and/or the likelihood these parasites are lost or established in the naïve population (Ewen et al., 2012). It is assumed that parasites are absent from the faunas of remote bioregions either because they have missed the boat (are absent from the introduced hosts) (Paterson et al., 1999) or they were drowned upon arrival (were present in the introduced hosts but failed to establish) (MacLeod et al., 2010). Therefore, if parasite loss positively affects host traits such as reproduction and survival, then the likelihood that an exotic host population can become established could be increased (also known as the Enemy Release Hypothesis) (Ewen et al., 2012; Keane & Crawley, 2002). Conversely, an exotic host species may benefit from the co-introduction of its parasites, if those parasites subsequently infect and cause population declines in native species that otherwise would compete with or predate upon exotic hosts (known as the Novel Weapon Hypothesis) (Daszak et al., 2000; Ewen et al., 2012).

My results have shown that introduced hosts and their parasites are having an effect on our native avian population, but how serious this is remains uncertain. The parasites transmitted between the introduced species and our native species are causing detrimental effects to our native populations' health, in the case of larval migrans; but how often are these introduced parasites infecting our native population and could our native species cope with this competition? From my study it can be presumed that our native species are struggling with adapting to new parasites, and competition between introduced and native species could be

contributing to the decline in our species. Introduced parasites and disease could also be a factor which explains why some exotic avian species are doing well within New Zealand, as they may be out-competing our native species through the use of the "Novel Weapon Hypothesis". How important this is remains uncertain, but management plans should be implemented to give New Zealand's native species the best chance to succeed and not be out-competed. The first step towards this would be to find out what parasites are natural parasites on/in our native species. From this point, management plans such as parasite surveys on introduced birds as well as routine surveys on our native species could be carried out.

Nonetheless, another point to consider is that the diagnosis of parasite infection, especially LM is extremely difficult. Most of the diagnoses in my study were made after necropsy had been completed on the birds. Most of my cases were also birds in the wild, and routine check-up of these animals does not occur nor treatment with anthelminthics. However, for captive birds or birds on crèche islands it could be a viable management strategy to modify their environment to minimise infection and/or to treat these birds with anthelminthics.

In conclusion, many parasite species can themselves cause harm outside their native range, and understanding how parasites succeed in colonising new regions such as New Zealand, is key to mitigating their spread and impact (MacLeod et al., 2010). Despite this, we have very little understanding of the mechanisms and processes that are important for determining the success of parasite colonisation in new regions following host introduction (MacLeod et al., 2010).

5.2 Further Research

Further studies are necessary in order to gain a better understanding of the nematodes that are infecting kiwi in New Zealand, especially identifying the nematodes that are causing larval migrans.

In light of our results, additional critical questions need to be addressed. Firstly, what are the common nematodes of all species of kiwi? And further what are the

nematodes causing visceral and neural larval migrans and at what life stage are these nematodes at? It is critical to identify all common nematodes within kiwi, especially to identify LM in kiwi, as a baseline is needed. There is also a massive gap in our knowledge to what these nematodes are that are native to our species. It is also important to identify the nematodes involved in LM within kiwi, as it has been shown by my study that it occurs regularly in kiwi populations, and may be an increasing problem with intensive conservation management. I suggest that based on my study, the most efficient method for identifying the nematodes in tissue sections is to use PCR with general nematode primers, specifically NC1 and NC2 as these have the largest span over the ITS-2 region (Hoste et al., 1995). If bands are observed, the PCR product could then be used for sequencing to identify what the parasite is. It could also be beneficial to look into other molecular methods such as genome studies, if funding is available, especially to identify the cutaneous LM nematode in the rowi species. Once we can identify the parasite involved it would enable further research into its life cycle, reservoir hosts and pathogenicity. A fuller understanding of the host, parasite and environmental factors that influence the syndromes of larval migrans in kiwi must begin with accurate identification of the parasite involved. Only then will effective management strategies for prevention and treatment of the disease become apparent.

Secondly, once the nematode is identified we can research what larval stage the parasite is at. This would help determine the infective role this nematode have. If adult nematodes are found it would determine that these are natural parasites of kiwi. Current research suggest that LM are caused by juvenile worms (Despommier, 2003), normally L3 stage (Taylor et al., 2007), as it is unable to develop into an adult.

Thirdly, in the case of the CLM in rowi it would be beneficial to definitively identify the nematode present, as if it is indeed within the *Trichostrongylus* species. This may be done by targeting more gene sequences or even whole genome analysis. This would enable future research to identify genes within this parasite and upload the findings on NCBI to enable others to use the data, and aid in their work.

