Nematode infections of kiwi (*Apteryx* spp.)

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Abstract

Overall, little is known about the nematodes of kiwi (*Apteryx* spp.) and there has, thus far, been little indication that such infections are associated with significant clinical disease in these species. However, over the past 15 to 20 years there has been increasing recognition of cases of nematode *larva migrans* identified in kiwi at necropsy, caused by the aberrant migration of nematode larvae within visceral organs and brain. The initial study of this research re-examines archived cases of *larva migrans* through DNA extraction and PCR using formalin-fixed, paraffin-embedded tissue sections. Sequencing and BLAST analysis of positive results showed 100% alignment to database sequences from *Toxocara cati*, a nematode parasite whose definitive host is the domestic cat, indicating an infection acquired from an invasive mammalian species. Following the success of this methodology, similar techniques were applied to archived biopsies from an outbreak of cutaneous nematodiasis in a geographically isolated population of juvenile rowi kiwi (*Apteryx rowi*). The resultant sequences aligned among members of the capillarid genus *Eucoleus* although without an exact match present in the database, and so the precise, species-level identification and original source of this unusual presentation of cutaneous capillariasis remains unknown. Concurrent with these investigations into aberrant nematode infections, an opportunistic, prospective survey that included 50 kiwi presenting for necropsy examination was performed, with the objective of examining as far as practicable the prevalence, diversity, and potential pathogenicity of gastrointestinal nematodes, including any potential association with aberrant migratory lesions. The results confirmed that gastrointestinal nematodiasis was common, with 94% of the kiwi examined infected to some degree, and at least five morphologically distinct nematode types were found. Species-level identification of the nematodes was not reached based on limited morphological and molecular evaluations. However, while such gastrointestinal nematode infections appeared on the whole to be well-tolerated by the kiwi hosts, occasional cases of significant ventriculitis were identified in association with gizzard infections, particularly in juvenile, captive-bred kiwi. Furthermore,

histological lesions consistent with nematode *larva migrans* were identified in around 43% of the survey kiwi in which visceral sections were also examined, indicating that this disease may be routinely under-diagnosed.

Preface

The primary objective of this research, as first introduced to me, was to identify the nematode(s) causing larval migratory lesions in the viscera and brain of kiwi. In pursuit of this aim I was genuinely surprised to find that – despite the iconic status of the hosts – there was actually very little known about their "normal" nematode fauna, and I became keen to try and address this deficit, as far as I was able, along the way. While I have not managed to produce The Definitive Work describing nematode infections in kiwi that I naively assumed would be in the realms of possibility when I first set out, I hope to have at least provided a few more building blocks for future wildlife clinicians, pathologists, parasitologists, and/or researchers to play with.

This thesis consists of six chapters. The literature review (Chapter 1) introduces the New Zealand kiwi, which is a fascinating and distinctive bird even without the deeply ingrained cultural significance it holds for this country, and while it would be easy to produce an entire review on its wonderful peculiarities alone the primary focus in this section attempts to remain focussed on such details that may be of relevance to its role as a host. The actual published material regarding specific nematode infections of kiwi is sparse, and a review of the current state of knowledge is thus able to be presented in a fairly comprehensive manner. Information regarding the cause and significance of the syndromes of nematode *larva migrans* is heavily weighted towards infection in people, and so while as far as possible the relevance to infections in avian hosts is addressed, a discussion of this disease necessarily dips into human literature; the same holds true for an overview of nematological diagnostic techniques, which considers also how these may (or may not) be employed in identifying the cause of *larva migrans* in kiwi.

The experimental chapters (Chapters 2-5) are all presented in the style of stand-alone, publishable papers and there is therefore some repetition of material throughout. Chapter 2 describes the investigation into the causative agent of nematode *larva migrans* in North Island

brown kiwi, and has been published in the International Journal for Parasitology: Parasites and Wildlife, available as an open access article:

French, A.F., Castillo-Alcala, F., Gedye, K.R., Roe, W.D., Gartrell, B.D., 2020. Nematode *larva migrans* caused by *Toxocara cati* in the North Island brown kiwi (*Apteryx mantelli*). Int. J. Parasitol. Parasites Wildl. 11, 221-228. DOI: https://doi.org/10.1016/j.ijppaw.2020.02.011.

Chapter 3 describes the investigation into the causative agent of cutaneous nematodiasis in juvenile rowi kiwi, and has also been published in the International Journal for Parasitology: Parasites and Wildlife, available as an open access article:

French, A.F., Castillo-Alcala, F., Gedye, K.R., Roe, W.D., Knox, M.A., Gartrell, B.D., 2020. Ventral dermatitis in rowi (*Apteryx rowi*) caused by cutaneous capillariasis. Int. J. Parasitol. Parasites Wildl. 13, 160-170. DOI: https://doi.org/10.1016/j.ijppaw.2020.10.003.

Both of these chapters are presented almost exactly as in the published articles although with minor modifications in formatting to fit the style of the thesis manuscript, and minor amendments to the methods and citations for additional information.

Chapter 4 describes results of an 18-month prospective survey of the prevalence and potential pathogenicity of gastrointestinal nematodes in kiwi including any possible association with nematode *larva migrans*, while chapter 5 is an extension of this research, applying molecular tools to assess further the identity and relationships of the nematodes found. It is anticipated that much of this information will be published in a modified format in the future.

Chapter 6, a general discussion, reviews some strengths and limitations of this research as a whole and suggests areas in which, perhaps, further study should be concentrated in the future. The reference list from each chapter has been condensed into a single bibliography presented at the end of the thesis, which follows the format of the International Journal for Parasitology: Parasites and Wildlife.

Authorship of chapters

I was the primary author of all chapters in this thesis. However, my supervisors Prof. Brett Gartrell, Dr. Fernanda Castilla-Alcala, Dr. Kristene Gedye, and Prof. Wendi Roe all contributed immensely to brain-storming the study designs and were instrumental in shaping and revising the chapters and the final presentation of data. Specific contributions from supervisors and other individuals to the experimental chapters are highlighted below.

Chapter 2

Dr. Kristene Gedye particularly assisted throughout this study with the implementation, interpretation, and troubleshooting of molecular results.

Chapter 3

Dr. Donald Martin reviewed histological images of nematodes to confirm their identification. Dr. Bronwyn Presswell and Jerusha Bennett provided material for use as a positive control. Dr. Kristene Gedye again provided invaluable molecular advice, particularly in the development of new primers. Dr. Matthew Knox created and interpreted the phylogenetic trees.

Chapter 4

The frequency and distribution map presented in Figure 4.1 was created by Prof. Brett Gartrell, who also took the image used in Figure 4.7. Stuart Hunter provided the image used in Figure 4.4 and performed the necropsy and histology described as “Additional case ~ juvenile kiwi A”. Dr. Megan Jolly was primary in the diagnostics and treatment of “Additional case ~ juvenile kiwi B” and shared the endoscopic video footage used to create the still image of Figure 4.22. Routine faecal flotations were performed by Barbara Adlington and Anne Tunnicliffe; Barbara Adlington also personally designed and performed the investigation into faecal flotation utilising differing concentrations of zinc sulphate solutions that finally allowed flotation of spirurid eggs in the case of “juvenile kiwi B”. Scanning electron microscopy images were taken by, or under the close supervision of, Raoul Solomon of the Massey Microscopy and Imaging Centre. Support and

advice regarding the microscopic examination of nematodes was received at various times from Barbara Adlington, Prof. Bill Pomroy, Dr. Ian Scott, and especially from Dr. Tony Charleston – however, any glaring errors in the descriptions of these organisms are my own.

Chapter 5

Dr. Matthew Knox provided advice in the creation of the phylogenetic trees – however, once again, any errors in their presentation or interpretation belong to me alone.

Acknowledgements

To my supervisors - unsurprisingly, none of this would have been possible without Brett, Fernanda, Kristene, and Wendi, not least because they decided to take the chance in allowing someone whose background is diagnostic pathology take the helm in research which veered almost immediately into the fantastical realm of molecular nematology (aka “Here Be Monsters”) – I hope they are not disappointed in the result. For my part, I could not have asked for a more relentlessly supportive group of supervisors. Thank you Brett, for the shiny new respect you’ve instilled in me for the native avian fauna of New Zealand and the people who care for it; Fernanda, for your unwavering enthusiasm, humour, and eye for detail; Kristene, for the patience with which you introduced me to the small gods of PCR and their vagaries; and Wendi, for being the calm voice of experience in navigating this strange and terrifying post-graduate research landscape. It’s been a journey, and I could not have made it this far without my own personal Fellowship of the Star Wars References and Cat Memes.

To Dr. George Mason – whose support of environmental research not only provided the funding for these studies, but who also took a personal interest in their progress and findings. It was a pleasure and a privilege to meet you.

To the Wildbase pathology service – most of the original necropsies recognising cases of aberrant nematode larval migration, from which the impetus for this research arose, were carried out by Ass. Prof. Maurice Alley (retired), Prof. Brett Gartrell, Ass. Prof. Kerri Morgan, and Stuart Hunter. Much gratitude to Stu especially, who was also invaluable in the collection of prospective specimens, contacting me when suitable necropsy material became available and allowing me to circle like a vulture, stealing bits and pieces; thanks must also extend to the Wildbase residents rotating through the pathology service during this time who similarly shared their necropsies with me and never failed to show interest and enthusiasm for this research –

to Jess McCutchan, Emily Kay, Kathryn Johnson, Nigel Dougherty, and any others I may have missed because my memory is not what it used to be.

To Dr. Megan Jolly, current senior practicing veterinarian of Wildbase Hospital – for provision of important case material included in Chapter 4. All the best investigations begin with a conversation in the corridor.

To Dr. Tony Charleston – my unofficial supervisor, for unwavering interest in and support of this work, advice on parasitological techniques, and microscopic examination of nematode specimens above and beyond the call of duty. My gratitude extends also to Mrs. Charleston for providing on more than one occasion lunch or afternoon tea to a starving post-grad student.

To Dr. Matthew Knox – for his very patient contribution to and instruction in the creation of phylogenetic trees.

To the Massey University School of Veterinary Science parasitology laboratory (and all who sail in her) – with extra-special thanks to Barb Adlington, to whom everything was interesting and for whom nothing was too much trouble; also to Anne Tunnicliffe, for her contribution to the faecal flotations, and to Prof. Bill Pomroy and Dr. Ian Scott for being willing to look at random nematodes from random places at random times.

To my friends in the Massey University School of Veterinary Science histology laboratory – to Evelyn Lupton, Saritha Gils, Petru Daniels, and Dr. Matthew Perrott, for not only providing all the histology processing, H&E recuts, and scrolls for PCR, but also moral support, morning teas, and even, temporarily, a job.

To the Massey Microscopy and Imaging Centre – for the use of and instruction in the fantastic equipment that is available to Massey research students; in particular to Dr. Matthew Savoian for meetings and e-mails discussing imaging options, and especial thanks to Raoul Solomon for so much patience and care in introducing me to the beauty that is scanning electron microscopy.

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To the international experts who willingly and enthusiastically responded to e-mail approaches for advice – Prof. Mark Blaxter from the University of Edinburgh, and Dr. Donald Martin, North America Head of Parasitology for Idexx Laboratories.

To New Zealand Veterinary Pathology-Idexx – especially Michael Engelander (then-laboratory manager for the Palmerston North laboratory) for the much-appreciated loan of a microscope.

To Dr. Bronwyn Presswell and Jerusha Bennett from the University of Otago – for providing technical advice and, most generously, material for use as a positive control in Chapters 3 and 5. I hope we one day get to meet again in person.

To all the post-grads I met along the way – in particular, with gratitude to both Gillian Dennis and Manjula Meda Gedara, each of whose own research allowed me to get out and experience the magic of field work since, as it turned out, I had none of my own; and with affection to all my various officemates – Bex Lucas-Roxburgh, Megan Scholtens, Kat Littlewood, Seer Ikurior, and Hilary Webb, from whom I gained much wisdom and with whom I drank much coffee.

And finally, to my parents – for their unconditional, if somewhat bemused, support of my choice to go back to school (again), at my advanced age.

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Animal Ethics requirements

As there were no manipulations of live animals during this study, Massey University Animal Ethics approval was not required.

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Abbreviations

BLAST	basic local alignment search tool
bp	base pair
COI	cytochrome oxidase *c* subunit I
DNA	deoxyribonucleic acid
DOC	Department of Conservation
FFPE	formalin fixed, paraffin-embedded
H&E	Haematoxylin and Eosin
HT	Haast tokoeka
ITS	internal transcribed spacer
LM	*larva migrans*
LSK	little spotted kiwi
MD	Meckel's diverticulum
mtDNA	mitochondrial DNA
NIB	North Island brown
ONE	Operation Nest Egg
PCR	polymerase chain reaction
rDNA	nuclear ribosomal DNA
SEM	scanning electron microscopy
SoVS	School of Veterinary Science
sp.	species (singular)
spp.	species (plural)
syn.	synonym

Parasitological terms

(Adapted from Deplazes et al., 2016)

Aberrant host: host in which a parasite cannot complete its development

Accidental host: host in which a parasite can develop but does not play a role in transmission

Definitive host: host in which sexual reproduction of the parasite occurs

Intermediate host: host in which the parasite passes through part of its development

Paratenic host: host harbouring infective larval parasite stages which do not develop further

Chapter 1: Literature review

1.1. The kiwi

"The kiwi is both a national bird and a biological oddity." (Peat, 2006; p. 8)

1.1.1. Systematics and biogeography

Kiwi (family Apterygidae, genus *Apteryx*) are nocturnal, flightless members of the avian clade Palaeognathae that are endemic to, and an internationally recognised symbol of, New Zealand. Historically, the palaeognaths were distinguished from all other avian families (the Neognathae) by anatomic features including the "reptilian" structure of their bony palate, and collectively they comprise less than 1% of extant avian species (Harshman et al., 2008; Castro and Morris, 2011). Included along with kiwi are the other flightless ratite families: ostriches (Struthionidae) in Africa; emus (Dromaiidae) in Australia; cassowaries (Casuariidae) in Australia and New Guinea; and rheas (Rheidae) in South America; as well as the flighted tinamous (Tinamidae) in Mexico, Central and South America (Harshman et al., 2008; Phillips et al., 2010). Recently extinct palaeognaths include the New Zealand moa (Dinornithidae) and the Madagascan elephant bird (Aepyornithidae), but although kiwi and moa historically shared the same landmass, phylogenetic studies have now shown that they were not closely related; kiwi are instead a sister taxon to the extinct elephant bird (Mitchell et al., 2014) while among the extant palaeognaths they group most closely with the emu and cassowary families, from which they diverged sometime between ~55 and 72 million years ago (Cooper et al., 2001; Haddrath and Baker, 2001; Phillips et al., 2010).

Five species of kiwi are currently recognised, occupying different geographic ranges within New Zealand (Weir et al., 2016; Germano et al., 2018) (Figure 1.1). *Apteryx mantelli* (North Island brown kiwi) inhabit the upper North Island and include four genetically and geographically distinct subpopulations, the precise taxonomic status of which are not yet established, identified as Northland, western (or Taranaki), eastern, and Coromandel lineages. *Apteryx haastii* (great spotted kiwi or roroa) live mainly in the highlands of the northern South Island. *Apteryx rowi*

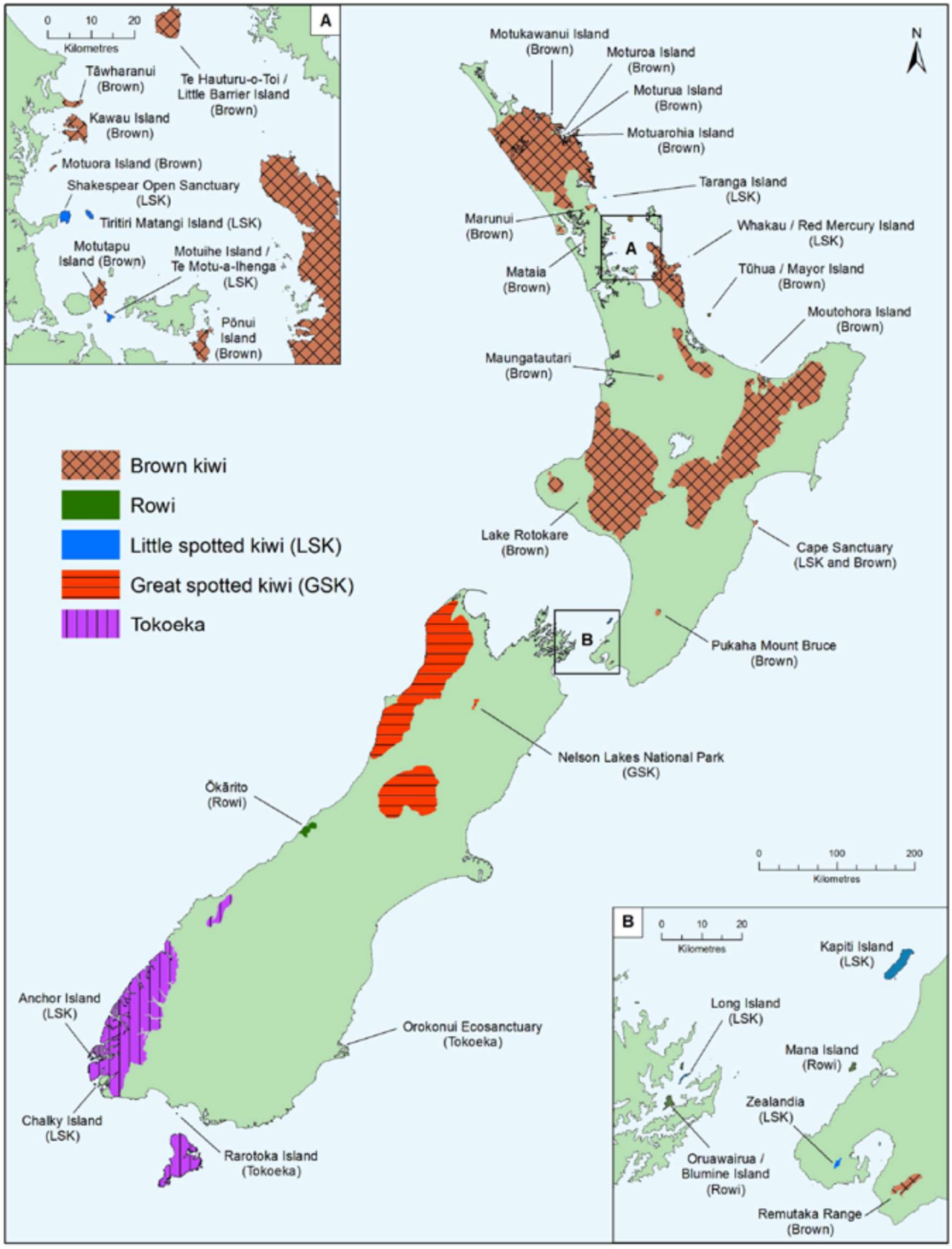

Figure 1.1. Distribution of the five extant kiwi species in New Zealand in March 2017. Source: Germano et al., 2018; reproduced with permission.

(Okarito brown kiwi or rowi) were reduced to a small remnant population at Okarito on the west coast of the South Island, but now include several newly established populations in the Omeroa ranges and on offshore islands. *Apteryx australis* (southern brown kiwi or tokoeka) in the

southern South Island also comprise four genetically and geographically distinct subpopulations occupying the Haast range, northern and southern Fiordland, and Stewart Island (Rakiura), with some genetic crossover where the two Fiordland lineages overlap. *Apteryx owenii* (little spotted kiwi or kiwi pukupuku), which suffered the most severe genetic bottle neck of all the kiwi species in the early to mid-1900s (Ramstad et al., 2013), are now established on several offshore islands and within two North Island sanctuaries.

1.1.2. Biology

Kiwi are slow-growing, taking three to five years to reach maturity, with a potential life span of 25 to 50 years or more depending on the species (Holzapfel et al., 2008). Different taxa also vary in their methods of breeding and parenting; in general, eggs are laid in burrows in clutches of one or two and require a long incubation period (65-85 days) which may be performed by the male alone (e.g. brown kiwi, little spotted kiwi), by both parents (e.g. tokoeka, rowi, great spotted kiwi), or by several members of a family group (e.g. Stewart Island tokoeka) (Castro and Morris, 2011). Chicks are precocial at hatching and are not fed by the parent, leaving the burrow to forage independently as early as three to five days post-hatch (Holzapfel et al., 2008). The basal metabolic rate and body temperature of kiwi are low relative to most other avian species (McNab, 1996); ratites in general exhibit some of the lowest body temperatures among birds, approaching the highest body temperatures of mammals (Clarke and Rothery, 2008), with kiwi additionally having the smallest body mass among the ratite families.

Primarily inhabiting native forest, kiwi have adapted to more diverse habitats including exotic forest, pastureland, and sand country (Sales, 2005). They forage, predominantly at night, by probing the soil and ground litter with their long bills; uniquely among birds, nostrils are located at the end of the bill and prey detection relies mainly on olfaction with some vibrotactile cues provided by a specialised 'bill-tip organ' (Cunningham et al., 2009). In the wild, kiwi diets are regionally and seasonally variable, but are believed to be predominantly (80-90%) composed of soil and surface-dwelling invertebrates, particularly earthworms (Sales, 2005). The kiwi

gastrointestinal tract, as for other ratites, lacks a crop (Angel, 1996), and includes two well-developed caeca which function as a site of fermentative digestion (Potter et al., 2006).

1.1.3. Conservation status and threats

Over the ~700 years of human occupation in New Zealand the distribution and numbers of kiwi have dramatically reduced but it is only relatively recently, within the last 30 years or so, that the extent of that population depletion has been recognised and acknowledged (Holzapfel et al., 2008). Historical contributors to the loss of kiwi include reduced habitat due to massive land-clearing (Holzapfel et al., 2008) as well as the hunting of individuals for food, feathers, and museum collections (Potts, 1872). However, the most significant contemporary cause for population decline is predation by introduced mammals including stoats and ferrets (family Mustelidae), cats (*Felis catus*), and dogs (*Canis familiaris*) (McLennan et al., 1996); having evolved in the absence of mammalian predators, kiwi are particularly poorly equipped to deal with this relatively modern threat.

Conservation efforts, both community-led and government driven, therefore concentrate predominantly on the reduction of predator numbers in known kiwi habitats through trapping or poisoning. In a further initiative by the New Zealand Department of Conservation, known as "Operation Nest Egg" (ONE), eggs or young chicks are located and removed from the wild to be hatched and reared in predator-free environments known as "crèches" until they reach a body size deemed less vulnerable to predation by stoats and cats, at which time they are released (Colbourne et al., 2005). While such endeavours have been successful in reversing the declining numbers of some populations, for example rowi, the status of other taxa remains precarious (Table 1.1).

In contrast to predation, infectious disease appears to have played a relatively minor role in the decline of kiwi at a population level, but the establishment of intensive conservation programmes such as ONE has been associated with outbreaks of clinically significant parasitic disease, particularly within crèche populations; for example, coccidiosis (Morgan et al., 2012)

Table 1.1. Conservation status of kiwi taxa according to the New Zealand Department of Conservation (DOC) and the IUCN Red List of Threatened Species (IUCN).

Species	Common name	DOC[a]	IUCN
A. mantelli	North Island brown kiwi	At Risk - Declining	Vulnerable - Stable[b]
A. australis	Tokoeka		Vulnerable - Decreasing[c]
A. australis "Haast"	Haast tokoeka	Threatened - Critical	
A. australis "northern Fiordland"	Northern Fiordland tokoeka	Threatened - Vulnerable	
A. australis australis	Southern Fiordland tokoeka	Threatened - Endangered	
A. australis lawryi	Rakiura tokoeka	Threatened - Endangered	
A. rowi	Rowi	Threatened - Vulnerable	Vulnerable - Increasing[d]
A. haastii	Great spotted kiwi	Threatened - Vulnerable	Vulnerable - Decreasing[e]
A. owenii	Little spotted kiwi	At Risk - Recovering	Near Threatened - Increasing[f]

[a]Robertson, 2016; [b]Birdlife International, 2017a; [c]Birdlife International, 2019; [d]Birdlife International, 2017b; [e]Birdlife International, 2016a; [f]Birdlife International, 2016b

and avian malaria (Banda et al., 2013). Crèches are rarely spelled and hold higher densities of young kiwi than wild habitats, increasing the potential for parasite magnification. Additionally, while they are designed to contain the flightless kiwi and keep mammalian predators out, their borders are porous to many flighted species of introduced birds, creating potential for spillover of introduced parasite species to the kiwi.

There is scant published evidence to implicate gastrointestinal nematode infections as a cause for significant disease in kiwi hosts; indeed, there is scant published information on kiwi nematode infections in general. There has, however, been increasing recognition of sporadic cases of nematode *larva migrans* (LM) identified at necropsy in both wild and captive or intensively managed kiwi (Reid and Williams, 1975; Boardman, 1995; Alley and Gartrell, 2003; Alley et al., 2004b; Alley and Gartrell, 2006; van Zyl, 2014; Gartrell et al., 2015). The identity of the nematodes involved in these cases has not yet been established.

1.2. Nematode infections of kiwi

"On sait que ce type (A mantelli) devient de plus en plus rare; négligée jusqu'ici, l'histoire de ses parasites offre done d'autant plus d'intérêt qu'elle sera bientôt impossible à poursuivre" (Chatin, 1884; p. 770)

("We know that this type (A mantelli) is becoming increasingly rare; neglected until now, the study of its parasites offers all the more interest that it will soon be impossible to pursue.")

1.2.1. Host-parasite relationships

Given their geographic isolation for millions of years, it stands to reason that kiwi have co-evolved along with their own host-specific parasitic fauna (e.g. Palma and Price, 2004). In more recent history, the introduction to New Zealand of humans accompanied by a variety of exotic

mammals and birds has additionally meant exposure to not only increased predators and competitors, but also a wide range of novel parasitic species (Holzapfel et al., 2008). The potential for establishment of patent infections in a novel host is limited by a multitude of factors, including host physiology and the host-specificity of the parasite (Poulin and Keeney, 2008). Such potential may be increased where the introduced and novel hosts share a relatively close phylogenetic relationship, and concern for the risk to kiwi was raised when the strongylid nematode *Libyostrongylus douglassii*, which parasitises the proventriculus and gizzard and may cause significant clinical disease to its host, was identified in farmed ostriches in New Zealand (McKenna, 2005). Conversely, it has also been suggested that the slightly unusual physiology of kiwi, primarily a body temperature that encroaches on the mammalian range, may put them at increased risk of infection by some pathogens more commonly encountered in mammals (Clark and McKenzie, 1982; Hill et al., 1995).

Despite potential health implications to birds being raised in captivity and other intensive management settings, as well as a rising interest in the preservation of native parasites along with wildlife species (Gomez and Nichols, 2013), current understanding of the nematodes that infect kiwi is limited. This shortfall could be attributed, at least in part, to a number of constraints that apply generally to the study of endoparasites in wild birds (Wobeser, 2008), not least of which is that the examination for presence of parasitic infections is typically restricted to necropsy of "salvage" specimens, i.e. those located by chance, often damaged or decomposed to some extent. Experimental infection and "sacrifice" studies are out of the question in an endangered species. Under these conditions, details of a host-parasite relationship can be extremely difficult to establish.

1.2.2. Gastrointestinal nematodiasis in kiwi

Only three nematodes from kiwi have been formally described in the literature (Table 1.2). The first, an ascarid nematode originating from the small intestine of a kiwi, was among specimens collected during the French "Transit of Venus" expedition to Campbell Island and New Zealand

in 1874 and returned to France for analysis. It was designated *Ascaris apterycis* with the findings reported in two bulletins (Chatin, 1884, 1885) which provide a limited morphological description but no images. The existence of this species of nematode has not been confirmed since its original description.

Around 90 years later, the identification of two further nematodes from a kiwi was established after specimens were submitted from the Whangarei Animal Health Laboratory, New Zealand, to the British Museum of Natural History in the United Kingdom for formal description (Harris, 1975); the laboratory of origin was erroneously attributed to the South Island of New Zealand in the original publication, and so the description of the original host (identified only as *Apteryx* sp.) as also originating from the South Island must be cast into doubt. Of the two nematodes, one was a spirurid parasitising the gizzard, designated *Cyrnea (Cyrnea) apterycis*, while the other was from the caecum, identified as an ascarid in the family Heterakidae, and originally designated *Heterakis gracilicauda*. Fifteen years after its original description, however, a collaborative publication including the original author re-evaluated *H. gracilicauda* (Inglis and Harris, 1990). Finding morphological features in common with the intestinal nematode *Hatterianema hollandei* originating from another ancient native New Zealand species, the tuatara (*Sphenodon punctatus*), it was proposed to erect a new family Kiwinematidae within the Heterakoidea superfamily to contain these two organisms, with the kiwi parasite as type-species of a new genus *Kiwinema* (Inglis and Harris, 1990). The proposed new taxonomic designation for the kiwi caecal nematode, *Kiwinema gracilicauda*, does not appear to have been widely adopted as yet, subsequently appearing in literature only as part of a discussion of native New Zealand parasites in which it remains attributed to the family Heterakidae (Yeates et al., 2012).[1]

[1] A subsequent publication by Inglis (1991) further erected subfamilies within Kiwinematidae, placing the genera *Kiwinema* and *Hatterianema* within Kiwinematinae, and proposing a new genus, *Mammalakis*, to reside within Mammalakinae. Citing this reference, a supplementary edition to the Keys to the Nematode Parasites of Vertebrates (Gibbons, 2010) acknowledges the new family Kiwinematidae, its subfamilies, and the new genera, but appears to erroneously place *Hatterianema* within the Mammalakinae rather than the Kiwinematinae.

Table 1.2. Nematodes identified from kiwi, referenced to their first mention in literature at either family or genus (+/- species) level. Names in bold indicate formal published identification. Unless otherwise referenced, superficial taxonomic placements (order, superfamily, family) have been extrapolated from Anderson et al. (2009). - = anatomic origin of nematode not specified in publication.

Order	Superfamily	Family	Genus +/- species	Anatomic origin
Ascaridida	Ascaridoidea	Ascarididae	***Ascaris apterycis*** (Chatin, 1884, 1885)	intestine
			Toxocara cati (Clark, 1982, 1983a, b)	intestine
			Porrocaecum ensicaudatum[a] (Clark, 1982, 1983a, b)	gizzard
		Anisakidae[a] (Boardman, 1995)		intestine
	Subuluroidea	Subuluridae	*"Primasubulura"* n. sp. (Clark, 1983a, b)	-
	Heterakoidea	Heterakidae/**Kiwinematidae** (n. fam.)	***Heterakis gracilicauda*** (Harris, 1975)/***Kiwinema*** (n. gen.) ***gracilicauda*** (Inglis and Harris, 1990)	caecum
		Heterakidae (Reid and Williams, 1975)		-
		Heterakidae	*Heterakis* n. sp A (Clark, 1983b)	-
			Heterakis n. sp D (Clark, 1983b)	-
			n. gen. "aff. *Spinacauda*" n. sp. C (Clark, 1983b)	-
			n. gen. "aff. *Spinacauda*" n. sp. E (Clark, 1983b)	-
		Aspidoderidae	n. gen. "aff. *Lauroia*" n. sp. (Clark, 1983b)	-
Spirurida	Spiruroidea	Spiruridae (Smith et al., 1973)		gizzard
	Habronematoidea	Spiruridae/Habronematidae[b]	***Cyrnea (Cyrnea) apterycis*** (Harris, 1975)	gizzard
		Habronematidae	*Cyrnea* n. sp. or spp. (Clark, 1983a, b)	-
		Tetrameridae	*Tetrameres* n. sp (Clark, 1983b)	-
	Acuarioidea	Acuariidae (Reid and Williams, 1975)		-
		Acuariidae	*Acuaria* (Orr, 1995)	gizzard
Enoplida	Trichinelloidea	Trichuridae (Reid and Williams, 1975)		-
		Trichuridae	*Capillaria* n. sp. or spp. (Clark, 1983a, b)	-

[a] some publications attribute *Porrocaecum* spp. to family Anisakidae (e.g. Bowman, 2014); [b] at the time of its initial description, genus *Cyrnea* was placed in the family Spiruridae (Harris, 1975) but is now attributed to family Habronematidae (Chabaud, 2009)

Although formally described kiwi nematodes are few in number, in the early 1980s Dr. Clark from the University of Canterbury, New Zealand, published two abstracts from conference presentations on kiwi parasites (Clark, 1983b) and kiwi nematodes specifically (Clark, 1983a) which suggest that our knowledge of the diversity of these agents remains rudimentary. Additional to *H. gracilicauda*, Clark proposed the existence of at least five new species from the Heterakoidea superfamily: two belonging in the genus *Heterakis*; two requiring placement in a new genus near *Spinicauda* but also showing affinity to *Hatterianema*; and one requiring a new genus near *Lauroia* (Clark, 1983a, b). These reports pre-date the proposed re-classification of *H. gracilicauda* to *K. gracilicauda* through recognition of its morphological commonalities with the tuatara nematode *Hatterianema hollandei*, and so the morphological attribution of other kiwi heterakoids close to genera more typically associated with infection of reptiles (*Spinicauda/Hatterianema*) is of particular interest.

Little detail was provided within the abstracts as to how these new species were differentiated morphologically; nor was it made clear whether the organisms were identified in homogeneous populations, perhaps reflecting co-evolution of kiwi and parasite within different geographic ranges, versus admixed populations in individual birds. With a similar lack of detail, the abstracts propose that kiwi spirurids include not only *C. apterycis* but also two or three further, unidentified *Cyrnea* species and a possible novel species of *Tetrameres*, along with the existence of one or two new *Capillaria* species and a species of *Primasubulura* (Clark, 1983a, b).

Additional to the introduction of multiple potentially novel nematode species, Dr. Clark described infections of kiwi by ascarids originating from other definitive hosts: *Toxocara cati*, whose definitive host is the domestic cat; and *Porrocaecum ensicaudatum*, a parasite of blackbirds and thrushes (*Turdus* spp.) (Clark, 1983a, b). A contemporary publication by Clark and McKenzie (1982) describes in more depth the discovery of organisms consistent with *T. cati*, as identified by detailed morphological evaluation, within the small intestine of a kiwi. Both adult

and larval forms were present, reflecting the first published finding of adult *Toxocara* in birds. The authors speculate that the reported lower body temperature of kiwi relative to similarly sized avian species might have contributed to the ability of this kiwi to behave as definitive host for an organism that is more typically adapted to a mammalian host. In contrast to the case of *T. cati*, only larval stages of *P. ensicaudatum* were described from the gizzard, and it was suggested that perhaps, as opposed to *Toxocara*, the lower body temperature of kiwi might actually inhibit maturation of this avian parasite (Clark, 1983a, b). The possible synonymy of one of these ascarid agents with Chatin's *A. apterycis* was discussed but, as the original specimens were unable to be located in the Museum National d'Histoire Naturelle (Paris, France), it was concluded that the true identity of this organism would most likely never be known for certain (Clark and McKenzie, 1982).

Other published accounts of kiwi nematode parasitism are scarce and brief. In a study of pneumoconiosis in kiwi (Smith et al., 1973), it is mentioned that some of the study birds had numerous nematodes from the Spiruridae family embedded in their gizzard wall. Reid and Williams (1975) provide a brief summary of endoparasites identified in kiwi which cites one of Chatin's publications (giving the parasite name in error as simply *Apterycis*), and also lists four other nematode families identified in kiwi: Trichuridae, Heterakidae, Acuariidae, and Spiruridae, referenced to a personal communication. Described in a New Zealand Animal Health Laboratory Surveillance report, a kiwi originating from a zoo had nematodes morphologically consistent with *H. gracilicauda* identified from the caecum and "histotrophic" nematodes present within sections of gizzard mucosa which were speculated to be *C. apterycis* (Anonymous, 1978). A later Surveillance report also describes necropsy findings in a kiwi with nematodes disrupting the koilin layer of the gizzard, considered in this case most likely to be *Acuaria* species as may be commonly found in other ground-feeding birds such as finches (family Fringillidae) (Orr, 1995).

Such published cases of nematode parasitism have been reported almost exclusively in brown kiwi, where specified, and although these mostly pre-date the molecular confirmation of three genetically distinct species among the brown kiwi (Burbidge et al., 2003), from the origin of the reports it is considered likely the majority were North Island brown. One further Surveillance report (Orr and Black, 1996) does describe a captive little spotted kiwi with a high burden of *Heterakis* eggs found on faecal examination.

Most recently, a retrospective study of the prevalence of nematode infections in kiwi examined the archived records in the National Wildlife and School of Veterinary Science (SoVS) pathology databases (Massey University, Palmerston North, New Zealand) between 1991 and 2012 (van Zyl, 2014). North Island brown kiwi were the most common species submitted to the facility for necropsy (551 of 699 cases) and were found to have a prevalence of ventricular nematodiasis of 26.3% and intestinal nematodiasis of 16.6% for kiwi in which these tissues were examined histologically and findings reported to the database (60 of 228 and 36 of 217 cases respectively). Of the other kiwi species for which histological results were available, findings were as follows: ventricular and intestinal nematodiasis were identified in two of nine (22.2%) and one of eight (12.5%) little spotted kiwi respectively, and in two of seven (28.6%) and two of 12 (16.7%) rowi respectively; intestinal nematodiasis was identified in only one of 20 (5%) great spotted kiwi, with no ventricular nematodiasis infections described from a total of 36 cases; and neither ventricular nor intestinal nematode infections were identified from 20 tokoeka.

Diagnosis of gastrointestinal nematodiasis in this study was based solely on the reporting of nematodes in histological sections, and so results likely underestimate the frequency of infection. It is also worth noting that differentiation of small intestinal versus caecal infections was not made, although it is probable that a majority of the positive cases were in fact caecal. The presence or absence of any gastrointestinal pathology reported in relation to the parasites was not directly addressed; in fact, in hardly any of the above references is the question of

potential pathogenicity of gastrointestinal nematodiasis to the kiwi host raised. Reid and Williams (1975) comment that although kiwi often presented with heavy parasite burdens, there was little known regarding the significance of such organisms to the host, while Clark (1983b, p. 93) makes passing mention to the fact that the heterakoid parasites were "rather benign", while the spirurids were "potentially the most harmful". It is likely, however, that the current lack of overt evidence that gastrointestinal nematodes in kiwi are of any major pathological significance to their hosts is a contributing factor to the relative scarcity of attention that they have so far received.

1.2.3. Nematode *larva migrans* in kiwi

The first published case of LM in kiwi is provided by Reid and Williams (1975) as an example of confirmed disease in association with nematode parasitism, briefly describing a three-month-old captive chick exhibiting "invasion of its brain tissue by nematodes" (p. 324), and suggesting the organism was a spirurid without further elaboration on how this identification was reached. A report discussing historical causes of mortality in brown kiwi at the Auckland Zoo also described a case of "cerebral nematodiasis" in a three-month-old chick (Boardman, 1995; p. 11). The same bird had a heavy gastrointestinal nematode burden, identified as *C. apterycis* in the gizzard, anisakids in the intestine, and *H. gracilicauda* in the caecum; the larvae in the brain remained unidentified. Both publications, as well as one of the New Zealand Animal Health Laboratory Surveillance reports, mention also that histological evidence of granulomas in other organs such as liver, consistent with the presence of migrating nematode larvae, had been identified in a number of other kiwi (Reid and Williams, 1975; Anonymous, 1978; Boardman, 1995).[2]

Between 2003 and 2006, three individual cases of visceral and neural LM in brown kiwi were described out of the Wildbase Pathology service at Massey University, Palmerston North, New

[2] It is considered probable that all three of these references are describing the same case(s), representing historical data originating from the Auckland Zoo, Auckland, New Zealand.

Zealand (Alley and Gartrell, 2003; Alley et al., 2004b; Alley and Gartrell, 2006), in addition to a conference presentation abstract that compiled a total of 10 cases in which parasitic granulomas were identified in gastrointestinal tissue, liver, lung, and brain (Morgan et al., 2005). The first individual case reported was a young, free-living male with granulomas present in the brainstem, cerebellum, and myocardium, with nematode larvae histologically confirmed in at least one brain lesion (Alley and Gartrell, 2003). Nematodes were also present in the gizzard and the caeca, the former unidentified, the latter consistent with *Heterakis* species. While the animal was found to have died from predation, it was suggested that the parasitic lesions may potentially have predisposed to this fatality. The second individual case was a three-month-old chick from an unfenced reserve presenting with granulomas in liver, lung, and cerebellum, the latter of which contained a larval nematode; a similar granuloma was also found in the proventricular wall, and heterakoid nematodes were again numerous in the caecum (Alley et al., 2004b). The final individually reported case was an adult female found alive in the wild suffering from paresis and disorientation, that died despite treatment; at necropsy, a granuloma histologically typical of the previous cases of LM was found in the mid-brain, although in this case no larva was described in the section (Alley and Gartrell, 2006). Additionally, granulomatous inflammation was present in the wall of the gizzard, and necrosis and loss of the caecal mucosa was described histologically in association with many intra-luminal nematodes – a rare suggestion of gastrointestinal pathology in association with nematode parasitism. *Heterakis* species were identified in the caecum, while *Cyrnea* species and other, unidentified nematodes were present in the gizzard. In none of these cases was the identity of the migrating larvae established.

As a unique addition to the multiple reports of visceral and neural LM, in 2013 there was an outbreak of crusting ventral dermatitis among a group of juvenile rowi being raised on a créche island (van Zyl, 2014; Gartrell et al., 2015). Biopsies were taken which identified nematodes migrating within the epidermis of affected skin, leading to a putative diagnosis of cutaneous LM;

these have been the only published cases of cutaneous LM in kiwi (or, to the authors' knowledge, any avian species) to date (Gartrell et al., 2015).

As well as establishing the prevalence of gastrointestinal nematode infections, the retrospective study by van Zyl (2014) also evaluated cases of visceral and neural LM diagnosed histologically and archived in the National Wildlife and SoVS Pathology databases (Massey University, Palmerston North, New Zealand) between 1991 and 2012. Visceral LM was diagnosed in 15 of 333 (4.5%) brown kiwi in which internal organs were examined histologically, with single cases only in little spotted kiwi (1 of 12, 8.3%) and rowi (1 of 16, 6.3%), and none in great spotted kiwi (0 of 33) or tokoeka (0 of 32). Of the tissues examined, liver and lung were most commonly affected. All cases of neural LM were in brown kiwi; out of 158 with brain histology reported there were six cases identified (3.8%). There was likely some overlap between the cases of visceral and neural LM, but the number of kiwi with both visceral and neural lesions was not specified.

The range of tissues examined histologically was obviously not uniform among these retrospective cases. The decision to perform histology following necropsy examination as well as the specific tissues examined are dependent on several factors including the degree of tissue autolysis and whether or not a gross diagnosis is obtained at necropsy. Where a definitive cause of death is grossly evident, for example predation or other trauma, histology is in many cases not routinely carried out and the presence of an underlying disease such as neural LM that could potentially have predisposed to death by misadventure may therefore be significantly underestimated.

1.3. Nematode larva migrans

"…the abnormal, aimless wandering of a hapless and frustrated, ill-fated worm, having no happier prospect than eventual death after an indefinite period of inflicting damage to a physiologically inadequate, over-responsive host." (Beaver, 1969; p. 3)

1.3.1. Definition

The term "*larva migrans*" was first introduced in human medicine in the 1920s to more formally describe skin lesions caused by hookworm larvae, a condition historically known as "creeping

eruption" (Beaver, 1969; Sprent, 1969). In the early 1950s, when lesions in internal organs of children were found that were attributable to the migration of larval *Toxocara canis*, an ascarid nematode whose definitive host is the domestic dog, the designation of "visceral *larva migrans*" was proposed to reflect that the host-parasite relationship was similar to that found in the cutaneous disease (Beaver et al., 1952). The term itself was broadly defined as "the prolonged migration of a larval parasite in the skin or internal organs of an abnormal host, usually man" (Beaver, 1956; p. 587). There followed some contention between authors as to the exact scope of the definition, ranging from encompassing any lesion in any tissue of the body of any human or animal caused by migration of a larval parasite (Sprent, 1969) to inclusion of only those larvae that undergo prolonged migration mimicking that expected in a normal intermediate or paratenic host (Beaver, 1969), a divergence which does not appear to have ever been formally resolved.

Accompanying the characterisation of visceral disease, cases of nematode larval migration primarily affecting the eye or the central nervous system were also recognised (e.g. Nichols, 1956a), eventually giving rise to the distinct syndromes of ocular and neural (alternatively neurological, cerebral, or cerebrospinal) LM (e.g. Fillaux and Magnaval, 2013). The potential that a number of different nematodes may be responsible for LM-type infections in humans was quickly acknowledged and explored (e.g. Beaver, 1956; Nichols, 1956b; Bowman, 1987), and the term has occasionally become even more loosely applied to aberrant nematode infections that may mature to adult forms e.g. *Capillaria hepatica* (syn. *Calodium hepaticum*)(Kumar et al., 1985) or lagochilascariasis (Bowman, 1987). A contemporary definition for LM is difficult to pin down, but the term remains most closely associated with the disease presentations it was first introduced to describe – cutaneous lesions caused by hookworm larvae and the visceral forms of infection by *Toxocara* and related ascarid species. Even there, with the increased ability to diagnose and confirm the specific cause of human infections, alternative aetiological descriptors for some forms of disease now appear to be in more common usage, e.g. ocular and neurological

(or cerebral) toxocariasis (e.g. Fillaux and Magnaval, 2013; Fan et al., 2015) and/or aetiological modifiers to the diagnosis have been recommended, e.g. hookworm-associated cutaneous LM (Caumes and Danis, 2004).

1.3.2. Aetiology

Tissue migration occurs as a part of the normal lifecycle of many parasitic nematodes although the origin and benefit of such migrations, which often begin and end in the intestinal tract, are not fully understood. Speculative origins include the historical loss of an intermediate host stage in which migration occurred with retention of the migratory pathway in the definitive host or, alternatively, evolution to oral infectivity from an historical route of infection via skin penetration, which would necessarily have required a migratory step to reach the goal organ (Read and Skorping, 1995). Whatever the origin, and despite the presumed energy cost to the parasite, studies have found faster growth and larger size (implying increased fecundity) in nematodes that have a juvenile tissue phase comparative to closely related nematodes that do not, suggesting that there are reproductive benefits in the evolutionary maintenance of migratory behaviour (Read and Skorping, 1995).

In contrast to the physiological restrictions limiting development of patent infections in a novel host, nematode larval stages that inhabit the tissues as part of their lifecycle appear commonly able to invade and survive in a wide range of novel hosts even where they may be rapidly expelled from the gastrointestinal tract (Read and Skorping, 1995). The first described cause of internal migratory lesions, the ascarid nematode *T. canis* remains the most common, "poster child" cause for human disease worldwide, although there is now increasing evidence that the closely related *T. cati* may represent an historically underdiagnosed cause of similar clinical syndromes (Fisher, 2003; Fillaux and Magnaval, 2013), and the life cycles of these zoonotic agents have been well studied (Figure 1.2). Infective larvae migrate through the wall of the intestine in their definitive host and enter the liver by the portal circulation, from there progressing to the lungs via the pulmonary artery. Within the lungs, larvae break out from the

capillaries into alveoli and may either be transported up the trachea to be swallowed and returned to the intestine to mature, or may return to the bloodstream and disseminate to various other tissue sites where they remain dormant with potential for reactivation and further migration under certain physiologic conditions (Strube et al., 2013; Deplazes et al., 2016). In the case of paratenic or aberrant (dead end) hosts, larvae follow the migratory route into tissue sites where they may survive for prolonged periods without any further development from the infective stage (Strube et al., 2013).

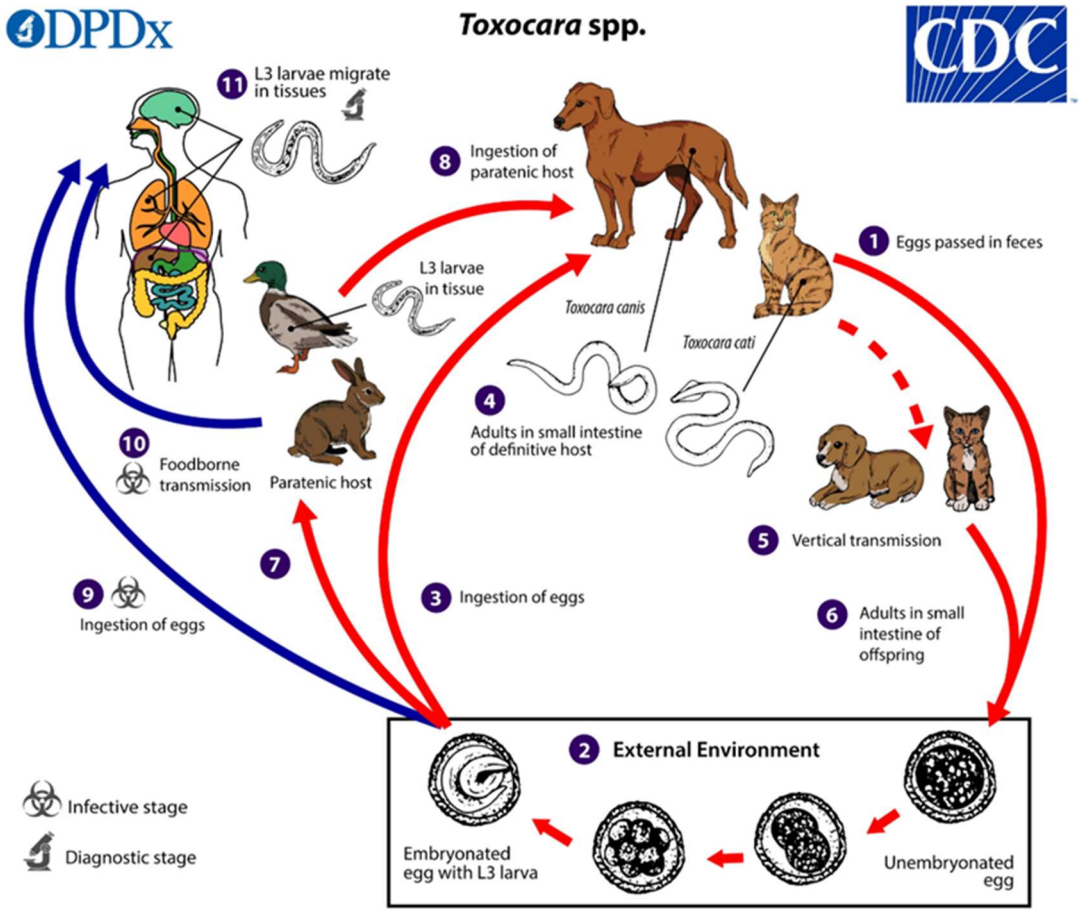

Figure 1.2. Life cycle of *Toxocara* spp. Source: Centers for Disease Control and Prevention (CDC); in the public domain. Note that use of this image for the purposes of this review does not constitute endorsement by the CDC. The original image and further information pertaining to it are available free of charge at: https://www.cdc.gov/parasites/toxocariasis/biology.html (accessed 25-01-2021).