Whilst it is important to identify the nematodes causing LM in kiwi, it is just as important to investigate further other helminths (cestodes, trematodes and nematodes) within the gastro-intestinal tract of the kiwi. Future research should aim to identify these parasites morphologically as well as with molecular identification. This would help identify what parasites are occurring naturally within kiwi and those that could be aberrant. Currently the list of parasites found within kiwi have not been up-dated since 2010 by (McKenna). Therefore with future research results could be compared to McKenna (2010) research, and new parasites could be added if needed.

Conservation efforts are becoming more important to the survival of our unique species. Knowing what parasites can affect kiwi will help aid with management plans and treatment of kiwi. As the need grows to determine the distribution of introduced parasites within our native fauna as well as identifying their insidious impacts (Adams, 2003) we also need a better understanding of what represents a natural parasite burden in our native fauna and this in turn will improve our management of kiwi.

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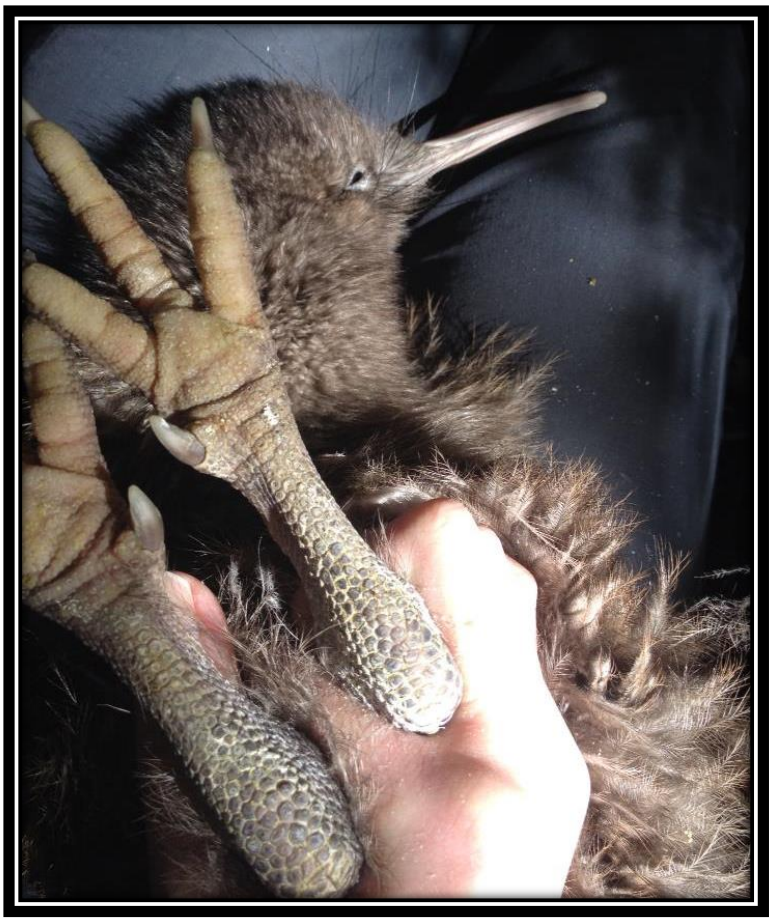

Appendix

Appendix I. Table showing multiple logistic regression of association of post mortem cases of nematodiasis in all species of kiwi. The numerator denotes the number of positive cases, and the denominator denotes the number of relevant tissues available within each group.