Larval migratory lesions may therefore be found not only in aberrant hosts such as humans but also within paratenic and definitive hosts (Barron and Saunders, 1966), although whether lesions in the latter cases should be termed LM depends on the strictness of the accepted definition. In any host the larval migration can cause pathological reactions, including inflammation, hypersensitivity, or vascular thrombosis (Read and Skorping, 1995). In most cases where there has been a long-standing association between host and parasite, the behaviour of the parasite (including migration patterns) is more precisely regulated, and theoretically more "benign", in comparison to that of non-host-adapted organisms; a clinically significant pathological response may therefore be more likely to develop in a newly encountered host (Sprent, 1969). Nevertheless, the line between LM as a disease process and lesions caused by the migratory routes of larvae within an established definitive or paratenic host is not clearly drawn.

1.3.3. Disease in avian hosts

Unsurprisingly, literature regarding LM concentrates primarily on humans and aberrant infection by larvae originating from several different nematode orders have now been established to cause the human syndromes (Table 1.3). In contrast, well-described agents leading to disease in avian species are relatively few. In the case of *Toxocara*, studies regarding infection in birds appear largely in the context of assessing the zoonotic potential to humans ingesting undercooked or raw muscle or liver, whereby the birds are performing the role of a paratenic host (Strube et al., 2013), rather than addressing any clinicopathological significance to the bird itself.

Table 1.3. Some causes of nematode *larva migrans* in humans (infections in which internal or cutaneous migration by infective-stage nematode larvae may occur in humans as an aberrant host).

Visceral, ocular, and/or neurological lesions	Cutaneous lesions (Caumes and Danis, 2004)
Toxocara canis and *Toxocara cati* (Fillaux and Magnaval, 2013)	"Hookworms" e.g. *Ancylostoma caninum*
Baylisascaris procyonis (Graeff-Teixeira et al., 2016)	"Threadworms" e.g. *Strongyloides stercoralis*
Ascaris suum (Schneider and Auer, 2016)	*Pelodera strongyloides*
Anisakis and *Pseudoterranova* spp. (Pravettoni et al., 2012)	*Gnathostoma* spp.
Gnathostoma spp. (Herman and Chiodini, 2009)	
Angiostrongylus cantonensis (Barratt et al., 2016)	

Avian species in which larval *T. canis* infection has been confirmed include chickens (*Gallus gallus domesticus*) (e.g. Dutra et al., 2014), pigeons (*Columba livia*) (Galvin, 1964), and Japanese quail (*Coturnix japonica*) (Pahari and Sasmal, 1991). Natural infection of other birds has been implicated by cases of human toxocariasis which developed following ingestion of raw liver, originating in one case from a duck (family Anatidae, species unspecified) (Hoffmeister et al., 2007), in another an ostrich (Noh et al., 2012). In the case of *T. cati*, the investigation of infection in avian species appears thus far restricted to chickens (e.g. Azizi et al., 2007; Oryan et al., 2010; Taira et al., 2011). The definitive hosts of *T. canis* and *T. cati*, the domestic dog and cat respectively, are relatively ubiquitous in New Zealand and, as ground feeding birds, kiwi would have ample opportunity for exposure to infection. Additionally, the single report of a kiwi presenting as an apparent definitive host of *T. cati* (Clark and McKenzie, 1982) does raise speculation as to what potential role the kiwi may play in the life cycle of this parasite.

Another ascarid nematode with a similar life cycle to *Toxocara* spp., *Baylisascaris procyonis* has been identified as an important, emerging cause for human LM in areas where the raccoon definitive host (*Procyon lotor*) is found, and similar syndromes have been confirmed in a wide range of both mammalian and avian hosts (Graeff-Teixeira et al., 2016), including at least 50 avian species from twelve different orders (reviewed by Russell, 2006). In contrast to *Toxocara*, many of these reports involve natural, clinically significant neurological infections which are often particularly devastating because in the case of *B. procyonis* the migrating larvae continue to grow in size as they migrate (Russell, 2006; Graeff-Teixeira et al., 2016). However, neither definitive host nor parasite are known to occur in New Zealand.

Considered by some authors to fall under the definition of LM, the strongylid nematode *Angiostrongylus cantonensis* is also being increasingly reported as a cause of neurological infection in humans as well as a range of mammals (Barratt et al., 2016), and has been described in several avian species in Australia (Monks et al., 2005; Reece et al., 2013). The tawny

frogmouth (*Podargus strigoides*) in particular is highly susceptible to infection and has been proposed as a wildlife sentinal in monitoring for the presence of this zoonotic parasite (Ma et al., 2013; Spratt, 2015). In the rat (*Rattus* spp.) definitive host, the infective larvae migrate to their final destination, the pulmonary circulation, via the central nervous system. In aberrant hosts larvae migrate preferentially to the brain where they are most commonly found within the meninges, only occasionally entering the neuroparenchyma (Barratt et al., 2016). While the range of angiostrongyliasis includes Australia and the Pacific Islands, the presence of this parasite in New Zealand has not been confirmed and no locally acquired cases have yet been reported.

Falling only under the broadest definition of LM, lesions attributable to aberrant larval migration may also potentially be found in association with natural infections of avian nematodes in their definitive host; for example, members of the ascarid genus *Ascaridia* (Deplazes et al., 2016). The life cycle of these parasites is direct, and maturation is generally restricted to the intestine of the host where larvae invade the intestinal wall for a period as they mature, but extra-intestinal migration to liver and lungs has been confirmed in some species, e.g. *A. columbae* in pigeons (Melendez and Lindquist, 1979). The involvement of a host-adapted nematode species presenting with some form of aberrant larval migration must therefore remain as a consideration for the lesions found in kiwi.

1.3.4. Clinicopathological significance

The significance of larval migration to any host is dependent on the localisation and number of larvae as well as the reaction of the host to their presence. Early studies on *T. canis* found that the distribution of larvae in the tissues of various, experimentally infected laboratory animals differ in a characteristic way between species (Beaver, 1969; Strube et al., 2013), and infections were often well tolerated, up to 10 larvae per gram of body weight in tissue other than the brain (Beaver, 1956). In humans, subclinical *Toxocara* infections are considered common, while

syndromes of relatively mild, non-specific illness have been coined “common” or “covert” toxocariasis (Fillaux and Magnaval, 2013).

Neurological manifestations of the disease are likely to be the most severe in any host. While *Toxocara* species do appear to differ in their affinity for invasion of neural tissue (Strube et al., 2013), experimental studies on mice have indicated that the number of *T. canis* larvae that reach the brain is dose dependent (Holland and Cox, 2001) and decreases with repeated exposure to infection (as may be the case with natural exposure) (Abo-Shehada et al., 1991), while in the case of *B. procyonis* larvae are not considered to be specifically neurotropic (Graeff-Teixeira et al., 2016) with only a low percentage of infective larvae (~5 to 7%) ever reaching the brain. However, whether neurotropic or not, there may be some evolutionary advantage to the parasites in their presence at this site, through host morbidity or behavioural changes that increase susceptibility to predation and potential for parasite transmission (Fan et al., 2015; Graeff-Teixeira et al., 2016). This effect could have increased consequence for an endangered species such as the kiwi, already significantly at risk from predators.

1.4. Nematode diagnostics and their application to the investigation of *larva migrans*

“I've come up with a set of rules that describe our reactions to technologies:
1. Anything that is in the world when you’re born is normal and ordinary and is just a natural part of the way the world works.
2. Anything that's invented between when you’re fifteen and thirty-five is new and exciting and revolutionary and you can probably get a career in it.
3. Anything invented after you're thirty-five is against the natural order of things.” (Adams, 2012; p. 95)

1.4.1. Faecal examination

Faecal examination for the presence of eggs is the most common parasitological method of assessing presence of gastrointestinal helminth infections in avian species (Greiner and Ritchie, 1994). Sample collection is minimally invasive in live patients, and testing is often applied not only to sick birds but as part of health checks performed routinely or prior to translocation. The principle behind faecal flotation is the utilisation of a solution with a higher specific gravity than water that allows less dense elements, such as nematode eggs and protozoal cysts, to rise to the surface via passive gravitation or centrifugation, while heavier faecal debris sinks (Ballweber et

al., 2014). Common flotation solutions include NaCl, $NaNO_3$, $ZnSO_4$, or sugar; different solutions may be more suitable than others depending on the organism of interest and should ideally be chosen with knowledge of the specific gravities of both the solution and the parasitic elements of interest (Greiner and Ritchie, 1994), as while solutions with a higher specific gravity would theoretically allow the flotation of a wider range of parasitic elements, they may also lead to morphological distortion and increased background debris (Ballweber et al., 2014).

Faecal flotation for the assessment of gastrointestinal nematode infections has its limitations. With some well-studied exceptions, there is a limit to the level to which nematode eggs can be identified based on morphologic or morphometric examination (e.g. genus, family, or order) and often no known correlation between the number of eggs present and the parasitic burden (Greiner and Ritchie, 1994). While sampling of faecal material is minimally invasive, in the case of wild birds particularly the collection of specimens from ground surfaces does run the risk of soil or other contamination. A further complication, particularly in the case of ground-feeding birds, arises from the uncertainty as to whether the presence of unusual or unexpected eggs in a sample reflects a true, patent infection or whether these may be originating from the ingestion of soil and/or the faeces of other species; coprophagy has been established in the dog to be a potential cause both for over-estimation of patent infections as well as for the presence on faecal examination of the eggs and oocysts of parasites for which dogs are not a definitive host (Nijsse et al., 2014). Additionally, a delay between collection and submission to a laboratory setting for analysis also runs the risk of eggs continuing to develop and mature within the sample, potentially increasing their specific gravity and/or altering their morphology, or even allowing their hatching into larval forms (Greiner and Ritchie, 1994).

Despite these limitations, faecal examination remains an indispensable tool for the assessment of gastrointestinal nematode infections in living birds. However, by the strictest definition of LM infections are non-patent, involving larval forms only which are incapable of maturation in the

abnormal host (Beaver, 1956). Given this, along with the localisation of larvae outside of the gastrointestinal tract, faecal testing is unlikely to be of primary use in diagnosing or identifying the causative agents of LM.

1.4.2. Morphological identification

Detailed examination of the external and internal anatomy has historically been, and probably remains, the most common approach for identification and taxonomic characterisation of nematodes. However, given a limited range of distinguishing morphological features, particularly among some groups of parasites, and the biochemical and genetic confirmation of morphologically indistinguishable (cryptic) species, it is becoming increasingly clear that this technique may in some cases be unreliable if used in isolation (e.g. Chilton et al., 1995; McManus and Bowles, 1996). Nematodes, including both free-living and parasitic forms, are one of the most abundant phyla on the planet and while the number of total species may be a million or more, a mere fraction of these (~23,000) have yet been described (Blaxter and Koutsovoulos, 2015).

Published keys to nematode identification are available (e.g. Anderson et al., 2009) but while a relatively small proportion of parasitic nematodes, particularly those occurring commonly in companion and food animals, have been well-studied and may be readily identified even by non-experts when occurring at the expected site in their expected hosts, the accurate morphological identification of parasites encountered in novel hosts, and those that have not been previously encountered or formally described at all, relies heavily on the experience and expertise of the examiner. Of concern, there has been a declining trend worldwide in the number of nematologists skilled in the area of traditional morphological taxonomy (Ferris, 1994).

Several studies have been published describing the morphological features of larvae considered the most common or potential causes of LM in humans (Nichols, 1956a, b; Bowman, 1987). The location and retrieval of entire larval organisms from tissue specimens is labour intensive, requiring tissue digestion (e.g. using a pepsin-hydrochloric acid solution) and the microscopic

detection of larvae from the digestive fluid (Deplazes et al., 2016), often with no guarantee of the presence of intact organisms within the specimen. This technique was trialled unsuccessfully in the case of cutaneous LM in rowi (Gartrell et al., 2015); however, the tissue samples digested had been previously fixed in 70% ethanol which may have interfered with the efficiency of the digestion process, and additionally there is some evidence that recovery is improved where viable larvae are present in the sample (Prociv, 1989a). Even with successful harvest, the specific identification of larval nematodes is further limited by their very small size and a lack of distinguishing external features, even more so than for adult forms (Nichols, 1956b; Li et al., 2007).

1.4.3. Histology

Particularly in necropsies where specific evaluation of the gastrointestinal tract for endoparasites is not performed, as well as where parasitic lesions are unexpected and/or not evident grossly (as for many cases of LM), diagnosis of infection may in some cases be solely via the chance presence of nematodes in histological section. Keys are available for characterising adult nematodes histologically (e.g. Chitwood and Lichtenfels, 1972; Gardiner and Poynton, 1999) but, given the serendipitous nature of such findings, in many cases the available plane of section may lack some or all of the morphological features required for identification, and even in the best of sections identification to species level is seldom possible based on histomorphology alone (Gardiner and Poynton, 1999). Histological identification of larval organisms has further limitations, primarily the relative lack of distinguishing morphological features in larvae as opposed to adults. The published morphological studies of larvae associated with human LM also cover their appearance in histological section (Nichols, 1956a, b; Bowman, 1987); again, a lack of control over the plane of the organism being viewed in diagnostic versus experimental sections may contribute towards the difficulty in histomorphological identification, particularly where species differentiation requires precise cross-sectional measurements (Nichols, 1956a).

While a definitive diagnosis of LM requires the histological confirmation of larvae, a presumptive diagnosis may be based on the identification of the typical histological lesion. For visceral disease, this is most commonly described in humans as an “eosinophilic granuloma” (Kaplan et al., 2001), consisting of a central focus of necrosis surrounded by a mixed inflammatory infiltrate including eosinophils and palisading epithelioid macrophages, but this finding alone is not pathognomonic for LM. In the case of *Toxocara* spp., the most extensively studied of the causes of human LM, where larvae are absent in the granuloma they may be found on additional sectioning either within inflammatory lesions or in relatively normal tissue ahead of the migration tract (Beaver, 1956); in fact, it has been speculated that larvae present within such inflammatory lesions are more likely to be dead (Prociv, 1989a). Studies evaluating the long-term survival of migrating *T. canis* larvae indicate well-developed strategies for host immune evasion, including the production of a mucin-rich coating over the surface of the cuticle that can be shed in response to adherence by host antibody and inflammatory cells, leaving the organism free to continue migration (Fillaux and Magnaval, 2013; Maizels, 2013). In necropsy samples, continued and sometimes atypical migration is also possible following death of the host (Prociv, 1989a). In the live host, once migration ceases the larva may become walled off and persist for some time in its infective form, or it may be killed and its structure degraded by the inflammatory response (Beaver, 1956; Parsons and Grieve, 1990; Fillaux and Magnaval, 2013). The inability to confirm the presence of larvae despite strong histological suspicion for LM may therefore be due to either continued larval migration out of the available tissue section or degradation of the organism beyond recognition, thus creating a diagnostic dilemma for the pathologist.

1.4.4. Immunohistochemistry

As a potential tool for confirmation of the causative organism in cases of LM diagnosed from histological sections, immunohistochemistry allows the identification of antigenic elements of specific organisms such as *T. canis* and may have the advantage of positive results even in the

absence of larvae. In one study (Musso et al., 2007), immunopositive staining was found in the central necrotic debris of granulomas and within the cytoplasm of the surrounding epithelioid macrophages, as well as staining larval fragments where present. However, the anti-*Toxocara* serum required for this test is not commercially available (Kaplan et al., 2001), and furthermore its utility requires a pre-existing suspicion of the specific organism involved – for example, based on history of exposure and/or serological testing – information that is not readily available in animals and generally inapplicable to wildlife cases presented for necropsy.

1.4.5. Serology

While the "gold-standard" for diagnosis of LM is the confirmation of larval presence via biopsy, given the invasive nature and low sensitivity of this test antemortem diagnosis in humans instead relies heavily on serological evidence of exposure (Poulsen et al., 2015); as for most serological tests, a positive result cannot be taken as confirmation of cause but must be interpreted as part of the whole suite of laboratory, imaging, and clinical results available. The most common serodiagnostic test for human toxocariasis is based on the detection of specific antibodies against the excretory-secretory antigens produced by the infective larvae of *T. canis* using an enzyme-linked immunosorbent assay (ELISA). Some cross-reactivity to other parasitic nematodes has been reported, and the addition of Western-Blot analysis as a confirmatory test has increased sensitivity (Fillaux and Magnaval, 2013), but a reliable serological method of differentiating infection with (or exposure to) *T. canis* versus *T. cati* remains elusive (Poulsen et al., 2015). Although the development of a serological assay for antemortem diagnostic purposes may be something to be considered in the future, serological testing is neither available nor applicable to a preliminary investigation into the cause of LM in kiwi.

1.4.6. Molecular analysis

1.4.6.1. Extraction of DNA

The small size of most nematodes makes fresh samples good candidates for the successful extraction of DNA, either by direct mechanical disruption of the organism or by proteinase digestion (Powers, 2004). However, in the majority of cases, diagnosis of LM is via histology and

the only samples of nematode tissue available are present within tissue that has been preserved in formalin, a fixative that causes cross-linkage between DNA and protein (Sengüven et al., 2014). The quality of DNA in formalin-fixed samples is known to degrade over the time spent in formalin, and degradation may continue at a low level even once the fixed tissue is processed and embedded within paraffin (Libório et al., 2005). Nonetheless, formalin remains the most common fixative used for pathological specimens and formalin-fixed, paraffin-embedded (FFPE) tissue blocks are the most commonly available archived pathological samples (Sengüven et al., 2014).

A variety of techniques have been investigated for the successful extraction of DNA from FFPE tissue and a number of commercial kits are now available, although the quality of DNA produced also relies heavily on pre-extraction factors including the condition of the fixative, fixation time, and post-fixation storage (Sengüven et al., 2014). Despite this, it has been shown that extraction, particularly of small molecular weight DNA fragments, can be feasible even from tissue blocks that have been stored for 40 years (Libório et al., 2005).

1.4.6.2. Polymerase chain reaction

While a number of biochemical and molecular techniques were already being utilised as an adjunct to the morphological identification of nematodes, the development of polymerase chain reaction (PCR) in the 1980s as a method for rapid amplification and identification of selected regions of DNA was rapidly adopted in the field of nematology as an important component of phylogenetic studies and species differentiation (McManus and Bowles, 1996). A major advantage of this technique is the requirement of only a very small amount of DNA, and in most cases it can be similarly applied to identify all life stages (e.g. adults, larvae, and eggs) (Powers, 2004).

The process involves multiple cycles of denaturation of the template DNA through heating, followed by a lowered temperature to allow the annealing of specific primers (short sequences of DNA complementary to the target region), and then a period of elongation, or template-

directed DNA synthesis, catalysed by a heat-resistent polymerase enzyme. Duplication of the target DNA in each cycle results in its exponential amplification (McManus and Bowles, 1996), and the detection of the resultant amplicons is commonly achieved through size separation by gel electrophoresis. The development of programmable thermocyclers has allowed this technique to become a relatively accessible, non-labour-intensive research tool (Powers, 2004).

1.4.6.3. Sequence comparison

PCR-based diagnostic tests for the identification of nematodes include the development of primer sets that assess species-specific size variability in the target DNA fragment; PCR-linked restriction fragment length polymorphism analysis, which requires the further step of digestion of the PCR product by restriction endonuclease enzymes producing a characteristic pattern of bands that reflect sequence variation within the target DNA; and the random amplification of polymorphic DNA, which analyses the pattern produced by the amplification of random fragments of genomic DNA (McManus and Bowles, 1996; Gasser, 2001). However, the development and commercialisation of rapid and reliable methods of DNA sequencing (e.g. as reviewed by Pareek et al., 2011) has allowed this to become a widely utilized tool which boasts the advantage that (in the absence of sequencing errors) data obtained from different sources and using different methods are directly comparable with newly obtained sequences (McManus and Bowles, 1996; Gasser, 2001).

To further this end, sequence similarity search programs such as the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990; Altschul, 2014) link directly to established databases of sequence information, e.g. GenBank® (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov). Limitations to the use of BLAST include the availability of relevant data as well as a fundamental reliance on the accuracy of the data submitted to the database, not only the integrity of the sequences but also the reliability of the accompanying identification (Harris, 2003). In general, databases of nematode genomics remain relatively

sparsely populated (Poon et al., 2017), exacerbated by the fact that, as of yet, there is no single target sequence that is universally agreed to allow reliable species-level identification.

1.4.6.4. Target DNA

For routine PCR a pair of primers is used, each delimiting one end of the chosen target DNA fragment (McManus and Bowles, 1996); some sequence information is therefore required prior to analysis, in order to apply appropriate primers. In trying to identify an unknown agent, primers may be extrapolated or designed from sequence information that is already available from related organisms, or from sequences that have been established to be conserved over a wide range of taxa (so called "universal" primers) (McManus and Bowles, 1996).

The most studied targets of nematode DNA lie within the mitochondrial genome (mtDNA) and nuclear ribosomal DNA (rDNA) (Powers, 2004). Both represent "high-copy" sequences, present as multiple copies and at multiple sites within the cell, and so the PCR amplicon may therefore be considered a consensus of multiple sites targeted by the chosen primers; while this increases the chance of successful amplification, interpretation relies to some extent on the assumption that all sites undergo concerted evolution to remain identical, supported for the most part by the fact that many of the target genes are necessary for successful function of the cell (Feliner and Rosselló, 2007). While it has been proposed in other phyla that low-copy nuclear genes may offer a better alternative for species-level phylogenies, there is so far little investigation into these alternatives, particularly in the field of nematology.

rDNA especially includes some of the most widely characterised of nematode gene sequences (Powers, 2004). rDNA consists of repeating units including three genes that code for ribosomal RNA, identified as subunits 18S (small subunit or SSU), 5.8S, and 28S (large subunit or LSU) and two intervening, non-coding spacer regions (internal transcribed spacer-1 and -2, or ITS-1 and ITS-2) (Powers, 2004). As a general rule, the gene regions evolve more slowly and are therefore more highly conserved than the spacer regions, although discrete regions of variability may be present. Most notably, the 18S gene has been applied to studies of nematode phylogeny, leading

to revision of the higher-level taxonomic classification system and increased understanding of evolutionary relationships within the entire phylum (e.g. Blaxter et al., 1998).

While 18S sequence information will generally allow the higher placement of organisms, the degree of conservation means that it may be insufficient for species identification (Powers, 2004). However, primer sets for relatively short segments of the 18S gene have been developed in the investigation of toxocariasis, which on DNA sequencing contain enough polymorphic sites to allow the differentiation of several different species of ascarid nematodes including *T. canis* (e.g. Pinelli et al., 2013). In contrast, it has been found that there is considerable variation in both the length and sequence of the ITS regions, leading some investigators to favour these as potentially more useful and specific markers for the identification of nematode species (Powers, 2004). Several different primer sets designed specifically for the differentiation of *Toxocara* spp. based on their ITS-2 region have been previously published (e.g. Jacobs et al., 1997; Li et al., 2007).

The mitochondrial genome consists of multiple genes encoding structural protein subunits, transfer RNAs, and ribosomal RNAs with some intervening non-coding DNA (Li et al., 2008). In general, mtDNA evolves more rapidly than nuclear DNA although there are varying degrees of conservation between genes; the most common regions sequenced in nematodes include the genes coding for NADH dehydrogenase subunit 4 and cytochrome oxidase *c* subunit I (COI) (Blouin, 2002). In part because of the increased rate of substitutions and gene rearrangements, universal primers applicable to a wide range of nematode species are less available for mtDNA than for rDNA sites, although these features may also make mtDNA a better target for species differentiation, particularly in closely related species (Blouin, 2002). The COI gene particularly has been suggested as a suitable target for DNA "barcoding" (a term applied to the identification of species using a short section of DNA from a specific gene or genes) across a wide range of taxa including nematodes, although as yet there remains a relative paucity of comparative COI

sequences from nematodes within the databases (Poon et al., 2017). However, complete mitochondrial genomes have been established for a number of nematodes including *Ascaris suum* (Okimoto et al., 1992), as well as for the *Toxocara* species *T. canis* and *T. cati* (Li et al., 2008).

1.4.6.5. Preliminary investigation of nematode larva migrans in kiwi

Molecular investigation into the causative agent of visceral and neural LM in kiwi has been previously performed by van Zyl (2014). Based on the histomorphology of some of the larvae, specifically the presence of lateral alae, *Toxocara* species were considered the primary organism of interest. Archived FFPE tissue from a total of 18 kiwi with histological evidence of LM underwent DNA extraction, and PCR was performed using primers specific to the ITS-2 region of rDNA for both *T. canis* and *T. cati* (per Jacobs et al., 1997). The results were negative across all the case samples, suggesting that neither *T. canis* nor *T. cati* were involved in these cases, although the possibility of false negatives due to the inherent difficulty in extraction of DNA from formalin-fixed samples was discussed (van Zyl, 2014).

A similar investigation was also performed on skin biopsies in which cutaneous LM had been diagnosed in rowi (van Zyl, 2014; Gartrell et al., 2015). In this case, following DNA extraction universal primers for the nematode ITS-2 region were used (per Gasser et al., 1993), producing an appropriate amplicon in all cases, and the PCR product was sequenced and subjected to BLAST analysis. The resultant sequence aligned among members of the genus *Trichostrongylus*, a group that has not previously been reported as a cause for LM in any species, and the authors concluded that the result should be viewed with caution as concurrent morphological evaluation was limited (Gartrell et al., 2015). This somewhat unexpected result suggests the need for a more integrative approach to nematode identification.

1.5. Conclusion

The kiwi is a unique bird, representing a much beloved but sadly endangered New Zealand icon. Although many aspects of its biology and the contemporary threats it faces have been well-studied, the diversity and impact of nematode infections to kiwi hosts have not previously received, or perhaps even warranted, much attention. However, the increasing recognition of lesions caused by the migration of nematode larvae within organs including skin and brain has brought the pathogenic potential of nematode infections in kiwi into the spotlight, and identification of the specific nematode or nematodes responsible is the first step toward understanding their source and significance to kiwi and their future management. Although the likelihood of an acquired infection is high, a potential origin from the native gastrointestinal nematode fauna of kiwi also cannot be overlooked, as little is currently known about the composition and behaviour of such infections.

Courtesy of extensive previous research into the aetiopathogenesis of nematode *larva migrans* in humans, there are tools already available that may be extrapolated into the investigation of this disease in kiwi. Limitations to the diagnostic material available from archived or even prospective necropsy cases suggests that an integrative approach may be required, concentrating on a combination of histomorphology and PCR-based analysis. In turn, the development and validation of a diagnostic technique that can be readily applied to the available samples, primarily archival formalin-fixed paraffin-embedded tissue specimens, will be of benefit not only to kiwi but also in future investigations of similar disease in wildlife or other species.

This thesis therefore aims not only to expand the current understanding of the nematodes causing gastrointestinal and aberrant infections in kiwi, but also to define the utility and limitations of techniques that may be applied to identifying these organisms.

Chapter 2: Nematode *larva migrans* caused by *Toxocara cati* in the North Island brown kiwi (*Apteryx mantelli*)

2.1. Abstract

Sporadic cases of visceral and neural nematode *larva migrans* have been diagnosed at necropsy in the endangered New Zealand kiwi (*Apteryx* spp.), but the causative organisms have not yet been definitively identified. From an initial group of five affected kiwi, PCR was performed on DNA extracted from archival formalin-fixed paraffin-embedded tissue sections in which larval nematodes had been histologically identified. Sequencing of positive results from four out of the five kiwi aligned with sequences from *Toxocara cati*, a nematode parasite whose definitive host is the domestic cat. PCR was then performed on a second group of 12 kiwi that had histologic inflammatory lesions consistent with *larva migrans*, but variable larval presence. Repeatable positive PCR results were only achieved in one tissue, in which larval organisms were histologically confirmed. This study supports the use of PCR as an alternative or adjunct to the morphological identification of nematode larvae in formalin-fixed histopathological samples, as well as showing that in investigation of *larva migrans*, PCR has greatest chance of success from sections where nematode larvae are evident histologically. The identification of *Toxocara cati* from lesions of *larva migrans* in kiwi reflects an indirect, parasite-mediated effect of an invasive mammalian species on a native species.

2.2. Introduction

The mammalian invasion of New Zealand has caused major declines in native fauna, primarily through direct predation. Although often overlooked, alterations in parasite ecology that accompany the establishment of introduced species may also have a significant impact on native ecosystems (Chalkowski et al., 2018). The spillover of parasites from introduced hosts into New Zealand avifauna has been studied for avian malaria (Schoener et al., 2014), but there has been little, if any, work performed on the effects of introduced nematodes.

Cases of visceral and neural nematode *larva migrans* (LM), which may be defined as the prolonged migration of a larval parasite in the internal organs of an abnormal host (Beaver, 1956), have been diagnosed by histopathology in the endangered New Zealand kiwi (family Apterygidae, genus *Apteryx*) with increasing recognition over the past 15 years (Reid and Williams, 1975; Anonymous, 1978; Boardman, 1995; Alley and Gartrell, 2003; Alley et al., 2004b; Alley and Gartrell, 2006; van Zyl, 2014). Kiwi are nocturnal, flightless palaeognaths, and are unique to New Zealand. Five genetically and geographically distinct species are currently recognised, all of which have suffered population decline of varying severity since the occupation of New Zealand by humans and predatory mammals (Holzapfel et al., 2008). The consequent establishment of intensive conservation programmes has been associated with outbreaks of parasitic disease such as coccidiosis (Morgan et al., 2012) and avian malaria (Banda et al., 2013); however, reported cases of LM have remained sporadic, involving both wild and intensively managed kiwi.

Based on histomorphology of the larval nematodes, *Toxocara* spp. have been proposed as the most likely cause, but a recent study was unable to confirm this by molecular analysis (van Zyl, 2014). *Toxocara canis* is the major cause for non-cutaneous LM in humans worldwide, and while *Toxocara cati* has only infrequently been associated with similar clinical syndromes, it is now considered likely to represent a greater zoonotic risk than has been previously recognised (Fisher, 2003; Fillaux and Magnaval, 2013). The adult nematodes inhabit the intestinal tract of

their definitive hosts, the domestic dog (*Canis familiaris*) and cat (*Felis catus*) respectively, both of which are common throughout New Zealand. Infection occurs through ingestion of the infective larvae either within the egg (e.g. ingestion of faecal-contaminated soil) or within a mammalian, avian, or invertebrate paratenic host. Following ingestion of infective eggs by the definitive host, larvae hatch and migrate from the intestine to the liver and lungs, then via the trachea returning to the intestine to mature. In older animals especially, somatic migration may also occur, where larvae are instead disseminated from the lungs through the blood stream into tissues (e.g. skeletal muscle) where they remain dormant but infective and may be re-activated e.g. if the animal is pregnant or lactating (Wang et al., 2018). In abnormal hosts such as humans, the infective larvae undertake prolonged, aberrant somatic migration (Fillaux and Magnaval, 2013).

Diagnosis of toxocariasis in humans is not straightforward, and may be based on a combination of history, clinical presentation, haematology, fluid analysis, imaging, and serological testing. Definitive diagnosis of infection requires the confirmation of larval presence via biopsy, but this procedure is not routinely performed because of its invasive nature and the low probability of obtaining a diagnostic sample (Fillaux and Magnaval, 2013). In contrast, diagnosis of LM in animals, particularly wildlife, is most commonly achieved via histology following necropsy, and available samples may be restricted to formalin-fixed, paraffin-embedded (FFPE) tissue blocks. In either case, identification of causative organisms has historically been based on morphology, a technique with acknowledged limitations due to the small size and relative lack of distinguishing features among larval nematodes (Nichols, 1956a). A further diagnostic dilemma occurs when inflammatory lesions consistent with LM are identified histologically, but no larvae are evident in the sections; in such cases, a pathologist may be suspicious of LM but unable to confirm the diagnosis (Kaplan et al., 2001).

A number of different studies have now demonstrated the utility of PCR in the identification of *Toxocara* DNA present in cerebrospinal fluid (Caldera et al., 2013), bronchoalveolar lavage fluid (Pinelli et al., 2013), and fresh tissue (e.g. Zibaei et al., 2017; Wang et al., 2018) as well as in the assessment of environmental contamination by *Toxocara* eggs (e.g. Borecka and Gawor, 2008). Although formalin fixation has a negative effect on the quality of DNA, PCR can be successfully performed using FFPE samples (Libório et al., 2005; Sengüven et al., 2014), and has been recently applied to confirm identification of *Baylisascaris procyonis* causing neural LM in FFPE sections of brain from a dog (Hazlett et al., 2018), thus providing an adjunct or alternative to histomorphology for the identification of larvae in fixed tissue. The use of PCR on FFPE sections has the additional benefit of allowing assessment of the association between the presence of the organism and histological evidence of disease.

The purpose of this study was to investigate further the cause of visceral and neural nematode LM in archival FFPE necropsy tissue from kiwi, and to validate the use of molecular techniques to isolate nematode DNA from tissue granulomas with and without larval sections present.

2.3. Methods

2.3.1. Case selection.

A retrospective search of the School of Veterinary Science Pathology database (Massey University, Palmerston North, New Zealand) was performed to identify cases in which histological examination of tissues from kiwi submitted for necropsy resulted in a diagnosis of confirmed (larvae present in section) or suspected (typical inflammatory lesions present but larvae not identified) nematode LM. Cases with lesions present only in gastrointestinal tissue or skin were excluded. The original Haematoxylin and Eosin (H&E)-stained sections were examined to confirm the diagnosis, or new sections cut and examined if the original slides were unavailable.

The archived FFPE tissue blocks of suitable cases were located and “sandwich” sections cut, comprised of a 4 µm section mounted on a slide and stained with H&E, followed by two or three

10 µm tissue scrolls taken for molecular analysis, then a further 4 µm section mounted and stained with H&E. The H&E-stained sandwich sections were examined to assess the probability of larval tissue being present within the tissue scrolls. An initial group of cases (group I) were chosen for molecular analysis using the following criteria: a) nematode larvae histologically confirmed to be present in at least one affected tissue in sandwich sections taken both before and after the tissue scrolls used for DNA extraction; and b) cases diagnosed within the past six years. The primary aim of the analysis of group I samples was the specific identification of the larval organisms associated with lesions of LM.

Following the results from group I, a further group of cases (group II) were chosen, removing the previous criteria, in order to further evaluate the utility of performing PCR on lesions without histologically identifiable larvae. This second group of tissues had confirmed characteristic inflammatory lesions present in histological sandwich sections but variable larval presence.

2.3.2. DNA extraction

DNA extraction was performed on the FFPE tissue scrolls using a commercial kit (Roche High Pure FFPET DNA isolation kit, Roche, Switzerland or NucleoSpin DNA FFPE XS kit, Macherey-Nagel, Germany), per the manufacturer's instructions with minor modifications, most notably variable extension of the lysis step in a 56 °C water bath from two or three hours to overnight. DNA was also extracted from fresh specimens of adult *T. canis* and *T. cati* for use as positive controls, using a commercial kit (NucleoSpin Tissue kit, Macherey-Nagel, Germany) per the manufacturer's instructions. The adult *Toxocara* were sourced from the gastrointestinal tract of a domestic dog and cat respectively at routine necropsy and identified morphologically by the School of Veterinary Science parasitology laboratory (Massey University, Palmerston North, New Zealand), based primarily on the size of the organisms and conformation of the cervical alae.

2.3.3. Molecular analysis

Primer sets were sourced from literature, including one set designed for the gender identification of kiwi tissues (Huynen et al., 2003) for the purpose of evaluating the quality of kiwi DNA present within the samples, and multiple sets targeting either the internal transcribed spacer (ITS)-2 region or 18S gene of nuclear ribosomal DNA of ascaridoid nematodes (Gasser et al., 1993; Jacobs et al., 1997; Li et al, 2006, 2007; Dangoudoubiyam et al., 2009; Pinelli et al., 2013), with target sequences of variable base pair (bp) length (Table 2.1). Each PCR contained 1X of 5X HOT FIREPol Blend Master Mix (10 mM $MgCl_2$,Solis Biodyne, Estonia), 300 nM each of forward and reverse primers (IDT, IA, USA), and 1 µl of template DNA (or nuclease free water), made to a total of 20 µl with nuclease free water.

PCR was performed on either a Labcycler (SensoQuest, Germany) or Mastercycler Nexus GX2 (Eppendorf, Germany). The protocol used for the kiwi gender-specific primer set (W5-W7) was as follows: initial activation of 15 minutes at 95 °C, 40 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds, followed by a final elongation at 72 °C for 7 minutes. A touchdown PCR protocol (Korbie and Mattick, 2008), which employs decreasing annealing temperatures beginning above the estimated melting temperature of the primers and transitioning to a lower, more permissive temperature in order to increase sensitively, specificity, and yield of DNA amplification, was used for all nematode primer sets under the following conditions: initial activation of 15 minutes at 95 °C, 12 cycles of 95 °C for 30 seconds, annealing for 30 seconds (starting at 60 °C , reducing by 0.5 °C per cycle), and 72 °C for 30 seconds, followed by a further 35 cycles of 95 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 30 seconds, and final elongation at 72 °C for 7 minutes. Positive controls, consisting of DNA extracted from *T. canis* and/or *T. cati*, and a negative (blank) control containing no DNA were run simultaneously. The PCR product was separated by electrophoresis on a 1% w/v agarose gel (Bioline, UK) using RedSafe (iNtRON Biotechnology, South Korea) and visualized with a gel image

Table 2.1. Primers used in this study, including a range of primers designed to amplify parts of the ITS-2 region or the 18S gene of nuclear ribosomal DNA of ascaridoid nematodes, and one set of primers designed to differentiate the gender of kiwi.

Primer name	Primer sequence (5'-3')	Target	Approximate amplicon size (bp)	Reference
W5 W7	AATCACCCTTTAAACAAGCTGTTAAAGCAA CCTTTCTCAAATCTCTCTTTTGTTCTAGACAC	Uncertain – Kiwi W-linked and Z-linked or autosomal	350 (males and females) +/- 200 (females only)	Huynen et al., 2003
NC2	TTAGTTTCTTTTCCTCCGCT	Nematode ITS-2 region	N/A (reverse primer)	Gasser et al., 1993
NC13	ATCGATGAAGAACGCAGC	Ascaridoid ITS-2 region	520 (with NC2)	Jacobs et al., 1997
XZ1	ATTGCGCCATCGGGTTCATTCC	Ascaridoid ITS-2 region	450 (with NC2)	Li et al., 2006
T cat1	GGAGAAGTAAGATCGTGGCACGCGT	*Toxocara cati* ITS-2 region	400 (with NC2)	Jacobs et al., 1997
YY1	CGGTGAGCTATGCTGGTGTG	*Toxocara canis* ITS-2 region	330 (with NC2)	Li et al., 2007
18SF 18SR	CCATGCATGTCTAAGTTCAA TTATTCTCCGTTACCCGTTA	Ascaridoid 18S gene	325	Dangoudoubiyam et al., 2009
Nemo 18S F Nemo 18S R	GGCTAAGCCATGCATGTC ACTTGATAGACACGTCGCC	Ascaridoid 18S gene	265	Pinelli et al., 2013

system (MultiDoc-It Imaging System, UVP, CA, USA). Size of PCR products was estimated in comparison to a HyperLadder™ 100 bp molecular ladder (Bioline, UK).

2.3.4. Sequencing and BLAST analysis

Amplicons of the appropriate size were cut from the gel, eluted overnight in elution buffer (10 mM Tris-HCl, pH 8.0), and the eluate submitted to the Massey Genome Service (Massey University, Palmerston North, New Zealand) for bi-directional Sanger sequencing. The resultant forward and reverse sequences were aligned using Geneious 10.2.3, manually trimmed, and subjected to BLAST (Basic Local Alignment Search Tool) analysis through the NCBI (National Center for Biotechnology Information) database (GenBank®). Nucleotide sequences obtained in this study have been deposited in GenBank under the accession numbers MN585764 to MN585772.

2.4. Results

2.4.1. Cases

Group I samples consisted of 16 tissues in total from five kiwi, necropsied between 2011 and 2017. Of these, six tissues (one from each of four kiwi, and two from one kiwi) had nematode larvae histologically confirmed in sandwich sections taken both before and after the tissue scrolls cut for molecular analysis (group Ia). Other tissues from the same birds in which lesions were present but larvae absent or only identified in one of the two histological sandwich sections were also subsequently analysed (group Ib). Group II samples consisted of 20 tissues from 12 kiwi, necropsied between 2004 and 2017, in which typical inflammatory lesions with or without larvae were confirmed present in both histological sandwich sections.

All selected kiwi were North Island brown (*Apteryx mantelli*), which comprise the vast majority of the kiwi species submitted to the necropsy service. They originated from various regions within the North Island of New Zealand, and the majority (15/17; 88.2%) were categorised as wild, from areas that practice no to variably extensive (but incomplete) predator control (Table 2.2). A majority (14/17; 82.4%) were females, and age cohorts (as reported in the database)

Table 2.2. Signalment and origin of kiwi in group I (#1 to 5) and group II (#6 to 17).

Kiwi #	Year of necropsy	Species	Age	Gender	Regional location	Origin	Tissues affected
1	2017	*A. mantelli*	Juvenile (30D)	Female	Hawkes Bay	Crèche[a]	Lung
2	2016	*A. mantelli*	Juvenile	Female	Northland	Wild	Lung, liver, brain
3	2016	*A. mantelli*	Adult (2Y)	Female	Waikato	Zoo	Lung, liver, brain, muscle, spinal cord
4	2015	*A. mantelli*	Adult	Female	Bay of Plenty	Wild	Liver, brain
5	2011	*A. mantelli*	Juvenile (6M)	Male	Northland	Wild	Lung, liver, brain
6	2017	*A. mantelli*	Subadult	Male	Coromandel	Wild	Brain
7	2016	*A. mantelli*	Adult	Female	Bay of Plenty	Wild	Liver
8	2013	*A. mantelli*	Juvenile	Female	Northland	Wild	Lung, brain
9	2012	*A. mantelli*	Adult	Female	Northland	Wild	Lung
10	2012	*A. mantelli*	Juvenile (5M)	Female	Hawkes Bay	Wild	Lung, brain
11	2011	*A. mantelli*	Juvenile	Male	Auckland	Wild	Liver, heart
12	2011	*A. mantelli*	Adult	Female	Northland	Wild	Lung
13	2009	*A. mantelli*	Adult	Female	Wanganui	Wild	Liver, heart
14	2006	*A. mantelli*	Adult	Female	Northland	Wild	Lung, liver, brain
15	2005	*A. mantelli*	Adult (2Y)	Female	Northland	Wild	Liver
16	2005	*A. mantelli*	Juvenile (6M)	Female	Northland	Wild	Lung, brain
17	2004	*A. mantelli*	Juvenile (3M)	Female	Northland	Wild	Lung, liver, brain

[a]a predator-free area used for raising juveniles

included an even mix of juvenile and adult birds along with a single subadult; the age of kiwi is impossible to estimate beyond these broad categorisations unless the bird has been tracked since hatch.

2.4.2. Histology

The characteristic histological lesion was a discrete granuloma of variable size with central accumulation of brightly eosinophilic, necrotic cellular and pyknotic nuclear debris and a peripheral rim of epithelioid macrophages and multinucleated giant cells (Figure 2.1A). Granulomata were most commonly identified in liver, lung, and/or brain, where they were focal, multifocal, or regionally clustered to confluent and appeared randomly located within the parenchyma. In a few cases, focal to regionally extensive acute inflammation was present in addition to these lesions within liver and lung sections, consisting of granulocytic infiltrates with or without acute necrosis. Less specifically, it was also common to see lymphoid aggregates adjacent to portal areas or airways, and brain sections sometimes included mild perivascular lymphoid cuffing and/or small foci of malacia and gliosis.

Larvae, where present, were most commonly found within the necrotic centre of a granuloma, more rarely within foci of acute inflammation. Cross-sections or near cross-sections of larvae were identified and measured in four of the five group I kiwi, ranging in diameter from 9.3 to 17.6 µm and exhibiting single, small, bilaterally symmetrical alae (Figure 2.1B). In the fifth kiwi (#4), where only longitudinal sections were identified, the larvae were of similar size but presence of alae could not be confirmed.

2.4.3. Molecular analysis

2.4.3.1. Group I

All 16 tissues from the five group I kiwi showed appropriate amplification using the kiwi gender-specific primers (W5-W7), confirming amplifiable kiwi DNA of up to 350 bp at least.

2.4.3.2. Group Ia

Touchdown PCR using the range of selected nematode primer sets was performed only on those tissues from each bird in group I in which larvae had been confirmed to be present in both

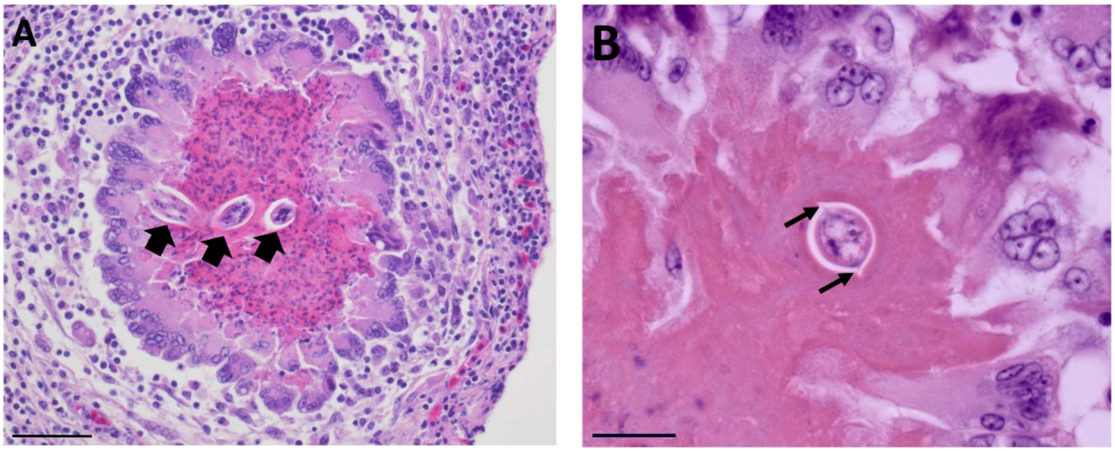

Figure 2.1. Histology. A: Typical inflammatory granuloma in the lung of a kiwi, containing several oblique nematode larval sections (arrows) (H&E, bar = 50 µm). B: Cross-section of a nematode larva within an inflammatory granuloma in the brain of a kiwi, showing bilateral alae (arrows) (H&E, bar = 20 µm).

histological sandwich sections (Table 2.3). The positive controls, *T. cati* and *T. canis*, amplified appropriately with all primer sets. One tissue (kiwi #1, lung) was PCR negative for all nematode primer sets. All other kiwi tissues tested produced amplicons of the appropriate size with two or more of the nematode primer sets. Successful amplification decreased with increasing target product size and all tissues were negative for the primer set with the largest target product (NC13-NC2, ~520 bp). Additionally, all tissues were negative for the *T. canis*-specific primer set (YY1-NC2).

From the positive results, representative amplicons were selected for sequencing and the resultant sequences were subjected to BLAST analysis. At least one of the nematode primer sets for each tissue yielded a sequence that aligned with the sequence from the *T. cati* positive control, as well as with sequences from *T. cati* present in GenBank (Table 2.4).

2.4.3.3. Group Ib

The four group Ia kiwi with positive nematode PCR results also had additional tissues in which characteristic inflammatory lesions were present but larvae were absent or identifiable in only one of the two histological sandwich sections. PCR was performed on these tissues using only the primer set with the smallest product (Nemo 18S F-Nemo 18S R, ~265 bp); based on group Ia

Table 2.3. PCR results from group Ia kiwi, tissues in which larval organisms were identifiable in histological sections taken before and after the sample used for molecular analysis, and for positive control organisms *T. cati* and *T. canis*. Includes a range of primers designed for amplification of nematode DNA, and one set of primers (forward primer W5) designed for differentiating the gender of kiwi. F = PCR positive female, M = PCR positive male, - = negative result, + = amplicon of appropriate size, +/- = faint and/or non-repeatable amplicon.

	Kiwi	Forward primer (approximate size of target)						
#	Tissue	NC13 (520bp)	XZ1 (450bp)	Tcat1 (400bp)	W5 (350bp (M&F) & 200bp (F))	YY1 (330bp)	18SF (325bp)	Nemo 18S F (265bp)
1	Lung	-	-	-	F	-	-	-
2	Lung	-	+/-	+	F	-	+	+
3	Brain	-	+/-	+	F	-	+	+
4	Brain2	-	-	-	F	-	+	+
5	Lung	-	+/-	+	M	-	+	+
5	Brain	-	-	+/-	M	-	+	+
	T. cati	+	+	+		-	+	+
	T. canis	+	+	-		+	+	+

Table 2.4. Results of BLAST analysis for group Ia kiwi, tissues in which larval organisms were identifiable in histological sections taken before and after the sample used for molecular analysis.

Kiwi # (Tissue)	Forward Primer	Sequence length	Aligned to GenBank#	Organism (Target)	Cover	Pairwise Identity	Bit score	E value
2 (Lung)	Tcat1	320	MH043958	*T. cati* (ITS-2)	100%	100%	592.048	4^{e-165}
2 (Lung)	18S	300	EF180059	*T. cati* (18S)	100%	100%	555.115	5^{e-154}
2 (Lung)	Nemo 18S F	215	EF180059	*T. cati* (18S)	100%	100%	398.150	6^{e-107}
3 (Brain)	Tcat1	305	MH043958	*T. cati* (ITS-2)	100%	100%	564.348	8^{e-147}
3 (Brain)	Nemo 18S F	212	EF180059	*T. cati* (18S)	100%	100%	392.610	3^{e-105}
4 (Brain2)	Nemo 18S F	260	EF180059	*T. cati* (18S)	100%	100%	481.249	7^{e-132}
5 (Lung)	Tcat1	322	MH043958	*T. cati* (ITS-2)	100%	100%	595.741	3^{e-166}
5 (Lung)	18SF	231	EF180059	*T. cati* (18S)	97%	100%	427.696	8^{e-116}
5 (Brain)	Nemo 18S F	261	EF180059	*T. cati* (18S)	100%	100%	483.096	2^{e-132}

results this was deemed the most likely to produce a positive result if nematode DNA was present (Table 2.5). Three out of four tissues in which larvae were identified in histological sections taken either before or after (but not both) the tissue scrolls collected for molecular analysis were positive, while one was negative. Sequencing and BLAST analysis of the positive amplicons in all three cases aligned with *T. cati* (EF180059, bit-scores 468.332-483.096), as for the group Ia tissues from the same birds. All six tissues in which larvae could not be identified histologically were negative, despite confirmation of the presence of characteristic inflammatory lesions in the histological sandwich sections.