		Neural Larval Migrans			Visceral Larval Migrans			Ventricular Nematodiasis			Intestinal nematodiasis		
Age	**Habitat →**	**Captive**	**Creche**	**Wild**	**Captive**	**Creche**	**Wild**	**Captive**	**Creche**	**Wild**	**Captive**	**Creche**	**Wild**
	Chick	0/24[1]	0/0[1]	0/5[1]	0/40[1]	0/0[1]	5/81	1/33	0/0[1]	2/10[1]	0/35[1]	0/0[1]	2/9
	Juvenile	0/21[1]	0/6[1]	3/36	0/43[1]	1/11	1/11	6/34	1/10	16/53	3/34	0/6[1]	7/36
	Adult	1/22	0/1[1]	2/34	3/60	0/1[1]	4/69	6/29	0/0[1]	18/52	6/45	0/0[1]	12/43
	Chi square P- value	Not Valid			Not Valid			Not Valid			Not Valid		
	Fisher's Exact Test P-value	Not Valid			Not Valid			Not Valid			Not Valid		
Sex	**Male**	1/31	0/5[1]	2/41	1/58	1/7	4/88	2/43	0/6[1]	22/64	6/45	0/4[1]	11/49
	Female	0/36[1]	0/2[1]	3/34	2/85	0/5[1]	6/72	11/53	1/4	14/50	3/69	0/2[1]	10/38
	Chi square P- value	Not Valid			Not Valid			Not Valid			Not Valid		
	Fisher's Exact Test P-value	Not Valid			Not Valid			Not Valid			Not Valid		
Species	**Brown**	1/54	0/5[1]	5/48	3/121	1/10	8/113	13/80	1/8	34/84	8/94	0/6[1]	18/64
	Great Spotted	0/8[1]	0/1[1]	0/7[1]	0/12[1]	0/1[1]	0/13[1]	0/10[1]	0/1[1]	0/12[1]	1/11	0/0[1]	0/5[1]
	Little Spotted	0/1[1]	0/0[1]	0/7[1]	0/1[1]	0/0[1]	1/11	0/0[1]	0/0[1]	2/9	0/0[1]	0/0[1]	2/8
	Rowi	0/1[1]	0/0[1]	0/8[1]	0/3[1]	0/0[1]	1/9	0/2[1]	0/0[1]	0/3[1]	0/3[1]	0/0[1]	1/6
	Tokoeka	0/3[1]	0/1[1]	0/5[1]	0/6[1]	0/1[1]	0/15[1]	0/4[1]	0/1[1]	0/7[1]	0/6[1]	0/0[1]	0/5[1]
	Chi square P- value	Not Valid			Not Valid			Not Valid			Not Valid		
	Fisher's Exact Test P-value	Not Valid			Not Valid			Not Valid			Not Valid		

[1] Variable not used in analysis due to lack of data

		Neural Larval Migrans			Visceral Larval Migrans			Ventricular Nematodiasis			Intestinal nematodiasis		
	Age →	**Chick**	**Juvenile**	**Adult**	**Chick**	**Juvenile**	**Adult**	**Chick**	**Juvenile**	**Adult**	**Chick**	**Juvenile**	**Adult**
Sex	**Male**	0/14[1]	0/34[1]	3/29	1/26	2/61	3/66	2/23	11/47	12/43	0/21[1]	6/38	11/39
	Female	0/15[1]	3/29	0/28[1]	0/25[1]	3/61	5/76	2/20	12/49	12/38	2/23	4/37	7/49
	Chi square P- value	Not Valid			Not Valid			0.7879			Not Valid		
	Fisher's Exact Test P-value	Not Valid			Not Valid			0.0160			Not Valid		
Species	**Brown**	0/22[1]	3/46	3/39	1/40	3/95	8/109	3/33	23/76	22/63	2/33	8/58	16/103
	Great Spotted	0/4[1]	0/9[1]	0/3[1]	0/5[1]	0/13[1]	0/8[1]	0/5[1]	0/12[1]	0/6[1]	0/5[1]	1/6	0/8[1]
	Little Spotted	0/0[1]	0/4[1]	0/4[1]	0/0[1]	1/5	0/7[1]	2/2	0/4[1]	2/5	0/0[1]	1/5	1/3
	Rowi	0/1[1]	0/1[1]	0/7[1]	0/2[1]	1/2	0/8[1]	0/2[1]	0/0[1]	0/3[1]	0/2[1]	0/2[1]	1/5
	Tokoeka	0/2[1]	0/3[1]	0/4[1]	0/4[1]	0/8[1]	0/10[1]	0/3[1]	0/5[1]	0/4[1]	0/4[1]	0/5[1]	0/2[1]
	Chi square P- value	Not Valid			Not Valid			Not Valid			Not Valid		
	Fisher's Exact Test P-value	Not Valid			Not Valid			Not Valid			Not Valid		

[1] Variable not used in analysis due to lack of data

		Neural Larval Migrans		Visceral Larval Migrans		Ventricular Nematodiasis		Intestinal Nematodiasis	
	Sex →	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
Species	**Brown**	3/51	3/56	7/121	5/122	25/80	23/91	12/84	14/79
	Great Spotted	0/6[1]	0/10[1]	0/12[1]	0/14[1]	0/10[1]	0/13[1]	0/7[1]	1/9
	Little Spotted	0/5[1]	0/3[1]	1/9	0/3[1]	1/6	1/3	1/5	1/3
	Rowi	0/4[1]	0/5[1]	0/6[1]	1/6	0/2[1]	0/3[1]	0/5[1]	1/4
	Tokoeka	0/6[1]	0/3[1]	0/14[1]	0/8[1]	0/9[1]	0/3[1]	0/6[1]	0/3[1]
	Chi square P- value	Not Valid		Not Valid		Not Valid		Not Valid	
	Fisher's Exact Test P-value	Not Valid		Not Valid		Not Valid		Not Valid	