2.4.3.4. Group II

In nine out of the 12 group II kiwi, larvae were identified in at least one tissue, in at least one of either the original diagnostic or sandwich histology sections, supporting the diagnosis of LM as the cause of the inflammatory lesions in these birds. Where cross-sections were present, the larvae resembled those previously described in group I. PCR was performed on all tissues using the kiwi gender-specific primers (W5-W7) and the nematode primer set with the smallest product (Nemo 18S F-Nemo 18S R, ~265 bp), and results compared with the presence or absence of larvae in the sandwich sections (Table 2.5).

The nematode PCR was performed three times on all samples to test repeatability of results and any with positive results were repeated a further three times. A total of nine tissues originating from seven different kiwi produced a positive amplicon of appropriate size in at least one of the initial three PCR runs, but only one was positive in all three, and subsequently all six total PCR runs (kiwi #10, lung). Larvae in this tissue were present in both histological sandwich sections, and the sequence of the product aligned with *T. cati* (EF180059, bit-score 448.010). Just one other kiwi (#14) had larvae confirmed in both histological sandwich sections, in all three tissues tested; however, this was also one of four birds in group II that failed to amplify using the kiwi gender-specific primers, suggesting overall poor-quality DNA in the FFPE samples. Only one of these three tissues was positive with the nematode primers, in only two out of six total PCR runs (once in the initial three runs, and once in the additional three runs). Sequencing results were of poor quality.

The remaining seven positives only amplified in one out of six total PCR runs: three in the first run, and two each in the second and third runs. Two were from tissues in which larvae were identified in a single sandwich section while the other five had no larvae identified in either sandwich section. Amplicons as visualised in the gel were very faint in several cases (e.g. kiwi #10, brain and kiwi #17, liver), and sequencing was either unsuccessful or not attempted. The 11 tissues that were negative in all three initial runs included seven tissues in which larvae were

Table 2.5. PCR results in relation to the presence or absence of larvae identified in histological sections taken before and after the sample used for molecular analysis, for all groups. Y = larvae identified histologically, F = PCR positive female, M = PCR positive male, + = amplicon of appropriate size in at least one PCR run (primer set Nemo 18S F-Nemo 18S R), - = negative result.

			Presence of larvae		PCR results		
Group	Kiwi #	Tissue	Before	After	Kiwi DNA	Nematode DNA	Sequencing result
Ia	1	Lung	Y	Y	F	-	
	2	Lung	Y	Y	F	+	*T. cati*
	3	Brain	Y	Y	F	+	*T. cati*
	4	Brain2	Y	Y	F	+	*T. cati*
	5	Lung	Y	Y	M	+	*T. cati*
	5	Brain	Y	Y	M	+	*T. cati*
Ib	2	Liver	Y	-	F	+	*T. cati*[d]
	2	Brain1	-	-	F	-	
	2	Brain2	-	Y	F	+	*T. cati*[d]
	3	Muscle1	-	-	F	-	
	3	Muscle2	-	-	F	-	
	3	Lung	Y	-	F	+	*T. cati*[d]
	3	Liver	-	-	F	-	
	4	Liver	-	-	F	-	
	4	Brain1	-	-	F	-	
	5	Liver	-	Y	M	-	
II	6	Brain	-	-	M	-	
	7	Liver	-	-	F	+[a]	*T. cati*[d]
	8	Lung	-	Y	F	+[a]	*T. cati*[d]
	8	Brain	-	-	F	-	
	9	Lung	-	Y	F	-	
	10	Lung	Y	Y	F	+[c]	*T. cati*[d]
	10	Brain	-	-	F	+[a]	Unsuccessful
	11	Liver	Y	-	-	-	
	12	Lung	-	-	-	-	
	13	Heart	-	-	F	-	
	13	Liver	-	-	F	+[a]	*T. cati*[d]
	14	Brain	Y	Y	-	-	
	14	Liver	Y	Y	-	-	
	14	Lung	Y	Y	-	+[b]	Poor quality
	15	Liver	-	-	F	-	
	16	Brain	-	-	-	+[a]	*T. cati*[d]
	16	Lung	-	Y	-	+[a]	Poor quality
	17	Liver	-	-	F	+[a]	No attempt
	17	Lung	-	-	F	-	
	17	Brain	-	-	F	-	

[a]positive in 1/6 PCR runs; [b]positive in 2/6 PCR runs; [c]positive in 6/6 PCR runs; [d]aligned with *T. cati* (EF180059, bit-scores ranging from 204.252 to 483.096)

not identified in either sandwich section and two tissues with larvae identified in a single sandwich section; one of each also failed to amplify using the kiwi gender-specific primers.

2.5. Discussion

This study provides strong evidence that the cause of visceral and neural LM in North Island brown kiwi is the nematode parasite *Toxocara cati* whose definitive host, the domestic cat, is an introduced and invasive species in New Zealand. In a previous study, molecular analysis failed to amplify *Toxocara* DNA in 29 tissues from 18 kiwi diagnosed histologically with LM (van Zyl, 2014). PCR was limited to the use of two species-specific primer sets for *T. canis* and *T. cati* with approximate target sizes of 390 bp and 400 bp respectively, and did not specifically evaluate the sections tested for the presence or absence of larvae, only for the presence of characteristic inflammatory lesions. Thus for this current study, it was decided to initially test only cases with a high probability of nematode DNA being present in the sections taken for molecular analysis, and to trial a range of primer sets of varying target size, including species-specific and more generic primers.

The predominant histological lesion associated with LM in kiwi resembles the "eosinophilic granuloma" described in human visceral LM (Kaplan et al., 2001). Although not pathognomonic for LM, 14 out of the 17 kiwi also had nematode larvae confirmed histologically in at least one tissue supporting the diagnosis of LM as cause for the lesions. Histomorphology of the nematode larvae in sections from affected kiwi, where cross-sections were available for evaluation, was consistent with that described for *T. canis* and *T. cati* based predominantly on the cross-sectional diameter and presence of single, small, bilaterally symmetrical alae (Nichols, 1956a; Bowman, 1987). Differentiating these two *Toxocara* species in histologic section relies on a minor size difference, with one study finding that cross-sections through the mid-body of *T. cati* larvae never exceeded 18 µm in diameter, while *T. canis* larvae were often greater than 18 µm but ranged in diameter from 14 µm to 20 µm (Nichols, 1956a). The nematodes in kiwi tissues were therefore more consistent with *T. cati*, but not definitively identifiable given the overlap in size

ranges and the fact that the level of the larvae measured in diagnostic (as opposed to experimental) sections can't be readily distinguished.

Based on the histomorphology, however, primer sets were chosen that had been designed for identification of ascaridoid nematodes, including one set each designed for the specific identification of *T. canis* and *T. cati*. All of the nematode primer sets evaluated amplified the control organisms *T. canis* and *T. cati* appropriately. It is worth noting that two further primer sets that were initially tested and which reliably amplified the controls were subsequently found to be unsuitable as they also amplified similarly sized target sequences from kiwi tissue (data not shown). This highlights the importance of sequencing to confirm validity of PCR results, especially when using primer sets on a previously untested species.

Successful amplification from FFPE tissue decreased with increasing product size, until no samples were positive for the product of ~520 bp length. Formalin fixation causes cross-linkage between DNA and protein (Sengüven et al., 2014); the quality of DNA in formalin-fixed samples degrades over time spent in formalin, and degradation may continue at a low level even after the fixed tissue is processed and embedded within paraffin (Libório et al., 2005). A number of commercial kits designed specifically for the extraction of DNA from FFPE samples are now available, but the quality of DNA extracted also relies heavily on pre-extraction factors including the condition of the fixative, fixation time, and post-fixation storage (Sengüven et al., 2014). Despite this, successful DNA extraction, particularly of small molecular weight fragments, has been proven feasible even from tissue blocks that have been stored for up to 40 years (Libório et al., 2005). In this small sample of cases, the tissue blocks had been stored for up to 14 years. Specific information regarding pre-extraction handling was not readily available although, as is common with wildlife submissions, the time from death to necropsy in the kiwi examined did vary widely which may also result in degradation of DNA quality due to decomposition prior to fixation. However, extraction of kiwi DNA fragments to a size of at least 350 bp was successful

from all cases archived for five years and less, but only four out of the eight earlier cases, using commercially available kits.

The only group Ia tissue that was negative for all nematode primer sets was one of the more recent cases (archived less than a year at the time of extraction) and PCR successfully amplified kiwi DNA up to ~350 bp in size. Despite the histological confirmation of larvae in both sandwich sections, it is possible that nematode DNA was not included within the extraction sample, or that the quality of nematode DNA present was not equivalent to that of the kiwi tissue. A further consideration is that the nematode present was not *T. cati, T. canis*, or other ascaridoid nematode likely to be amplified by the chosen primers, although the morphology of the larvae in section was compatible with *Toxocara* spp. as previously described.

Results from group I otherwise suggested that PCR is only likely to be successful where there are histologically identifiable nematode larvae in sections directly adjacent to the tissue scrolls taken for molecular analysis, with no amplification from any of the six tissues in which no larvae were evident. Studies evaluating the long-term survival of migrating *T. canis* larvae indicate well-developed strategies for host immune evasion, including the production of a mucin-rich coating over the surface of the cuticle that can be shed in response to adherence by host antibody and inflammatory cells, leaving the organism free to continue migration (Fillaux and Magnaval, 2013; Maizels, 2013). It is likely that such mechanisms reduce the chance that recoverable nematode DNA will be present in tissue migration tracts once the larvae have transited. However, inevitably, a proportion of the larvae will become 'trapped' and destroyed by the host's immune response (Parsons and Grieve, 1990; Fillaux and Magnaval, 2013).

Results from the second group of kiwi did not entirely support the hypothesis that PCR is of no diagnostic value if no larvae are identified in histological sections. The one repeatable positive case was the only tissue in which larvae were present in both histological sandwich sections and that also produced successful amplification of kiwi DNA. There were, however, five tissues that

were positive despite a lack of histologically identifiable larvae. Each tissue was positive in only one out of three initial duplicate PCR runs. Because of this inconsistency, PCR was repeated on all positives a further three times and was negative in each of these five cases. Considerations for this finding would have to include the possibility of sample contamination causing the original positive results or, in the cases where sequencing was unsuccessful, potential non-specific amplification. Alternatively, it may be that very small amounts of nematode DNA of an amplifiable size were present in the extractions but not consistently included in aliquots taken for PCR. This was suspected to be the case for two tissues with larvae identified in a single sandwich section and one tissue with larvae confirmed in both sandwich sections but with evidence of poor-quality DNA, which showed similarly inconsistent positive results.

Two of the positive tissues with no identifiable larvae contained large numbers of granulomata +/- extensive acute inflammation histologically, and it is possible that larval sections were obscured or simply missed at histological evaluation, especially if not intact. Additionally, the small size of *Toxocara* larvae (less than 20 µm diameter) could allow for the possibility of larvae or larval fragments present in samples taken for molecular analysis but not in histological sandwich sections. Nonetheless, while it may be worth attempting PCR in cases lacking histologically identifiable larvae, the chances of a reliable positive result appear to be low, and replication of PCR results is recommended.

In all cases subjected to BLAST analysis, the nematode sequences from kiwi tissues with lesions of LM aligned with sequences from *T. cati* available in the GenBank database. In three of the group Ia kiwi, this included sequences from both the ITS-2 region and 18S gene of nuclear ribosomal DNA, lending weight to the identification. The ITS-2 primer set Tcat1-NC2 was specifically designed for the differentiation of *T. cati* from other ascaridoid nematodes (Jacobs et al., 1997); in contrast there are only two bases different between the sequences of *T. cati* and *T. canis* in the smaller product amplified by the generic 18S primers Nemo 18S F-Nemo 18S R

(Pinelli et al., 2013). There is, as yet, no single target sequence that is universally agreed to allow species-level identification, although most investigations in the field of molecular nematology have centred on the "high-copy" sequences present in nuclear ribosomal and mitochondrial DNA (Seesao et al., 2017). Use of BLAST for organism identification relies heavily on the accuracy of the data submitted to the database, including both the integrity of the sequences and the reliability of the accompanying identification (Harris, 2003). The scope of available data is also somewhat limited as, in general, databases of nematode genomics remain relatively sparsely populated. The possibility of a nematode closely related to *T. cati* but unrepresented in GenBank could not be entirely ruled out. A number of other, lesser known *Toxocara* spp. have been described from wildlife but lack molecular studies and remain of uncertain zoonotic potential; for example, *Toxocara pteropodis* in fruit bats (*Pteropus* spp.) (Clark and McKenzie, 1982; Prociv, 1989b), and *Toxocara mackerrasae* in the Australian bush rat (*Rattus fuscipes*) (Warren, 1970) (neither of which have been documented in New Zealand).

The identification of *T. cati* as a cause for LM in kiwi is both biologically and ecologically feasible, given the extensive range of the definitive host, the domestic cat, throughout New Zealand (Aguilar et al., 2015). One study into the potential for spread of parasites by feral cats found that 67% of feline faecal samples collected within an area of New Zealand farmland contained *T. cati* eggs (Langham and Charleston, 1990). Another known parasite of cats, *Toxoplasma gondii*, has also been shown to spill over into New Zealand fauna, including kiwi as well as other birds, marine mammals, and shellfish (Roe et al., 2013; Howe et al., 2014; Coupe et al., 2018). The indirect effect of invasive species on native species and ecosystems through co-invading parasites is gaining increasing attention, and the variety of influences that such parasites may have is still being explored (Chalkowski et al., 2018). While LM due to *T. cati* is most likely a sporadic cause of mortality and illness in kiwi and is unlikely to be driving population level changes, its significance may increase where kiwi populations are reduced and managed intensively for conservation.

Infective larvae of *Toxocara* spp. develop within the egg after it is passed in faeces, and remain viable under the right environmental conditions for 6 to 12 months, potentially longer (Macpherson, 2013). Kiwi feed by probing the surface litter and deeper soil with their long bills and would have a high potential for exposure in contaminated areas. Additionally, the kiwi diet in the wild is composed predominantly of soil and surface-dwelling invertebrates, particularly earthworms (Sales, 2005) which have been shown to act as either transport or paratenic hosts for *T. canis* (Pahari and Sasmal, 1991). Those cases arising in captivity or where kiwi inhabited predator-free sanctuaries at the time of death would have to be investigated on a case-by-case basis for the potential of exposure to *Toxocara* eggs: for example, the length of time the kiwi had been present at the controlled site; the potential for contamination by cat faeces around the perimeter of the controlled site; or any transfer of surface litter or insects from uncontrolled sites.

To the authors' knowledge, this is the first report of *T. cati* causing LM in an avian species other than the chicken, where published reports predominantly describe experimental infection (Azizi et al., 2007; Oryan et al., 2010; Taira et al., 2011), largely driven by the zoonotic potential for humans ingesting undercooked or raw muscle or liver from infected animals. The significance of LM to any host depends on the localisation and number of migrating larvae. Early studies on *T. canis* indicated that infections in various laboratory animal species were often well tolerated, up to 10 larvae per gram of body weight in tissue other than the brain (Beaver, 1956), and in humans, subclinical infections are considered common (Fillaux and Magnaval, 2013). Species differences in larval distribution have also been also described (Beaver, 1956). In one six-month study of *T. cati* larval migration in chickens, larvae were most commonly identified in liver, lung, and muscle but seldom in the brain (Taira et al., 2011); no clinical signs or changes in behaviour were observed in this or another long-term study (Oryan et al., 2010), however, it has been suggested that the severity of disease may be dose dependant and more significant in natural infections (Azizi et al., 2007).

In this small retrospective study of necropsy cases, brain lesions were histologically confirmed in 10 of the 17 kiwi, however, the tissues examined were not uniform in each case and based on the original necropsy reports it appears that only 12 of the kiwi may have had brain histology performed. Other potential predilection sites are not routinely collected at necropsy, and only a single kiwi in this study (#3), which had presented with ataxia prior to death, had sections of spinal cord and skeletal muscle taken for histological evaluation. While subclinical visceral infections may also be common in kiwi, neural infections are likely to have the most impact with the potential to contribute to mortality either directly or through neurological deficits that could interfere with the ability to forage or predispose to death by misadventure. Experimental studies with *T. canis* have also demonstrated lower levels of risk and predator aversion in infected mice than control animals (Holland and Cox, 2001); this is thought to be a pathological side effect rather than true host-manipulation, but is still an effect that could have increased consequence for an endangered species such as the kiwi, already significantly at risk from predation.

There is a single report of a kiwi presenting as a possible definitive host for *T. cati* (Clark and McKenzie, 1982). Nematodes consistent with *T. cati*, as identified by detailed morphological examination, were found at necropsy within the small intestine of a North Island brown kiwi. Both larval and adult forms were present, apparently the first published finding of adult *Toxocara* in birds. The authors speculate that the reported lower body temperature of kiwi relative to similarly sized avian species might have contributed to the ability of kiwi to behave as a definitive host for an organism that is more typically adapted to a mammalian host. None of the pathology reports in the cases from the study presented here describe adult *Toxocara* in the intestinal tract, although a complete parasitological examination is rarely performed for a routine necropsy. The nature of most cases of LM is that migrating larvae are unable to complete their life cycle in an abnormal host and do not mature to adult nematodes. While somatic migration can occur as part of the normal life cycle of *T. cati* in the definitive host, it is worth noting that none of the larvae in the kiwi tissues presented here showed any indication of

development beyond the size of the infective larval stage. Further research is required to characterise the nematodes inhabiting the kiwi gastrointestinal tract, to more precisely define the role of kiwi as a host for *T. cati*.

2.6. Conclusion

The results of this study provide strong evidence for *T. cati* as a cause for visceral and neural LM in the North Island brown kiwi (*Apteryx mantelli)*, and additionally support the utility of PCR using archival FFPE tissue blocks in the investigation of this disease. PCR is most likely to yield a meaningful result when larvae can be identified histologically in sections directly adjacent to those used for molecular analysis, and when primer sets with a small target product (e.g. less than 400 bp) are used. The identification of *T. cati* as a cause of LM in native New Zealand kiwi represents an indirect parasite-mediated effect of an invasive mammalian species, the domestic cat.

Chapter 3: Ventral dermatitis in rowi (*Apteryx rowi*) caused by cutaneous capillariasis

3.1. Abstract

In 2013 there was an outbreak of crusting ventral dermatitis among a group of juvenile rowi (*Apteryx rowi*), a species of the endangered New Zealand kiwi, that were being raised on an off-shore island sanctuary. Biopsies taken at the time found nematodes migrating within the epidermis of affected skin but the specific identity and origin of the organisms was not established, and sporadic cases of similar skin disease continue to occur on the island. On examination of additional sections from the original skin biopsies, adult nematodes and eggs were identified, the histomorphology of which was consistent with *Capillaria sensu lato*. PCR was performed on DNA extracted from archived formalin-fixed, paraffin-embedded tissue blocks of skin from eight affected rowi, using primers targeting the 18S region of nuclear ribosomal DNA and the COI gene of mitochondrial DNA of capillarid nematodes. The 18S sequences from all rowi samples were identical and matched sequences from members of the genus *Eucoleus*. In contrast, two distinct capillarid COI sequences were obtained, in one case both from the same rowi skin biopsy. While there were no close matches, both COI sequences also aligned nearest to sequences identified as *Eucoleus* spp. It is considered unlikely that two different nematode species are involved in the rowi skin lesions and the possible amplification of a COI pseudogene or "numt" is discussed. A species-level identification of the capillarid nematodes causing skin disease in rowi was not obtained, however, based on histological evaluation the infections include reproductively-active adult nematodes. This finding indicates the possibility of perpetuation of the skin disease in the absence of the original source, as well as raising potential for the transfer of infection from the island when the juvenile rowi are translocated to their new habitats.

3.2. Introduction

The kiwi family (Apterygidae) comprises five recognised species that occupy different geographic ranges within New Zealand. Of these, rowi (*Apteryx rowi*, also known as Okarito brown kiwi) were the most recently confirmed as a distinct species through phylogenetic testing (Burbidge et al., 2003; Tennyson et al., 2003). In 2018, rowi were estimated to number around 600 and were classified by the New Zealand Department of Conservation (DOC) as "threatened: nationally vulnerable", up-graded from their initial classification of "nationally critical" as a direct result of successful conservation management programmes (Robertson et al., 2016; Germano et al., 2018). The IUCN Red List (BirdLife International, 2017b) also classifies rowi as "vulnerable" with an increasing population trend; at the present time, however, their continued recovery remains conservation dependent.

Prior to the arrival of humans and predatory mammals to New Zealand, the distribution of rowi is believed to have included the northwest coast of the South Island and the southern tip of the North Island (Germano et al., 2018). At its lowest numbers, the extant population was restricted to a small area of lowland forest at Okarito on the west coast of the South Island, but now includes some newly established populations in the Omoeroa ranges and on two predator-free offshore islands, following translocations of birds as their numbers have slowly increased. Rowi have more than doubled in population size since the Operation Nest Egg (ONE) programme, managed by DOC, was initiated in 1994 (Robertson et al., 2016). In brief, eggs are located and removed from their burrows in the wild, artificially incubated and hatched, and the chicks raised in predator-free sanctuaries known as crèches. Once they reach a size where they are deemed to have a greater chance of survival against predators, they are released back into the wild (Colbourne et al., 2005).

In the case of rowi, ONE chicks are raised on a single off-shore crèche island where they are intermittently monitored by DOC rangers. In 2013, 15 out of 30 juvenile rowi on the crèche island developed a crusting dermatitis affecting the ventral abdominal skin and vent margin (Gartrell

et al., 2015). Histology of skin biopsies taken from several of the affected birds showed nematodes present within the epidermis, suggesting the condition cutaneous *larva migrans*. Molecular sequencing of nematode DNA extracted from the biopsies aligned among *Trichostrongylus* spp; however, the authors concluded that this result should be viewed with caution as concurrent morphological evaluation was limited and members of this nematode genus have not previously been associated with cutaneous migratory lesions (Gartrell et al., 2015).

The full investigation, management, and outcome of the 2013 outbreak of cutaneous nematodiasis in juvenile rowi have been described elsewhere (Gartrell et al., 2015). Sporadic cases of similar skin lesions have been diagnosed on the crèche island in the years since. The purpose of this study was to investigate further, through histomorphological and molecular evaluation, the identity of the nematodes causing skin disease in juvenile rowi.

3.3. Methods

3.3.1. Case selection

A retrospective search of the School of Veterinary Science (SoVS) pathology database (Massey University, Palmerston North, New Zealand) was performed to identify cases of ventral dermatitis occurring in rowi in which nematodes were identified histologically in the lesional skin. Skin biopsies from the cohort of affected rowi taken during the 2013 investigation in which larval organisms were not identified in the original histological sections were also included in the initial selection. To obtain comparative sequences from avian capillarid nematodes previously identified histologically in New Zealand, a retrospective search was also performed to identify cases of oral or oesophageal/crop infection in kahu (Australasian harrier hawk, *Circus approximans*), as described by Alley et al. (2004a).

Where sufficient tissue was available from the rowi samples, the majority of which were small skin biopsies, multiple “sandwich” sections were cut from the archived formalin-fixed, paraffin-embedded (FFPE) tissue blocks, comprised of a 4 μm section mounted on a slide and stained

with Haematoxylin and Eosin (H&E), followed by a 10 µm tissue scroll for molecular analysis, a further 4 µm section mounted and stained with H&E, a further 10 µm tissue scroll, and a final 4 µm section mounted and stained with H&E. The original diagnostic slides (where available) and the three H&E-stained sandwich sections were examined for the presence of nematodes for the purposes of describing histomorphology of the organisms and the associated histopathology, as well as to assess for the probability of nematode DNA being present in the tissue scrolls taken for molecular analysis. For the larger necropsy samples from kahu, a single 4 µm H&E stained section to confirm histological presence of organisms followed by a 10 µm tissue scroll for molecular analysis were taken from the archived FFPE blocks.

For use as a positive control, DNA from an unidentified capillarid nematode retrieved from the proventriculus of a red-billed gull (*Chroicocephalus novaehollandiae scopulinus*) originating from Otago, New Zealand, was supplied by Jerusha Bennett and Dr. Bronwen Presswell from the Department of Zoology, University of Otago, New Zealand. For use as an alternative positive control in the internal transcribed spacer (ITS)-2 PCR, a *Trichostrongylus axei* nematode was sourced from the intestine of a sheep (*Ovis aries*) at routine necropsy, identified morphologically by the Massey University SoVS parasitology laboratory (Palmerston North, New Zealand), and preserved in 70% ethanol prior to DNA extraction.

3.3.2. DNA extraction

DNA extraction was performed on rowi and kahu FFPE tissue scrolls using a commercial kit (NucleoSpin DNA FFPE XS, Macherey-Nagel, Germany) per the manufacturer's instructions, with the exception of the lysis step which was carried out in a 56 °C water bath overnight. For extraction of DNA from the *T. axei* positive control, a mix of 100 µl DirectPCR Lysis Reagent (Tail) (Viagen Biotech Inc., USA) and 2.5 µl Proteinase K solution (20 mg/ml, Ambion, CA, USA) was prepared and 10 µl of this solution added to a single nematode in a PCR tube. This was incubated in an Applied Biosystems GeneAmp PCR system 2400 thermocycler (Thermofisher, USA) for 16 hours at 55 °C followed by 1 hour at 90 °C.

3.3.3. Molecular analysis

Primers used for molecular analysis are presented in Table 3.1 and include (sourced from literature) a primer set designed for the gender identification of kiwi tissue (Huynen et al., 2003); a primer set targeting the ITS-2 region of nuclear ribosomal DNA (rDNA) of nematodes, as used in the previous investigation (Gasser et al., 1993; Gartrell et al., 2015); and a primer set targeting the 18S region of rDNA of capillarid nematodes (Fischer et al., 2018).

Several literature-sourced primer sets targeting the cytochrome oxidase *c* subunit I (COI) gene of mitochondrial DNA (mtDNA), which had been used successfully on trichurid and/or capillarid nematodes of mammals (Zhu et al., 2000; Di Cesare et al., 2012; Guardone et al., 2013), were also trialled with no result, or with production of sequences aligning to avian, insect, or bacterial DNA. Only a single forward sequence that aligned most closely to a capillarid nematode sequence (*Eucoleus boehmi*, KX027312) was obtained using the primer set JB3-JB4.5 (Zhu et al., 2000) on one case of crop capillariasis from a kahu (#11). As no COI sequences originating from avian *Eucoleus* species were available in the National Center for Biotechnology Information database (GenBank®) at the time of investigation, this sequence was used in the design of a degenerate COI primer set (Eu COI F1B-Eu COI R1) for the purpose of this study. The primers were created manually using Geneious v. 10.2.3 (Kearse et al., 2012) following alignment of COI sequences from the mammalian capillarids *Eucoleus aerophilus* and *E. boehmi* available in GenBank (KC341988 to KC341992, KR186213 to KR86215, and KX027311 to KX027314), along with the single forward sequence obtained from the kahu.

For all primer sets, each PCR contained 1X HOT FIREPol Blend Master Mix (10 mM $MgCl_2$, Solis Biodyne, Estonia), 300 nM each of forward and reverse primers (IDT, IA, USA), and 1 µl of template DNA, made to a total of 20 µl with nuclease free water. A negative (blank) control containing 1 µl of nuclease free water in place of template DNA was run simultaneously. PCR was repeated on all rowi samples with the capillarid 18S primers, and on all positive rowi and kahu samples with the capillarid COI primers, in order to confirm sequences.

Table 3.1. Primer sets used in this study, including one designed for the gender identification of kiwi, and others targeting the ITS-2 region or 18S gene of nuclear ribosomal DNA and the COI gene of mitochondrial DNA of nematodes.

Primer name	Primer sequence (5'-3')	Target	Approximate amplicon size (bp)	Reference
W5	AATCACCCTTTAAACAAGCTGTTAAAGCAA	Uncertain – Kiwi W-linked and Z-linked or autosomal	350 (males and females) +/- 200 (females only)	Huynen et al., 2003
W7	CCTTTCTCAAATCTCTCTTTTGTTCTAGACAC			
NC1	ACGTCTGGTTCAGGGTTGTT	Nematode ITS-2 region	330	Gasser et al., 1993
NC2	TTAGTTTCTTTTCCTCCGCT			
Kt875351.1	CCCTAGTTGCGACTTTAAACGA	Capillarid 18S gene	290	Fischer et al., 2018
Capillaria 18S1R	TCCACCAACTAAGAACGGCC			
JB3	TTTTTTGGGCATCCTGAGGTTTAT	Capillarid COI gene	450	Zhu et al., 2000
JB4.5	TAAAGAAAGAACATAATGAAAATG			
Eu COI F1B	GGTCCWYTAGGWATAATYTATGC	Capillarid COI gene	290	Designed for this study
Eu COI R1	ARATCTAAAGATGCATTRGAAAG			

PCR was performed on a Labcycler (SensoQuest, Germany) using a touchdown PCR protocol. For the kiwi gender, ITS-2, and 18S primer sets, the following conditions were used: initial activation of 15 minutes at 95 °C, 12 cycles of 95 °C for 30 seconds, annealing for 30 seconds (starting at 60 °C , reducing by 0.5 °C per cycle), and 72 °C for 30 seconds, followed by a further 35 cycles of 95 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 30 seconds, and final elongation at 72 °C for 7 minutes. For the COI primer set a similar touchdown PCR protocol was used but with lower annealing temperatures, starting at 51 °C and reducing by 0.5 °C per cycle for 12 cycles, then 35 cycles at the final annealing temperature of 45 °C.

The PCR product was separated by electrophoresis on a 1% w/v agarose gel (Bioline, UK) using RedSafe (iNtRON Biotechnology, South Korea) to stain the DNA, and visualized with a gel image system (MultiDoc-It Imaging System, UVP, CA, USA). Size of PCR products was estimated in comparison to a HyperLadder™ 100 bp molecular ladder (Bioline, UK).

3.3.4. Sequencing and BLAST analysis

Amplicons of the appropriate size were cut from the gel, eluted overnight in elution buffer (10 mM Tris-HCl, pH 8.0), and the eluate submitted to the Massey Genome Service (Massey University, Palmerston North, New Zealand) for bi-directional Sanger sequencing. The resultant forward and reverse sequences were aligned using Geneious v. 10.2.3; where identical sequences were obtained from repeat PCR on individual template DNA, these were all aligned together. The alignments were manually trimmed and the resultant sequences subjected to BLAST (Basic Local Alignment Search Tool) analysis. Only those sequences from capillarids identified to species level are considered in the BLAST results discussion.

Nucleotide sequences obtained in this study have been deposited in GenBank under the accession numbers MT678491 to MT678507 (18S) and MT782133 to MT782145 (COI).

3.3.5. Phylogenetic analysis

To further assess the relationship between the rowi, kahu, red-billed gull, and published capillarid sequences, phylogenetic trees were created. To minimise the inclusion of potentially

misidentified sequences in the GenBank database, as well as to exclude sequences that lacked significant cover of the study sequences, the top 100 discontiguous megaBLAST results for the 18S and COI sequences obtained in this study were filtered to include only sequences identified as from the nematode families Capillariidae or Trichuridae, giving 99 sequences included in the 18S analysis, and 47 sequences in the COI analysis. As only a small cluster of four unidentified *Trichuris* sp. sequences were incorporated into the COI tree using this methodology, four further COI sequences originating from identified *Trichuris* species were selected from GenBank and included in the COI analysis, in order to cover a similar taxonomic scope as the 18S analysis. A sequence originating from *Enoplus* species was used in each case as an outgroup. Sequences were aligned using MAFFT (Katoh and Standley, 2013) employing the E-INS-i algorithm in Geneious v. 10.2.6 (Kearse et al., 2012) resulting in alignments of 293 and 261 nucleotides for 18S and COI respectively. For both alignments, maximum likelihood analysis was performed using PhyML version 3.0 (Guindon et al., 2010), available on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/phyml/). Phylogenetic trees were inferred employing Subtree Pruning and Regrafting (SPR) branch-swapping and nucleotide substitution models determined by Smart Model Selection (SMS) (Lefort et al., 2017). Branch support was assessed using an approximate likelihood ratio test (aLRT) with the Shimodaira–Hasegawa-like procedure. The 18S tree was produced using the Kimura 80 model with invariable sites and gamma distribution (K80+G[0.87]+I[0.5]). The COI tree was produced using a general time-reversible model with invariable sites and gamma distribution (GTR+G[0.208]+I[0.159]). Tree visualization was performed with Interactive Tree Of Life (https://itol.embl.de) (Letunic and Bork, 2019).

3.4. Results

3.4.1. Cases

Ten (nine biopsy and one necropsy) cases of ventral dermatitis in rowi were identified in the database, diagnosed between 2013 and 2018. Of these, eight had confirmed nematode organisms identified histologically in the original diagnostic sections, while two that were lacking

histologically identifiable nematodes were initially included as they were biopsies taken from the same cohort confirmed to be affected by cutaneous nematodiasis in the 2013 investigation. One of these two cases was subsequently excluded as there was insufficient tissue remaining in the FFPE block for molecular analysis. Of the nine remaining cases, seven had confirmed nematode organisms identified in histological sections both before and after the two tissue scrolls taken for molecular analysis, including the one remaining biopsy case that had been negative for organisms in the original diagnostic section. Conversely, one biopsy that had confirmed organisms in the original diagnostic section was negative in all sandwich sections and was subsequently excluded from molecular analysis. The final biopsy (rowi #8), which did contain nematode organisms in the original diagnostic section, had insufficient tissue for sandwich histology sections but remaining tissue was sufficient for DNA extraction and molecular analysis (Table 3.2).

Additional to the skin disease, one necropsy case from the affected 2013 rowi cohort also had a single larval nematode identified histologically in the liver; unfortunately, the organism was not present in histological sandwich sections from around the scroll taken for DNA extraction, and so molecular analysis was not performed on this tissue.

Thirteen cases of oral or crop capillariasis were identified in kahu, diagnosed between 2002 and 2010, originating from around the central North Island. In one case the archived H&E slides and FFPE blocks could not be located. Eleven of the remaining cases (eight from oral cavity and three from crop) had confirmed presence of capillarid organisms present histologically in H&E-stained sections adjacent to the tissue scroll taken for DNA extraction and were included in the nematode molecular evaluations (Table 3.2).

3.4.2. Histology

In areas of affected skin, the epidermis was mildly to moderately hyperplastic, typically characterised by papillary hyperplasia of the basal epidermis, acanthosis, and compact to

Table 3.2. Case information and PCR results for all samples tested in this study. Case information includes: sample type (biopsy and necropsy samples both in the form of archived formalin-fixed, paraffin-embedded tissue blocks); year the sample was taken; anatomic origin of the sample within the host; and geographic origin of the host within New Zealand. For PCR results: F = PCR positive female; - = negative result; + = positive result (produced amplicon of appropriate size, confirmed by sequencing); A or B = rowi or kahu capillarid COI sequences A or B as produced on duplicate PCR runs.

Case information					PCR results (approximate size of target)			
Source ID	Sample type	Year	Sample origin	Geographic origin	Kiwi gender (350bp (M&F) & 200bp (F))	Nematode ITS-2 (330bp)	Capillarid 18S (290bp)	Capillarid COI (290bp)
Rowi #1	Biopsy	2013	Skin	Crèche island	F	-	+	+A/A
Rowi #2	Biopsy	2013	Skin	Crèche island	F(200 only)	-	+	-
Rowi #3	Biopsy	2013	Skin	Crèche island	F	-	+	+A/B
Rowi #4	Biopsy	2013	Skin	Crèche island	-	-	+	+B/-
Rowi #5	Biopsy	2013	Skin	Crèche island	F	-	+	+A/A
Rowi #6	Necropsy	2013	Skin	Crèche island	F	-	+	+A/A
Rowi #7	Biopsy	2015	Skin	Crèche island	F	-	+	+A/A
Rowi #8	Biopsy	2018	Skin	Crèche island	F	-	+	+B/B
Kahu #1	Necropsy	2010	Oral	Hawke's Bay			-	-
Kahu #2	Necropsy	2007	Crop	Manawatu			+	+A/A
Kahu #3	Necropsy	2005	Oral	Manawatu			+	-
Kahu #4	Necropsy	2003	Oral	Wellington			-	-
Kahu #5	Necropsy	2003	Oral	Manawatu			+	+A/A
Kahu #6	Necropsy	2003	Oral	Whanganui			+	+A/A
Kahu #7	Necropsy	2002	Oral	Manawatu			+	+A/-
Kahu #8	Necropsy	2002	Oral	Whanganui			+	+A/A
Kahu #9	Necropsy	2002	Crop	Manawatu			+	+B/B
Kahu #10	Necropsy	2002	Oral	Manawatu			-	-
Kahu #11	Necropsy	2002	Crop	Manawatu			+	+B/B
Red-billed gull	Nematode DNA	2019	Proventriculus	Otago		-	+	+

laminar orthokeratotic hyperkeratosis. In some sections there was mild to moderate spongiosis, multifocal small intra-epidermal pustules, and/or superficial serocellular crusting. There were mild to moderate superficial dermal perivascular to interstitial infiltrates of lymphocytes and granulocytes (presumably eosinophils), and frequent exocytosis of lymphocytes and granulocytes into the overlying epidermis. Multifocal nodular lymphoid aggregates were present in the mid to deep dermis (Figure 3.1A). Two biopsies included junction of skin and cloacal mucosa, with some focal erosion of the mucosal epithelium and mild to moderate infiltration of the submucosa by lymphocytes and granulocytes.

Nematode sections were predominantly located within the epidermis, less commonly within the superficial keratin or appearing free over the skin (or, in one case, the cloacal) surface. In the single post-treatment (necropsy) case included in the study, one nematode was present at or just underlying the dermo-epidermal junction, but dermal localisation was otherwise not a feature. Both male and female adult nematodes were identifiable, as well as some suspected larval forms. Occasional free eggs were identified within the epidermis or superficial keratin. Cross-sectional or near cross-sectional diameters of adult nematodes ranged from ~50 to 75 µm through levels with identifiable sex organs, with no dramatic difference in size of males versus females noted. Musculature was coelomyarian and bilateral bacillary bands were apparent (Figure 3.1B). Sections through the oesophageal region contained large cells with granular basophilic cytoplasm consistent with stichocytes (Figure 3.1C). Eggs measured variably ~62-72 µm long by ~25-32 µm wide and were non-embryonated with a thick shell and bipolar plugs (Figure 3.1D).

3.4.3. Molecular analysis

3.4.3.1. Kiwi gender-specific primers

Seven out of eight extracts from the rowi samples produced appropriate amplicons using the kiwi gender-specific primers (W5-W7); all positives were consistent with females, although one (rowi #2) yielded only the 200 bp amplicon and not the 350 bp amplicon, suggesting overall

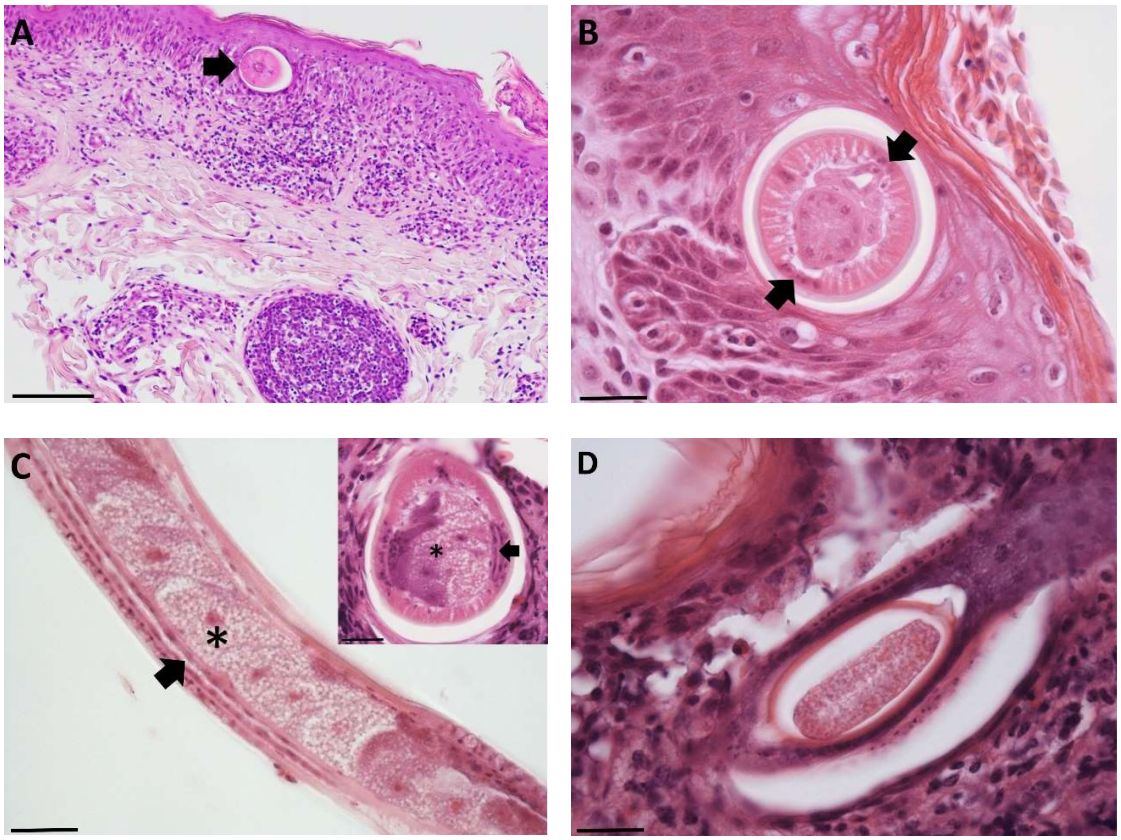

Figure 3.1. Histological images of capillarid nematodes in rowi skin biopsies. A: Cross-section of a nematode within the epidermis (arrow), associated with epidermal hyperplasia and dermal inflammatory infiltrates (H&E, bar = 100 µm). B: Cross-section of a capillarid nematode within the epidermis, demonstrating low, bilateral bacillary bands (arrows) (H&E, bar = 20 µm). C: Longitudinal (main image) and cross-section (inset) of nematodes through the level of the oesophagus (arrow) and stichocytes (asterisk) (H&E, bar = 20 µm). D: Near-longitudinal section of a non-embryonated egg within a nematode present in the epidermis, demonstrating the typical barrel-shape, thick shell, and bipolar plugs (H&E, bar = 20 µm).

lower quality DNA. The one extract that was negative (rowi #4) was reportedly a male, and so only a 350 bp amplicon would have been anticipated (Table 3.2).

3.4.3.2. Nematode ITS-2 primers

None of the eight rowi skin samples or the red-billed gull capillarid control DNA amplified with the ITS-2 primers (NC1-NC2). The *T. axei* positive control produced an amplicon of ~330 bp which on sequencing and BLAST analysis showed 100% homology with sequences from *T. axei* in GenBank (e.g. KC998725).

3.4.3.3. Capillarid 18S primers

All rowi extracts produced an amplicon of appropriate size (~290 bp) using the capillarid 18S primers (Kt875351.1-Capillaria 18S1R), including the sample that was negative with the kiwi

gender-specific primers. Trimmed sequence lengths were 270 to 279 bases and on alignment had identical nucleotide sequences apart from a single degenerate base within the sequence from rowi #5. On BLAST analysis, the consensus of all eight sequences aligned 100% with GenBank sequences from a range of *Eucoleus* species identified as *E. aerophilus* (MF599385), *E. garfiai* (LC484432), *E. perforans* (LC424998), *E. boehmi* (JX456628), and *E. dispar* (EU004821), and additionally showed only a single base difference to a sixth *Eucoleus* sequence identified as *E. contortus* (LC424996) (Table 3.3).

Eight out of 11 kahu extracts produced an amplicon of the appropriate size using the capillarid 18S primers, with final sequence lengths of 239 to 287 bases. On alignment, seven of the eight had identical nucleotide sequences, with one sequence (kahu #3) containing a single base difference (repeat PCR to confirm was not performed). Alignment of the rowi capillarid 18S consensus and the consensus of all eight kahu capillarid 18S sequences was identical, and BLAST results were therefore very similar (Table 3.3).

The control capillarid DNA extraction derived from a red-billed gull also produced an amplicon of the appropriate size using the capillarid 18S primers, with a final sequence length of 287 bases that showed a single base different to the rowi and kahu capillarid 18S consensus sequences, confirmed on repeat sequencing; this was at a different position to the base substitution within the sequence from kahu #3. On BLAST analysis, the seagull capillarid 18S sequence aligned with 100% pairwise identity to the sequence from *E. contortus* (LC424996) (Table 3.3).

3.4.3.4. Capillarid COI primers

Seven out of eight rowi extracts produced amplicons of an appropriate size (~290 bp) using the capillarid COI primers (Eu COI F1B-Eu COI R1); the one negative case (rowi #2) had only produced the 200 bp amplicon with the kiwi gender-specific primers suggesting overall poorer quality DNA, although an amplicon of ~290 bp was obtained with the capillarid 18S primers (Table 3.2). Sequencing identified two distinct COI sequences with ~11% difference in base composition; one

Table 3.3. Top BLAST results for consensus sequences obtained in this study from capillarids of rowi, kahu, and red-billed gull origin.

Sequence	BLAST program	Sequence length (bp)	Aligned to organism (GenBank #)	Cover	Pairwise identity	Bit-score	e-value
Rowi capillarid 18S sequence	Megablast	279	*Eucoleus aerophilus* (MF599385) *Eucoleus garfiai* (LC484432) *Eucoleus perforans* (LC424998) *Eucoleus boehmi* (JX456628) *Eucoleus dispar* (EU004821)	100%	100%	516.336	2.50^{e-142}
Kahu capillarid 18S sequence	Megablast	287	*Eucoleus aerophilus* (MF599385) *Eucoleus garfiai* (LC484432) *Eucoleus perforans* (LC424998) *Eucoleus boehmi* (JX456628) *Eucoleus dispar* (EU004821)	100%	100%	531.109	8.68^{e-147}
Red-billed gull capillarid 18S sequence	Megablast	287	*Eucoleus contortus* (LC424996)	100%	100%	531.109	8.68^{e-147}
Rowi capillarid COI sequence A	Megablast	260	*Eucoleus aerophilus* (KC341990)	100%	86.2%	281.811	8.70^{e-72}
Rowi capillarid COI sequence B	Megablast	255	*Eucoleus aerophilus* (KC341989)	100%	85.8%	270.731	1.84^{e-68}
Kahu capillarid COI sequence A	Megablast	260	*Capillaria aerophila* (JQ905059)	87.3%	89.4%	287.351	1.85^{e-73}
Kahu capillarid COI sequence B	Discontiguous megablast	260	*Eucoleus boehmi* (KC341992)	100%	83.1%	271.792	8.90^{e-69}
Red-billed gull capillarid COI sequence	Discontiguous megablast	260	*Eucoleus boehmi* (KR186213)	95%	81.0%	236.626	6.35^{e-58}

(rowi capillarid COI sequence A) from five rowi, and one (rowi capillarid COI sequence B) from two. On repeat PCR to confirm results, one sample (rowi #3) which had amplified sequence A the first time subsequently amplified sequence B from the same template DNA, and one other (rowi #4, which had also failed to amplify with the kiwi gender-specific primers) failed to amplify the second time. The trimmed COI sequences ranged from 167 to 271 bases in length. On alignment, all A sequences were identical, as were all B sequences. On BLAST analysis, both A and B consensus sequences aligned most closely with sequences from GenBank identified as *E. aerophilus* (sequence A to KC341990 and sequence B to KC341989) with 100% cover but only ~86% pairwise identity (Table 3.3). Rowi capillarid COI sequence B showed a consistent five base gap when aligned with all other capillarid COI sequences obtained from this study and with capillarid COI sequences available in GenBank; possibly as a result of this, the three sequences designated as rowi capillarid COI sequence B were not accepted on submission to GenBank (aligned rowi capillarid COI sequences are presented in appendix F).

Seven out of 11 kahu samples produced amplicons of the appropriate size using the capillarid COI primers. Two distinct sequences with ~15% difference in base composition were produced, one from five birds (kahu capillarid COI sequence A), the other from two (kahu capillarid COI sequence B), which in six out of the seven birds was confirmed on repeat PCR (kahu #7 failed to amplify the second time) (Table 3.2). The two B sequences were identical. Of the five A sequences, one (kahu #5) included 4 individual degenerate bases, while another (kahu #7, which did not amplify on repeat PCR) showed a single base difference from the remaining three. Similar to the rowi sequences, BLAST analysis found no close matches; the kahu COI consensus sequence A aligned nearest to a GenBank sequence identified as *Capillaria aerophila* (JQ905059, syn. *E. aerophilus*) and the kahu COI consensus sequence B aligned nearest to a sequence identified as *E. boehmi* (KC341992), with only ~83 to 89% pairwise identity (Table 3.3).

The control capillarid of red-billed gull origin also produced an amplicon of appropriate size. On BLAST analysis, closest alignment was to a sequence from *E. boehmi* (KR186213) with only ~81% pairwise identity (Table 3.3).

3.4.3.5. Phylogenetic analysis

Maximum likelihood trees comparing the sequences generated in this study with near relatives from GenBank confirmed their grouping within the capillarid group. The 18S analysis (Figure 3.2) placed the study sequences within a clade containing several identical sequences identified as members of the *Eucoleus* genus (a list of all GenBank accessions included in the 18S tree is presented in appendix N). Other members identified as *Capillaria* genus also grouped together with the study sequences, which were distinct from additional capillarid genera (*Aonchotheca, Baruscapillaria, Calodium, Pearsonema, Pseudocapillaria*). Members of the *Trichuris* genus grouped separately from the capillarid sequences. Results strongly suggest that the study sequences belong in *Eucoleus*/*Capillaria.*

Unlike the 18S sequences generated in this study, which showed >99% similarity, the COI sequences were all relatively distinct from one another (79-90% similar). As expected from this, and a lack of close matches on BLAST analyses, each of the COI sequences were distinct from others and grouped within a diverse clade including several members of the capillarid group (Figure 3.3). These results support 18S findings by placing the study sequences within the capillarid group, separate from *Trichuris*. A species-level identification based on COI data could not be reached due to the lack of close matches in GenBank; however, results show sequences representing novel genotypes with levels of divergence consistent with other, distinct species in the capillarid group.