[1] Variable not used in analysis due to lack of data

Appendix II.Table showing the calculated Tamura and Nei (1993) genetic distance matrix for all sequences examined in Chapter 4

	A.cantonesis	*C.elegans*	*G. binucleatum*	*G. spinigerum*	*L.douglassi*	*Rowi cosensus*	*T.colubriformis*	*T.canis*
A.cantonesis		0.352	0.6	0.527	0.416	0.362	0.36	0.501
C.elegans	0.352		0.442	0.587	0.508	0.474	0.481	0.482
G. binucleatum	0.6	0.442		0.139	0.39	0.418	0.407	0.564
G. spinigerum	0.527	0.587	0.139		0.612	0.402	0.409	0.462
L.douglassi	0.416	0.508	0.39	0.612		0.141	0.146	0.454
Rowi cosensus	0.362	0.474	0.418	0.402	0.141		0	0.438
T.colubriformis	0.36	0.481	0.407	0.409	0.146	0		0.547
T.canis	0.501	0.482	0.564	0.462	0.454	0.438	0.547	
T.cati	0.493	0.556	0.49	0.6	0.475	0.55	0.545	0.216
T.colubriformis	0.371	0.418	0.427	0.423	0.143	0.028	0.032	0.553
T.leonina	0.445	0.587	0.409	0.465	0.449	0.615	0.396	0.415
T.probolurus	0.502	0.527	0.443	0.479	0.211	0.085	0.09	0.514
T.retortaeformis	0.369	0.516	0.41	0.418	0.155	0.016	0.02	0.433
T.rugatus	0.319	0.535	0.442	0.401	0.155	0.03	0.033	0.52
T.tenuis	0.483	0.563	0.478	0.391	0.165	0.084	0.089	0.541
T.vitrinus	0.382	0.532	0.52	0.416	0.157	0.032	0.036	0.593
T.vitulorum	0.569	0.479	0.495	0.618	0.506	0.608	0.612	0.209
U. lucasi	0.311	0.521	0.444	0.524	0.332	0.334	0.331	0.574
U. stenocephala	0.314	0.521	0.595	0.513	0.329	0.302	0.307	0.496

	T.cati	*T.colubriformis*	*T.leonina*	*T.probolurus*	*T.retortaeformis*	*T.rugatus*	*T.tenuis*	*T.vitrinus*	*T.vitulorum*	*U. lucasi*	*U. stenocephala*
A.cantonesis	0.493	0.371	0.445	0.502	0.369	0.319	0.483	0.382	0.569	0.311	0.314
C.elegans	0.556	0.418	0.587	0.527	0.516	0.535	0.563	0.532	0.479	0.521	0.521
G. binucleatum	0.49	0.427	0.409	0.443	0.41	0.442	0.478	0.52	0.495	0.444	0.595
G. spinigerum	0.6	0.423	0.465	0.479	0.418	0.401	0.391	0.416	0.618	0.524	0.513
L.douglassi	0.475	0.143	0.449	0.211	0.155	0.155	0.165	0.157	0.506	0.332	0.329
Rowi cosensus	0.55	0.028	0.615	0.085	0.016	0.03	0.084	0.032	0.608	0.334	0.302
T.colubriformis	0.545	0.032	0.396	0.09	0.02	0.033	0.089	0.036	0.612	0.331	0.307
T.canis	0.216	0.553	0.415	0.514	0.433	0.52	0.541	0.593	0.209	0.574	0.496
T.cati		1.148	0.401	0.48	0.417	0.582	0.507	0.62	0.136	0.552	0.45
T.colubriformis	1.148		0.428	0.089	0.027	0.032	0.078	0.034	0.484	0.313	0.315
T.leonina	0.401	0.428		0.516	0.453	0.495	0.424	0.433	0.373	0.559	0.544
T.probolurus	0.48	0.089	0.516		0.082	0.076	0.087	0.082	0.484	0.441	0.415
T.retortaeformis	0.417	0.027	0.453	0.082		0.019	0.081	0.027	0.579	0.321	0.308
T.rugatus	0.582	0.032	0.495	0.076	0.019		0.087	0.035	0.527	0.311	0.325
T.tenuis	0.507	0.078	0.424	0.087	0.081	0.087		0.076	0.433	0.43	0.359
T.vitrinus	0.62	0.034	0.433	0.082	0.027	0.035	0.076		0.501	0.335	0.318
T.vitulorum	0.136	0.484	0.373	0.484	0.579	0.527	0.433	0.501		0.417	0.584
U. lucasi	0.552	0.313	0.559	0.441	0.321	0.311	0.43	0.335	0.417		0.103
U. stenocephala	0.45	0.315	0.544	0.415	0.308	0.325	0.359	0.318	0.584	0.103	