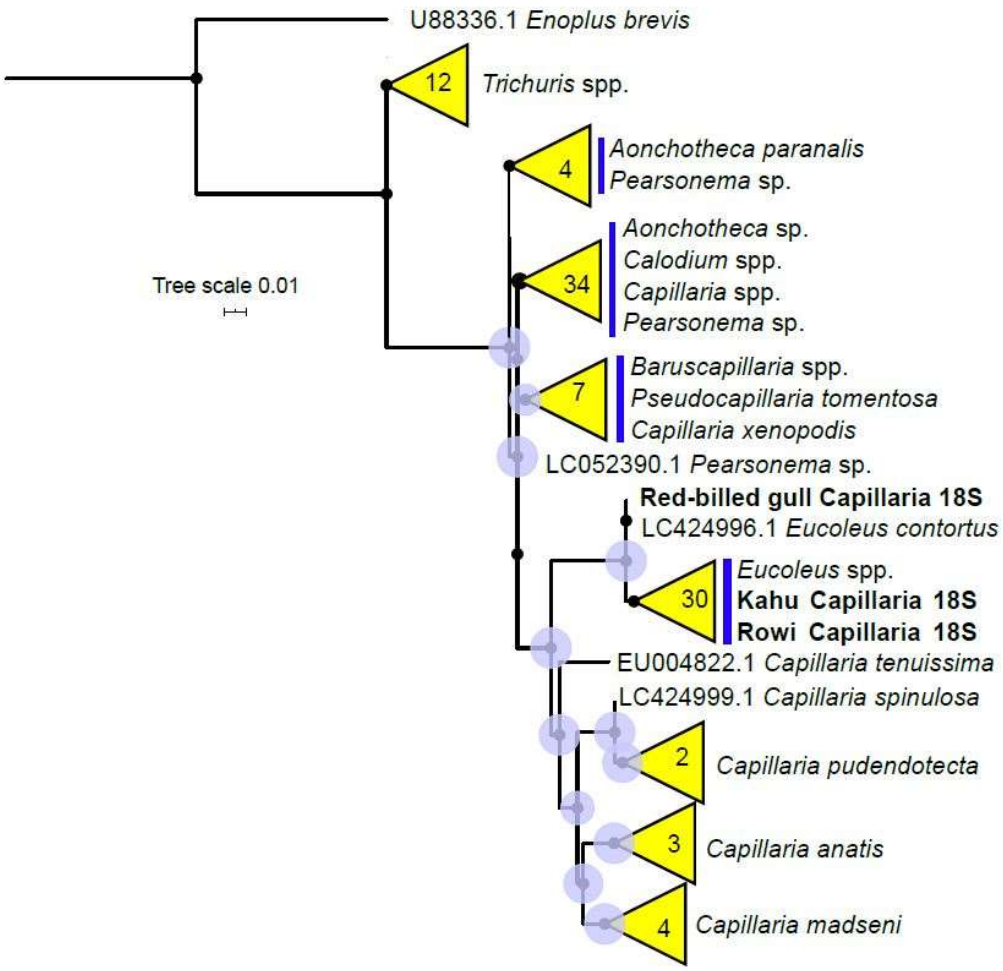

Figure 3.2. Maximum likelihood 18S phylogenetic tree of capillarid taxa recovered from rowi, kahu, and red-billed gull with GenBank representatives. Yellow triangles are collapsed nodes containing multiple closely related sequences (all GenBank accession numbers included within the uncollapsed 18S tree are presented in appendix N). Numbers within triangles represent the number of sequences. Blue vertical lines are used to partition multiple taxa present within collapsed nodes. Coloured nodes indicate branch support determined with an approximate likelihood ratio test (aLRT) at >80%. An *Enoplus brevis* 18S sequence was used as outgroup taxon.

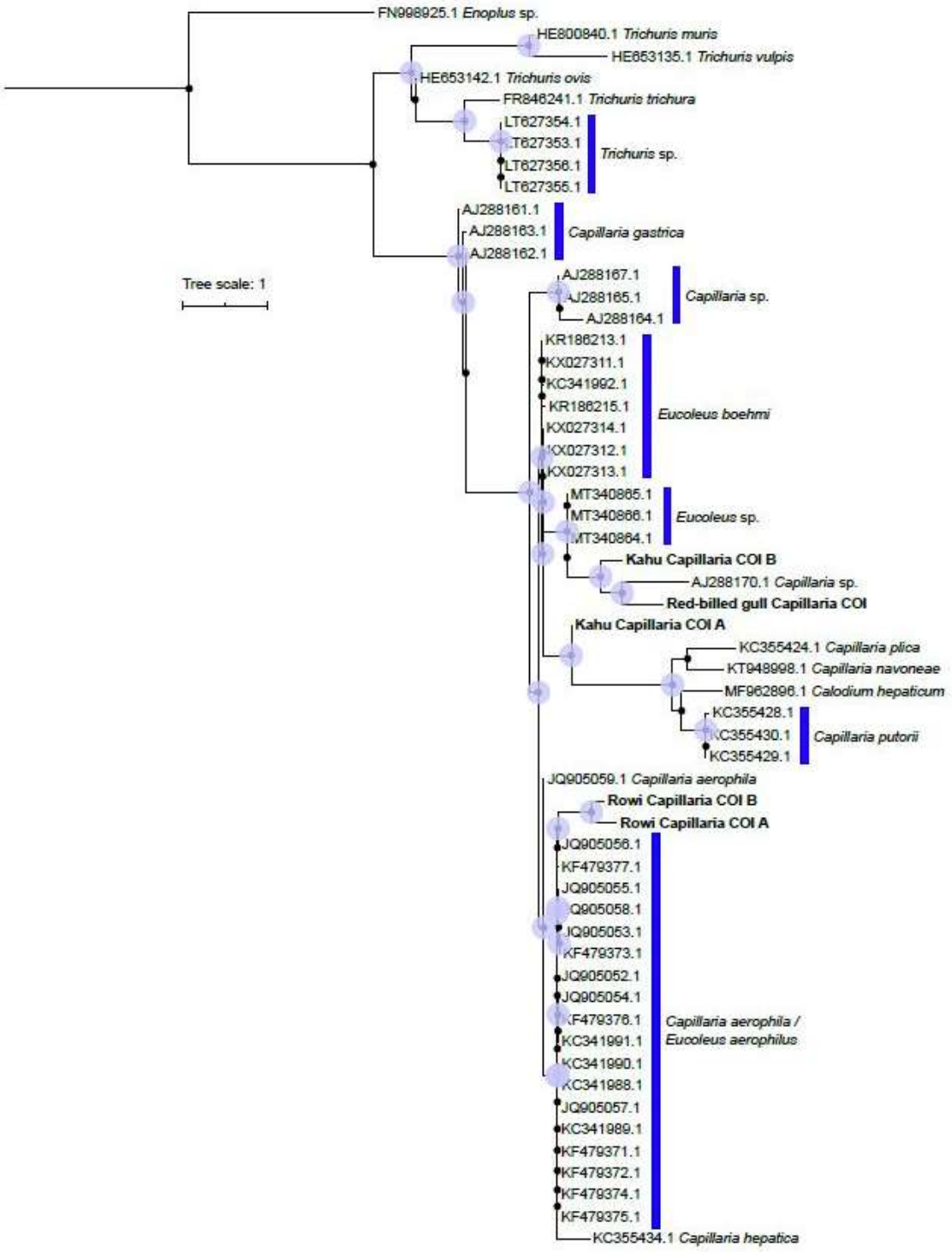

Figure 3.3. Maximum likelihood cytochrome oxidase *c* subunit I (COI) phylogenetic tree of capillarid taxa recovered from rowi, kahu, and red-billed gull with GenBank representatives. Blue vertical lines are used to show taxonomic information for multiple sequences. Coloured nodes indicate branch support determined with an approximate likelihood ratio test (aLRT) at >80%. An *Enoplus* sp. COI sequence was used as outgroup taxon.

3.5. Discussion

Cutaneous *larva migrans*, historically known as “creeping eruption”, can be broadly defined as the migration of a larval parasite in the skin of an abnormal host in which the parasite is unable to mature and complete its life cycle (Beaver, 1956; Caumes and Danis, 2004). Various nematode species originating from animal definitive hosts have been associated with cutaneous lesions in humans, including hookworms (e.g. *Ancylostoma* spp.) and threadworms (*Strongyloides* spp.), where the infective larvae, free-living in soil, penetrate and migrate within the skin. Cutaneous or subcutaneous lesions may also develop following oral infection and internal migration by other agents, for example cutaneous gnathostomiasis which is most commonly associated with the ingestion of infective *Gnathostoma* spp. larvae in raw or undercooked seafood. Larvae of the free-living nematode *Pelodera strongyloides* have also been reported to cause skin disease in humans (and, more commonly, dogs), again via percutaneous infection.

The skin disease in juvenile rowi cannot strictly be termed cutaneous *larva migrans* as the organisms identified histologically commonly included reproductively-active adults. In histological section, the nematodes lacked lateral cords, instead demonstrating hypodermal bacillary bands characteristic of the nematode class Adenophorea (aka Aphasmidia), as well as a stichosome associated with the oesophagus, a feature of the aphasmid superfamily Trichinelloidea that contains several important parasites of animals including the capillarid group (Gardiner and Poynton, 1999; Deplazes et al., 2016). Both histomorphology and molecular analysis in this case conclude that the nematode causing ventral dermatitis in juvenile rowi is a species of *Capillaria sensu lato* which, based on available sequences in GenBank, aligns among members identified as of the genus *Eucoleus*. The diagnosis of “cutaneous capillariasis” may therefore be more accurately applied to this disease.

The capillarids have a complicated and contentious taxonomy that has undergone numerous incarnations and revisions (Skrjabin et al., 1970; Moravec, 1982; Anderson and Bain, 2009). They have been classified as a subfamily, Capillariinae (e.g. Anderson and Bain, 2009), within the

Trichuridae family or a family, Capillariidae, in their own right (e.g. Moravec, 1982), and have at different times been consolidated into the single genus *Capillaria* or differentiated into up to 27 proposed genera (Gibbons, 2010), all with variable acceptance by contemporary taxonomists and authors. Over 300 species have been described, parasitising all classes of vertebrates. The lack of a universally accepted taxonomic scheme is a result, at least in part, of disagreements regarding the morphological delineations between proposed genera (Moravec, 1982; Anderson and Bain, 2009). For the purposes of this study, the term "capillarid" is used to describe species of *Capillaria sensu lato*.

Within their hosts, adult capillarids most commonly burrow within the lining of the gastrointestinal or respiratory tracts, but may also be found in liver, urinary bladder, and cutaneous sites (Yabsley, 2008; Anderson and Bain, 2009). Following the classification system proposed by Moravec (1982) up to 10 genera including *Eucoleus* have to date been identified in avian species (Yabsley, 2008; Tamaru et al., 2015), infecting sites within the gastrointestinal tract. To the authors' knowledge, cutaneous infections have not previously been reported in any host by species attributed to the *Eucoleus* genus. However, there are other capillarid species which do cause skin lesions in their hosts, e.g. *Pseudocapillaroides xenopi* (syn. *Capillaria xenopodis*) in South African clawed frogs (*Xenopus laevis*) (Iglauer et al., 1997), and *Paratrichosoma recurvum* in crocodiles (*Crocodilus moreletii*) (Charruau et al., 2017).

The previous PCR and sequencing results implicating a nematode aligning among *Trichostrongylus* spp. (Gartrell et al., 2015) were unable to be replicated. The reason for this is uncertain but raises the possibility of sample contamination in the original study. Molecular characterisation of the organisms present in rowi skin lesions in the current study found that sequences from the 18S gene of rDNA were 99.6 to 100% identical to sequences obtained from lesions of oral and crop capillariasis in kahu and from a capillarid nematode originating from the proventriculus of a red-billed gull, as well as from a variety of organisms identified as *Eucoleus*

spp. present in GenBank including some which parasitise the upper gastrointestinal tract of birds (*E. perforans*, *E. dispar*, and *E. contortus*) and some associated with mammalian hosts (*E. aerophilus* and *E. boehmi* from the respiratory tract, and *E. garfiai* in the tongue). These results support previous findings that the 18S region shows little genetic variability between closely related capillarid species (Guardone et al., 2013). This is, however, likely to have been exacerbated by the relatively short sequences obtainable from FFPE tissue samples in this study, as other studies which evaluated longer 18S sequences (~1800 bp) of capillarids did find sufficient variation to allow assessment of interspecific relationships within the group (Tamaru et al., 2015; Sakaguchi et al., 2020).

The COI gene of mtDNA is generally understood to show greater interspecies variation and has been applied as a target for genetic studies including DNA barcoding across a wide range of taxa; however, it is not without its own potential pitfalls (Derycke et al., 2010; Deagle et al., 2014). The capillarid COI PCR performed on rowi skin lesions produced two sequence types with greater than 10% divergence. The consistency of the two sequences between each bird and in duplicate PCR runs makes polymerase or sequencing errors unlikely. In no case did there appear to be co-amplification of the two sequences within the same reaction; however, in one (rowi #3) the same template DNA produced one of each sequence on duplicate PCR runs. The degree of divergence between the two sequences raises the possibility of two separate nematode species (Blouin, 2002), causing similar lesions within the same population and even, in the case of rowi #3, present in the same bird. While this is potentially feasible, it is considered biologically unlikely due to the small host population size and restricted geographical area in which the disease has been identified.

Among the concerns raised against the use of a single mitochondrial gene locus such as COI for barcoding purposes is the potential for over-estimation of species diversity: for example, due to heteroplasmy; gene duplication within the mitochondrial genome; or the integration of mtDNA

into the nuclear genome (Song et al., 2008; Derycke et al., 2010). The presence of non-functional copies of mtDNA within the nucleus (aka nuclear mitochondrial pseudogenes, or "numts") especially appears to be widespread among eukaryotes (Song et al., 2014). Following integration into the nucleus, the pseudogenes and their mitochondrial counterparts evolve independently and, apart from confounding barcoding studies, they have been found useful in phylogenetic analysis (Hay et al., 2004). Primer design may unwittingly favour the amplification of numts, where universal primers targeting relatively conserved regions of mitochondrial DNA are considered more likely to coamplify numts, but use of taxon-specific primers does not eliminate their presence entirely (Song et al., 2008; Moulton et al., 2010). Examination of the sequence composition can reportedly be used to identify the presence of pseudogenes, specifically the presence of indels, point mutations, and stop codons, but these are not always apparent even in relatively long COI sequences (Moulton et al., 2010).

COI pseudogenes do not appear to have been previously reported in nematodes, but other pseudogenes and gene duplications have been described within the mitochondrial genome of *Caenorhabditis* spp. (Raboin et al., 2010) and at least one parasitic nematode (Tang and Hyman, 2007). Presence of in-frame stop codons could not be confirmed in the relatively short sequences obtained from FFPE tissue in either rowi capillarid COI sequence A or B through translation using the invertebrate mitochondrial code by Geneious (v. 10.2.3). However, the rowi capillarid COI sequence B does include a five base gap evident when the sequence was aligned with other capillarid COI sequences, which could represent a microindel; a deletion such as this would lead to a reading frame shift. It is therefore tempting to speculate that rowi capillarid COI sequence B may reflect a numt or similar mitochondrial gene duplication/mutation, but techniques that may be of use to further investigate this hypothesis, for example next generation sequencing or isolation of mitochondrial DNA, are limited by the quality of DNA extracted from the available FFPE tissue samples as well as cost.

Two distinct COI sequences with ~15% divergence were also obtained from the kahu samples. In this instance the presence of two different species is more plausible. On repetition of the PCR, the two sequences were never identified in a single bird and there was no indication of co-amplification. One sequence was found in two out of the three tissue sections originating from the crop, while the other was identified in all of the oral samples and the remaining crop sample. In contrast to the two rowi capillarid COI sequences, on phylogenetic analysis the two kahu capillarid COI sequences did not cluster together, with the kahu capillarid COI B sequence aligning nearer to the sequence obtained from the red-billed gull capillarid. The relatively short COI sequences obtainable from FFPE tissue and lack of capillarid COI sequences of avian origin present in GenBank for comparison does limit interpretation of this observation.

In fact, nematode COI sequences in GenBank remain relatively sparse in general (Poon et al., 2017); at the time of investigation, COI sequences from *Eucoleus* spp. were few in number and all originated from mammalian hosts. This paucity of data may be a result, at least in part, of the difficulty in design of universal nematode COI primers given that the mtDNA of some nematode groups has been shown to have a significantly higher mutation rate than that of many other animal taxa (Blouin et al., 1995; Anderson et al., 1998; Ahmed et al., 2019). Although the capillarid COI sequences obtained from the rowi, kahu, and red-billed gull samples in this study do suggest the recognition of up to four (or potentially five) different capillarid species, no species-level identification was possible for any of these sequences through BLAST analysis. Additionally, while the 18S sequences obtained in this study clearly grouped with members identified as from genus *Eucoleus*, it is possible that this may also be an artefact of limited sequence material available for comparison, and as increased genetic data becomes available resolution of where these parasites belong among the *Capillaria sensu lato* may be possible.

As the specific identity of the nematode causing skin lesions in rowi could not be established, its origin remains uncertain. The juvenile rowi are raised on an island sanctuary where introduced

pests were eradicated in the 1990s and so the possibility of exposure to a nematode of mammalian origin is limited, although marine mammals are known to frequent the island. An avian parasite may be more likely as the borders of the island sanctuary remain porous to many flighted species. While *Eucoleus* species in birds typically parasitise the upper gastrointestinal tract, the atypical site in rowi, migrating within the epidermis around the vent, could be related to some biological peculiarities of kiwi; for example, kiwi lack a crop and have a lower resting body temperature and metabolic rate than most birds of a similar size (Sales, 2005). One report of infection of the lung and airsacs of a Peregrine falcon (*Falco peregrinus*) with nematodes identified morphologically as possible *E. aerophilus* suggests that infection of aberrant hosts, including infection of birds by a capillarid of primarily mammalian origin, may occur (Larrat et al., 2012).

The possibility of a novel capillarid species must also be considered. It is worth noting that the gastrointestinal parasites of kiwi themselves have been relatively poorly described. The presence of one or two novel species of *Capillaria* infecting kiwi has been previously postulated; however, the only published information, in the form of two conference abstracts (Clark, 1983a, b), is limited with no detail as to anatomical or geographical origin of the capillarids. No reports of the histological diagnosis of oral or oesophageal infection by capillarids in rowi or any other kiwi species were found in the SoVS pathology database, and skin lesions as seen in the crèche population of juvenile rowi have not been reported in rowi from other sites, nor in any other kiwi species. However, capillarid-type eggs are not uncommonly identified during routine faecal examinations from kiwi around the country, and are of uncertain origin and pathological significance. During the 2013 investigation of skin disease in rowi, seven of the 15 affected birds had capillarid-type eggs identified on faecal flotation (Gartrell et al., 2015) although their relationship to the cutaneous lesions remains unknown. The possibility that the juvenile rowi could have carried endemic parasites to the crèche island is low, however, given that they were

previously raised from eggs in a controlled environment, and the aberrant migration of a parasite originating from another host is a more likely scenario.

Whatever the original source, histological confirmation of reproductively-active female nematodes and eggs within the epidermis of the cutaneous lesions raises a possibility that the infection may be, or have potential to become, self-sustaining within the island population, as well as the potential for spread of the nematode if infected birds are transferred off the island crèche. For other capillarids inhabiting skin, eggs are laid within the epidermis and pass into the environment as the superficial layers are sloughed as part of natural (or accelerated) turnover of cells (Charruau et al., 2017). At the time of the 2013 outbreak, the rowi chicks were provided with artificial burrows which had not been moved or spelled for some years prior to the first observation of skin disease (Gartrell et al., 2015). In contrast to most other kiwi populations rowi tend to share burrows in small groups, and so contamination of the burrow soil and environs may lead to increased potential for transmission of infection between birds. Following the outbreak, it was recommended that the artificial burrows should be moved every six months and, anecdotally, the incidence of the skin disease has subsequently decreased.

The life cycles of different *Eucoleus* species are variably reported as direct (through oral ingestion of infective eggs) and/or indirect (requiring or utilizing an earthworm intermediate host) (Anderson, 2000; Yabsley, 2008). The potential for percutaneous infection by infective capillarid larvae has not been established, even in those species known to parasitise skin (Charruau et al., 2017). Oral infection with cutaneous invasion around the vent following migration from the intestinal tract seems the more likely route of infection in rowi, particularly as the necropsy finding of a larval nematode in the liver of one of the 2013 rowi cohort raises the possibility that some aberrant internal migration may also occur. Unfortunately, it was not possible to confirm through molecular analysis that this was the same organism as those in the skin, and the larval section included no distinguishing histomorphological features. However,

while visceral *larva migrans* caused by *Toxocara cati* has been reported in North Island brown kiwi (French et al., 2020b) this is not a valid differential in the juvenile rowi given that the crèche island has been free of the feline definitive host (*Felis catus*) for over 20 years and, similarly, the previous elimination of rodents and predatory mammals from the island reduce the possibility of hepatic capillariasis due to *Calodium hepaticum*.

Ideally, further investigation into the specific identification of the capillarids causing skin disease in juvenile rowi would benefit from the isolation of whole nematodes from lesional skin, for expert morphological examination as well as more extensive molecular analysis. However, as changes to the management of burrows on the crèche island appear to have reduced incidence of the disease, which is now well-recognised, future biopsy or necropsy specimens are unlikely to be forthcoming. Collection and comparative molecular analysis of capillarid nematodes originating from other species present on the crèche island may be of some benefit in identifying a potential original source of the infection. Additionally, molecular analysis of faecal samples containing capillarid eggs, both from the juvenile rowi and from other origins around New Zealand, may aid in expanding the currently sparse understanding of capillarid nematode infections in the kiwi.

3.6. Conclusion

Histomorphological and molecular studies indicate that the nematode causing skin disease in rowi is a species of *Capillaria sensu lato* and, with the caveat of limited GenBank sequences available for comparison, suggest a potential member of the *Eucoleus* genus. While two distinct capillarid COI sequences were obtained from the rowi samples, it is considered more likely to be a single species causing the lesions with possible concurrent amplification of a COI pseudogene. A species-level identification could not be reached, but comparison of molecular results suggests a different species to capillarid organisms derived from several kahu (Australasian harrier hawks) and one red-billed gull originating from elsewhere in New Zealand. The cutaneous nematode infections in rowi appear patent, raising the potential for perpetuation of infection in the

absence of the original source, and of translocation of the parasite if infected rowi are removed from the island.

Chapter 4: A prospective survey of gastrointestinal nematodes in kiwi (*Apteryx* spp.)

4.1. Abstract

Little is known about the common gastrointestinal nematodes of kiwi (*Apteryx* spp.). A prospective survey was undertaken; over a period of 18 months, the gastrointestinal nematodes from 50 kiwi presented for necropsy examination were collected along with histological samples of the gastrointestinal tract and selected organs to evaluate for evidence of tissue pathology in association with nematode presence, including any lesions suggestive of visceral and/or neural *larva migrans*. Results confirmed that gastrointestinal nematodiasis was common, with 94% of the kiwi examined infected to some degree, and at least five morphologically distinct nematode types were found. The most common sites parasitised were the gizzard and caeca, from which the recovered nematodes morphologically resembled the previously described species *Cyrnea apterycis* (Spirurida: Habronematoidea) and *Heterakis* (syn. *Kiwinema*) *gracilicauda* (Ascaridida: Heterakoidea) respectively. Additionally, lower prevalence and burdens of two different, unidentified nematodes from the order Spirurida were found originating in the proventriculus and the small intestine, along with rare infection by capillarid (Enoplida: Trichinelloidea) nematodes in the small intestine. Of concern, despite the recognition of three different types of spirurid nematodes, spirurid-type eggs were never identified on routine faecal flotation. Gastrointestinal histopathological changes included mild and localised to occasionally more severe and extensive inflammation associated with nematode invasion of the gizzard mucosa, and the formation of discrete mural nodules in the small intestine containing immature spirurids. Histological lesions suggestive of nematode *larva migrans* were identified in liver, lung, and/or brain of around 43% of kiwi in which visceral sections were examined, supporting previous supposition that this disease may be under-diagnosed.

4.2. Introduction

Kiwi (*Apteryx* spp.) are unique to and a national icon of New Zealand. However, despite potential implications to the health and management of kiwi, as well as a growing interest in the preservation of native parasites along with wildlife species (Gomez and Nichols, 2013), there is surprisingly little known about the resident gastrointestinal nematodes of kiwi or their pathological significance.

Only three nematodes have been formally identified from kiwi. The existence of the first of these, an ascarid nematode designated *Ascaris apterycis*, has not been confirmed since its initial published descriptions in the 1880s (Chatin, 1884, 1885). In contrast, *Cyrnea apterycis* from the gizzard and *Heterakis gracilicauda* from the caeca, both described some 90 years later (Harris, 1975), have been more widely acknowledged although generally earning only brief and/or incidental mentions in disease surveillance or case reports (e.g. Anonymous, 1978; Boardman, 1995; Alley and Gartrell, 2003; Alley et al., 2004b). Following these initial formal identifications, some progress was made in examining the diversity of gastrointestinal nematodes in kiwi: in the 1980s, Clark (1983a, b) presented findings that indicated the likely existence of multiple as yet undescribed nematodes including one or two additional species of *Cyrnea*; five additional, novel heterakoid species (some requiring the creation also of new genera); one or two novel capillarids; and potential new species of *Tetrameres* and *Primasubulura.* Additional to these multiple undescribed agents was the discovery of kiwi carrying ascarid nematodes acquired from other hosts: *Toxocara cati*, a parasite of the domestic cat (*Felis catus*); and *Porrocaecum ensicaudatum*, a parasite of blackbirds and thrushes (*Turdus* spp.) (Clark and McKenzie, 1982). However, with the exception of the subsequent re-evaluation and re-classification of *H. gracilicauda* into a proposed new genus, *Kiwinema* (Inglis and Harris, 1990), there has been no follow up to this potentially widely expanded diversity of gastrointestinal fauna, and few attempts to address the questions of prevalence in or potential pathogenicity to the kiwi hosts of such infections.

In one of his conference abstracts, Clark (1983b) does intimate that heterakoid nematode infections are generally considered benign while the spirurid infections are potentially harmful, the latter statement likely reflecting the fact that *Cyrnea* and related spirurid genera are known to burrow within the mucosa of the gizzard, proventriculus, or intestine of their avian hosts with variable associated pathology (Niemuth et al., 2013). Some reports that mention *C. apterycis* or unnamed spirurid gizzard infections in kiwi do confirm their presence within the koilin or superficial mucosa (e.g. Smith et al., 1973; Anonymous, 1978) but do not specifically comment on the presence or absence of associated pathology. There is a single report (Orr, 1995) describing histological evidence of ventriculitis in association with numerous nematodes disrupting the koilin layer, although in this case the agents were considered most likely to be *Acuaria* sp., members of a different spirurid family as may be commonly found in other ground-feeding birds such as finches (family Fringillidae); how this identification was reached is not detailed. Accounts of caecal heterakoid infections have also largely lacked any descriptions of a pathological reaction to their presence, with the exception of a single report in which there was histological evidence of a concurrent necrotising typhlitis (Alley and Gartrell, 2006).

A retrospective study of the prevalence of nematode infections in kiwi was recently undertaken, based on archived records from the National Wildlife and School of Veterinary Science (SoVS) pathology databases (Massey University, Palmerston North, New Zealand) between 1991 and 2012 (van Zyl, 2014). North Island brown (NIB) kiwi (*Apteryx mantelli*) were the most common species submitted to the facility for necropsy (551 of 699 cases) and were found to have a prevalence of ventricular nematodiasis of 26.3% and intestinal nematodiasis of 16.6% for kiwi in which these tissues were examined histologically and findings reported to the database (60 of 228 and 36 of 217 cases respectively). However, as diagnosis of gastrointestinal nematodiasis in this study was based solely on the reporting of nematodes in histological sections, results likely significantly underestimate the frequency of infection. Differentiation of small intestinal versus caecal infections was not made but given current knowledge of the nematodes recognised in

kiwi, it is probable that the majority of the positive cases were caecal. The presence or absence of any gastrointestinal pathology reported in relation to the presence of parasites was not addressed.

The retrospective survey of van Zyl (2014) was driven in part by increasing recognition of cases of nematode *larva migrans* (LM) in kiwi, which has brought attention also to the deficiencies in knowledge regarding their "normal" gastrointestinal nematode fauna. Molecular analysis performed on archived formalin-fixed, paraffin-embedded (FFPE) tissue sections has now established that *T. cati* is the likely causative agent of many cases of visceral and neural LM in NIB kiwi (French et al., 2020b; Chapter 2). By its strictest definition, LM is caused by the aberrant migration of larvae within the internal organs and brain of an abnormal host, in which the larvae are unable to mature to their adult form; however, as previously mentioned, there has also been a single report of both adult and larval forms of *T. cati*, as identified by detailed morphological examination, parasitising the small intestine of a kiwi (Clark and McKenzie, 1982). This finding raises speculation as to what exact role kiwi may play as host to this parasite and adds another dimension to the potential pathogenicity of gastrointestinal nematodiasis, in that associated pathological lesions may not necessarily be restricted to the gastrointestinal tract.

The purpose of this study was to investigate further the prevalence, diversity, and potential pathogenicity of the common gastrointestinal nematodes found in New Zealand kiwi, through parasitological and histopathological evaluation.

4.3. Methods

4.3.1. Collection of specimens

Over a period of 18 months, from February 2017 to October 2018, histological and parasitological specimens were collected from the gastrointestinal tract of kiwi submitted to the Wildbase pathology service (Massey University, Palmerston North, New Zealand) for necropsy examination.

The gastrointestinal tract from proventriculus to terminal rectum or cloaca, if not required for diagnostic purposes, was collected en bloc during necropsy. In some cases, sections of the tract had been opened for gross evaluation and/or sections taken for diagnostic purposes prior to the parasitological exam, in which case as much of the tract and contents that remained were collected. If not collected for diagnostic purposes, or in some cases in addition to diagnostic sampling, gastrointestinal sections were taken and fixed in 10% neutral buffered formalin for histological evaluation, originating from: proventriculus and ventriculus (gizzard), where possible including the proventricular-ventricular junction; duodenum (including pancreas); mid-small intestine (including Meckel's diverticulum (MD) where identifiable); ileum and caeca (consisting of a section through the proximal caeca including the terminal ileum centrally and in the majority of cases also additional sections from the distal caeca); and any grossly evident gastrointestinal lesions (e.g. mural nodules in the small intestine). Visual examination of internal organs for evidence of gross lesions was performed and reported as part of the diagnostic necropsy, and a formalin-fixed section of liver and, if available, lung and/or brain was collected either additional to or in lieu of diagnostic sections.

The gastrointestinal tract was opened in sections. The luminal contents were collected and the mucosa scraped with a scalpel blade for each of the following: proventriculus, gizzard, duodenum, jejunum, ileum, and caeca; small intestinal and caecal mucosal scraping was not consistently performed, particularly in the initial cases as the protocol was being developed and, after that, depending on the degree of tissue autolysis. After the initial eight necropsies, gizzard contents and mucosal scrapings were collected separately where possible, to assess whether different nematodes may be present free in the lumen versus those found embedded in the koilin, and samples from the small intestine were collected separately from the three sections with divisions defined as follows: duodenum as the loop of small intestine surrounding the pancreas; jejunum up to MD; and ileum as the remainder of small intestine to the caecal junction. Contents from both caeca were collected together.

The material collected from each segment of the tract was examined under a dissecting microscope (Nikon SMZ-U, Japan) and, where present, as many nematodes as possible collected and placed into 70% ethanol for potential future morphological and/or molecular analysis. Because nematode collection was invariably incomplete (due to sections taken for histological evaluation, contents lost during diagnostic examination, and, in some cases, time constraints), the number of nematodes present in gizzard and caeca is described in very broad categories of few (<20), moderate (20-100), or many (>100). If available, faecal samples were collected for routine faecal flotation either from the cloaca or, if no faecal material was present in the cloaca, from the rectum. Due to its short length and the frequency in which contents were harvested for faecal flotation, parasitological examination of the rectal contents for nematodes was excluded from the protocol.

4.3.2. Evaluation of gastrointestinal nematodes

4.3.2.1. Light microscopy

Low magnification morphological evaluation of nematodes was performed under the dissecting microscope at the time of collection. Notes were made where nematodes were identified on initial collection that were obviously, or suspected to be, different from the majority of "typical" nematodes being found at that site. Closer morphological evaluation of selected, individual parasites was performed by light microscopy (Olympus CX 41, Japan), with or without prior clearing using lactophenol. Small nematodes (e.g. those originating from gizzard and caecum), were placed on a glass slide within a drop of 70% ethanol, or covered by a large drop of lactophenol, and a coverslip placed over the top; those within ethanol were examined immediately, while those placed into lactophenol were left for a period of several hours to days to clear prior to examination. Large nematodes were placed into small jars filled with lactophenol and left to clear for several weeks prior to evaluation. Digital photographs were taken using a microscope digital camera (Olympus DP22, Japan). Morphology was compared with the available published formal descriptions of kiwi nematodes (Chatin, 1884, 1885; Harris, 1975; Inglis and Harris, 1990).

4.3.2.2. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed by the Massey Microscopy and Imaging Centre (Massey University, Palmerston North, New Zealand). Ethanol-fixed specimens were submitted and were transferred to 100% ethanol overnight before being critical point dried using liquid CO_2 as the CP fluid and 100% ethanol as the intermediary (Polaron E3000 series II critical point drying apparatus). The samples were mounted onto aluminium stubs and sputter coated with approximately 100 nm of gold (BAL-TEC SCD 005 sputter coater) and viewed in a FEI Quanta 200 scanning electron microscope at an accelerating voltage of 20 kV.

4.3.2.3. Faecal flotation

Routine diagnostic faecal flotation was performed by the SoVS parasitology laboratory (Massey University, Palmerston North, New Zealand). Flotations were performed using 0.5 g of the submitted faeces or rectal contents (or the entire sample if less than this amount was available), mixed with a 33% zinc sulphate solution (specific gravity of 1.18-1.20), and passed through a fine mesh sieve to remove coarse particulate matter. The filtrate was transferred to a 15ml centrifuge tube which was filled to establish a slight positive meniscus and a coverslip placed on top, and the tube was centrifuged at 314 g for five minutes. The coverslip was then removed and placed on a glass microscope slide for examination. Only those findings pertaining to animal-parasitic nematode eggs are considered in the results.

Following the results of the initial survey, an investigation of alternative faecal flotation solutions was performed on a single, pre-treatment faecal sample submitted to the parasitology laboratory from an unwell juvenile male NIB kiwi with endoscopically confirmed gizzard nematode infection. Multiple additional faecal flotations were performed as above, trialling solutions of saturated sugar (specific gravity within the range of 1.24-1.25) and graded concentrations of zinc sulphate (60%, 68%, and 80%, with corresponding specific gravities of 1.30, 1.318, and 1.385).

4.3.2.4. Histological evaluation

The tissue sections fixed in 10% formalin were trimmed and processed routinely, and 4 µm sections cut, mounted on slides, and stained with Haematoxylin and Eosin (H&E) by the SoVS histology laboratory (Massey University, Palmerston North, New Zealand) to assess for the presence of nematodes within the section, along with their histomorphology and any evidence of specific histopathology in relation to their presence. For cases in which diagnostic histology was performed the written reports were reviewed and, if relevant lesions were described, the diagnostic slides were also sourced and examined. Necropsy cases with potentially significant histology findings related to gastrointestinal nematode infection that occurred during the same time period, but in which parasitological exam was not performed, were also reviewed.

4.4. Results

4.4.1. Cases

Over the period of 18 months, samples from a total of 50 kiwi were examined (Table 4.1). Sample collection was not complete in every case, particularly during the beginning of the collection period as the protocol was being developed and finalised, but also for several cases where the post-mortem conditions affected the completeness or quality of sample collection (e.g. severe intestinal autolysis, sections of gastrointestinal tract missing due to predation) or where collection of samples for diagnostic purposes took precedent.

The majority of kiwi examined (41/50, 82%) were identified as NIB kiwi, originating from various regions within the North Island as well as three from captive facilities in the South Island. Of the 38 NIB kiwi originating from the North Island: 17 were submitted from Northland with one from the adjacent Auckland region; 12 were from the East Coast (six each from Bay of Plenty and Hawkes Bay regions); three originated from the West Coast (Taranaki region); a further three originated from the Manawatu-Whanganui region; and there was one each from South Waikato and the Coromandel Peninsula (Figure 4.1). Additional to the NIB kiwi, there were seven little spotted kiwi (LSK), one from an island sanctuary and six from a mainland sanctuary in the North

Island, and one each of a juvenile Haast tokoeka (HT) from a South Island reserve and a juvenile rowi from an island sanctuary (crèche).

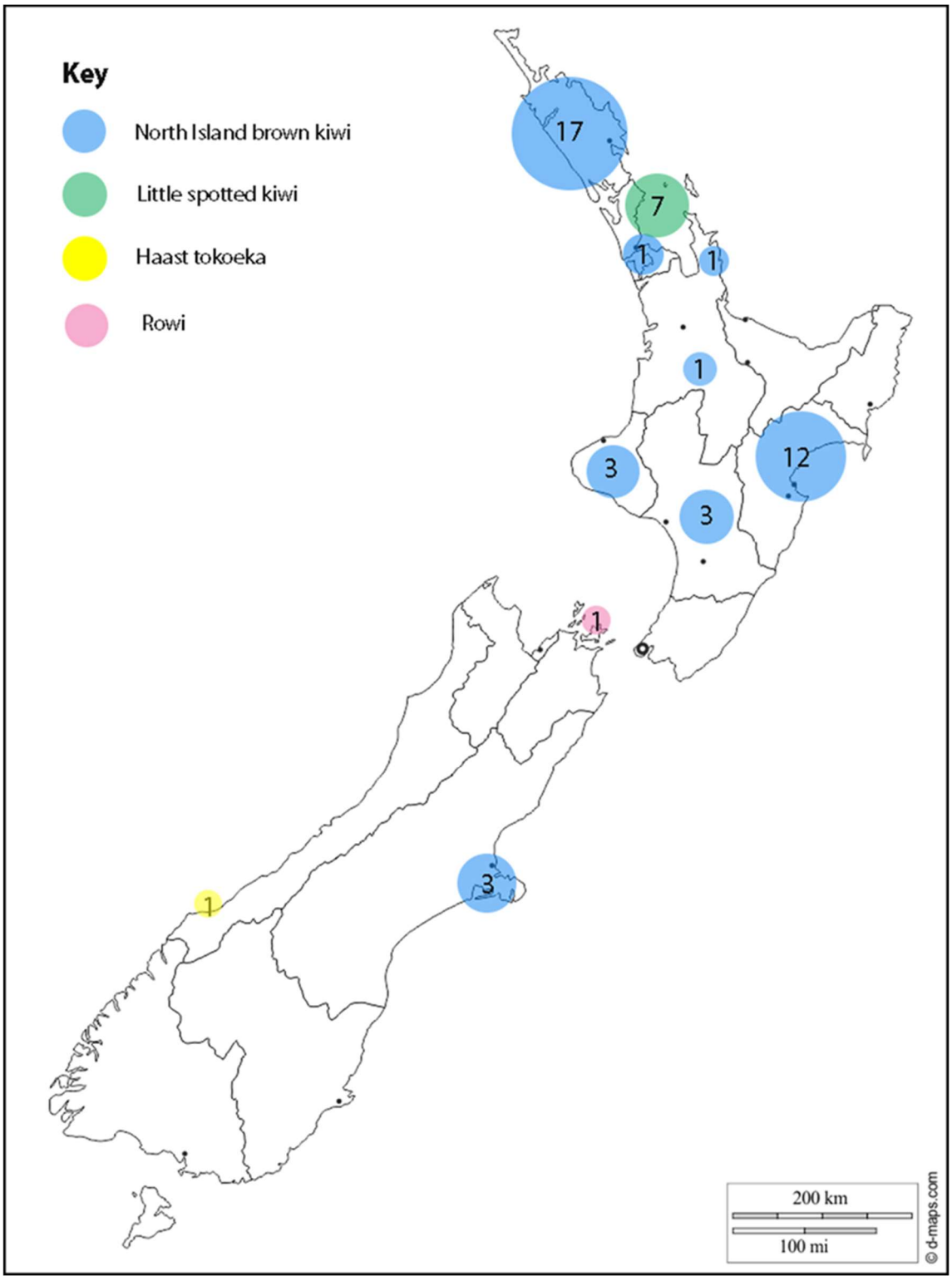

Figure 4.1. Map of New Zealand showing the regional and frequency distributions of the kiwi included in this survey. Created by Brett Gartrell.

Table 4.1. Signalment and origin data of kiwi included within this study, along with faecal flotation and gross parasitological examination results. For signalment data: NIB = North Island brown; HT = Haast tokoeka; LSK = little spotted kiwi; J = juvenile (e.g. out of burrow and up to 180 days of age); SA = subadult (e.g. 180 days to 3.5 years or first breeding); A = adult (e.g. reproductively active or >3.5 years); D = days; M = months; Y = years; ? = unknown. For origin data: Sanctuary = predator-free habitat (including crèches); Captive = captive facility/zoo; Wild = areas practicing no to variably extensive (but incomplete) predator control. For parasitological results: X = not performed; PV = proventriculus; G = gizzard; SI = small intestine; C = caeca; - = negative result (no eggs or nematodes found); + = few (<20); ++ = moderate (20-100); +++ = many (>100); (+) = the only nematodes found were typical for an adjacent site (likely displaced).

Kiwi #	Species	Age	Sex	Region	Origin	Faecal flotation (nematode eggs/g)	Nematode counts			
							PV	G	SI	C
1	NIB	J (4M)	M	Hawkes Bay	Sanctuary	-	X	+++	-	+++
2	NIB	J (39D)	F	Hawkes Bay	Sanctuary	-	-	-	-	++
3	NIB	J (78D)	F	Hawkes Bay	Sanctuary	4 Heterakis	-	+	-	-
4	NIB	SA	M	Northland	Wild	-	-	+	-	-
5	LSK	A (20+Y)	F	Auckland	Sanctuary	X	-	+	-	-
6	NIB	?	?	Tongariro	Wild	-	-	+	-	-
7	NIB	SA	M	Bay of Plenty	Wild	2 Ascarid + 2 Capillaria	-	++	-	+
8	NIB	SA	M	Taranaki	Wild	X	X	++	-	++
9	NIB	SA (10M)	F	Auckland	Wild	-	-	+	+[b]	-
10	NIB	A	F	Bay of Plenty	Wild	X (soil contamination)	-	+	-	+++
11	NIB	J	M	Bay of Plenty	Captive	X	-	++	-	+++
12	NIB	A	F	South Island	Captive	X	-	-	-	+++
13	NIB	A	F	South Island	Wild	X	-	++	-	+
14	NIB	A	M	Hawkes Bay	Wild	2 Ascarid	(+)	++	-	+++
15	NIB	SA	F	Coromandel	Wild	396 Ascarid + 14 "Pinworm"	+	++[a]	+[b]	+++
16	NIB	J	?	Bay of Plenty	Wild	X (soil contamination)	-	++	X	X
17	NIB	A (5Y)	M	Hawkes Bay	Wild	-	-	++	-	-
18	NIB	J	?	Northland	Wild	X	-	-	-	-
19	NIB	J	M	Northland	Wild	6 Ascarid, 2 Strongylid	-	++	(+)	+++
20	HT	J (6W)	F	South Island	Wild	30 Heterakis + 2 Capillaria	-	-	(+)	+++
21	NIB	J (4M)	F	Taupo	Sanctuary	-	-	+++	+[c]	+
22	NIB	A	F	Northland	Wild	-	-	++	+[b]	+
23	NIB	A	F	Northland	Wild	2 Capillaria	-	++	+[b]	++

[a]both proventricular-type and gizzard-type nematodes identified; [b]large spirurids; [c]capillarids; [d]unidentified/suspected environmental nematodes

Table 4.1 continued.

Kiwi #	Species	Age	Sex	Region	Origin	Faecal flotation (nematode eggs/g)	Nematode counts			
							PV	G	SI	C
24	NIB	A	M	Northland	Wild	-	-	+[b]	+[b]	+
25	NIB	A	F	Northland	Wild	-	-	++	+[b]	+
26	NIB	A (3Y)	F	South Island	Captive	5 Heterakis	-	-	-	+++
27	NIB	SA	F	Wairarapa	Wild	X	-	++	X	X
28	NIB	J (7W)	M	Hawkes Bay	Sanctuary	-	-	-	-	-
29	NIB	SA	F	Northland	Wild	-	-	++	+[c]	-
30	NIB	A (8Y)	M	Tongariro	Wild	17 Heterakis	-	++	(+)	+++
31	LSK	A	F	Auckland	Sanctuary	-	-	-	-	+
32	LSK	SA	F	Auckland	Sanctuary	-	-	++	(+)	++
33	LSK	SA	M	Auckland	Sanctuary	X	-	++	-	-
34	LSK	A	F	Auckland	Sanctuary	X	-	-	-	-
35	NIB	SA	M	Northland	Wild	-	-	++	-	+++
36	NIB	SA	F	Northland	Wild	50 Heterakis	+	+++	+[b]	+++
37	NIB	SA	M	Northland	Wild	340 Heterakis + 6 Capillaria	+	+++	+[b]	+++
38	Rowi	J	?	South Island	Sanctuary	2 Capillaria	-	+	-	-
39	NIB	SA	F	Northland	Wild	2 Heterakis + 2 Capillaria	-	+	-	+++
40	NIB	SA	M	Northland	Wild	14 Heterakis	-	+	-	+++
41	NIB	SA	F	Taranaki	Wild	?Heterakis larvae	-	++	(+)	++
42	NIB	SA	F	Northland	Wild	X	-	+++	-	-
43	NIB	A	M	Bay of Plenty	Wild	4 Heterakis eggs + ?larvae	-	+	-	-
44	NIB	A	F	Bay of Plenty	Wild	6 Capillaria	-	++	X	+
45	LSK	SA	M	Auckland	Sanctuary	X	X	++	-	-
46	NIB	SA (1Y7M)	M	Taranaki	Wild	56 Capillaria + 28 Heterakis	-	+++	-	+++
47	NIB	SA	M	Northland	Wild	-	+	+++[a]	(+)	+++
48	NIB	A	F	Northland	Wild	76 Heterakis	+	+[a]	+[b]	+++
49	LSK	A	M	Auckland	Sanctuary	-	-	+	+[d]	-
50	NIB	SA	M	Northland	Wild	150 Heterakis eggs + ?larvae	(+)	++	+[b]	+++

[a]both proventricular-type and gizzard-type nematodes identified; [b]large spirurids; [c]capillarids; [d]unidentified/suspected environmental nematodes

Thirty-four of the 50 total kiwi (68%) were categorised as wild, living free within sites that practice no to variably extensive (but incomplete) predator control; thirteen (26%) originated from predator-proof sanctuaries; and three (6%) were from captive facilities. Ages were reported in the database as juvenile (11/50; 22%), subadult (20/50; 40%), or adult (18/50; 36%). The youngest juvenile kiwi for which the age was specified was 39 days, and the oldest adult was a LSK that was over 20 years of age at the time of death; however, for most cases a precise age was not known and the age categorisation was subjectively based on necropsy examination findings (primarily body size and the gross appearance of the gonads). Twenty-five of the 50 kiwi (50%) were females and 21 (42%) males, while in three cases (6%, all juvenile kiwi) the sex could not be determined (or was not described) at necropsy. In one case (#6) neither age nor sex data were recorded in the database.

Most kiwi (47/50; 94%) carried some burden of gastrointestinal nematodes. Only three (6%) had no nematodes identified (#18, 28, and 34; Table 4.1), however, in several other cases the total burden was very low, with 10/50 kiwi (20%, all age groups represented) having few (<20) total nematodes including three with only a single nematode found in either gizzard or caecum. Nematode infections were most frequently encountered in the gizzard and the caeca, with co-infections of gizzard and caecal nematodes found in 27/48 kiwi (~56%) for which both were available for gross evaluation (Table 4.1).

4.4.2. Proventriculus

4.4.2.1. Prevalence

The proventriculus was typically empty of contents apart from scant mucoid material overlying the mucosa, and nematodes were never evident grossly. Out of 47 kiwi from which proventricular mucosal scrapings were examined, seven (~15%) contained nematodes, which were all few in number (less than 20 nematodes in total and all but one less than 10) (Table 4.1). In two of the seven cases, only two to three nematodes were found that were morphologically compatible with the nematodes found commonly in the gizzard (see section 4.4.3.2), considered

likely to represent displacement from this site. The other five cases included nematodes that were morphologically distinct from those of gizzard origin, in some cases in conjunction also with those that did resemble gizzard-type nematodes. All were subadult or adult NIB kiwi with four of the five originating from Northland and the fifth from the Coromandel; of note, the four Northland birds consisted of two sets of two birds (#'s 36 and 37, and #'s 47 and 48) each of which had been submitted at the same time from the same location.

4.4.2.2. Morphology

The recognition of proventricular-type nematodes was based primarily on the presence of those that were longer (up to ~1.2 cm in length) than the gizzard-type nematodes (see section 4.4.3.2), with an overall more slender body diameter (~130-170 µm) and a tail that tapered to a long point (Figure 4.2B & D); on closer examination several of these worms were confirmed to be adult females by the presence of eggs identified on microdissection. Eggs measured ~50-55 x 25-30 µm, were larvated, and had a distinct, asymmetrical ovoid shape with broad, flattened caps at each pole (Figure 4.2E). While alae were not obvious under light microscopic examination (Figure 4.2A), on SEM low bilaterally symmetrical alae (or longitudinal ridges) were apparent along the anterior body (Figure 4.2C). The presence of morphologically distinct, proventricular-type male nematodes was not discerned by superficial morphological examination. Egg morphology and oral conformation of the adult female, as far as could be examined on SEM, were considered consistent with a nematode of the order Spirurida. No further attempt at morphological identification was made.

4.4.2.3. Histology

Mild to severe autolytic change, predominantly affecting the superficial proventricular mucosa, was noted throughout the cases. Of 46 kiwi in which proventriculus was evaluated histologically, only three (~7%) contained nematodes within the examined section(s) (Table 4.2). This included one of the five cases in which the proventricular-type nematodes were identified on gross evaluation (#36), in which several cross- and oblique sections were identified histologically,

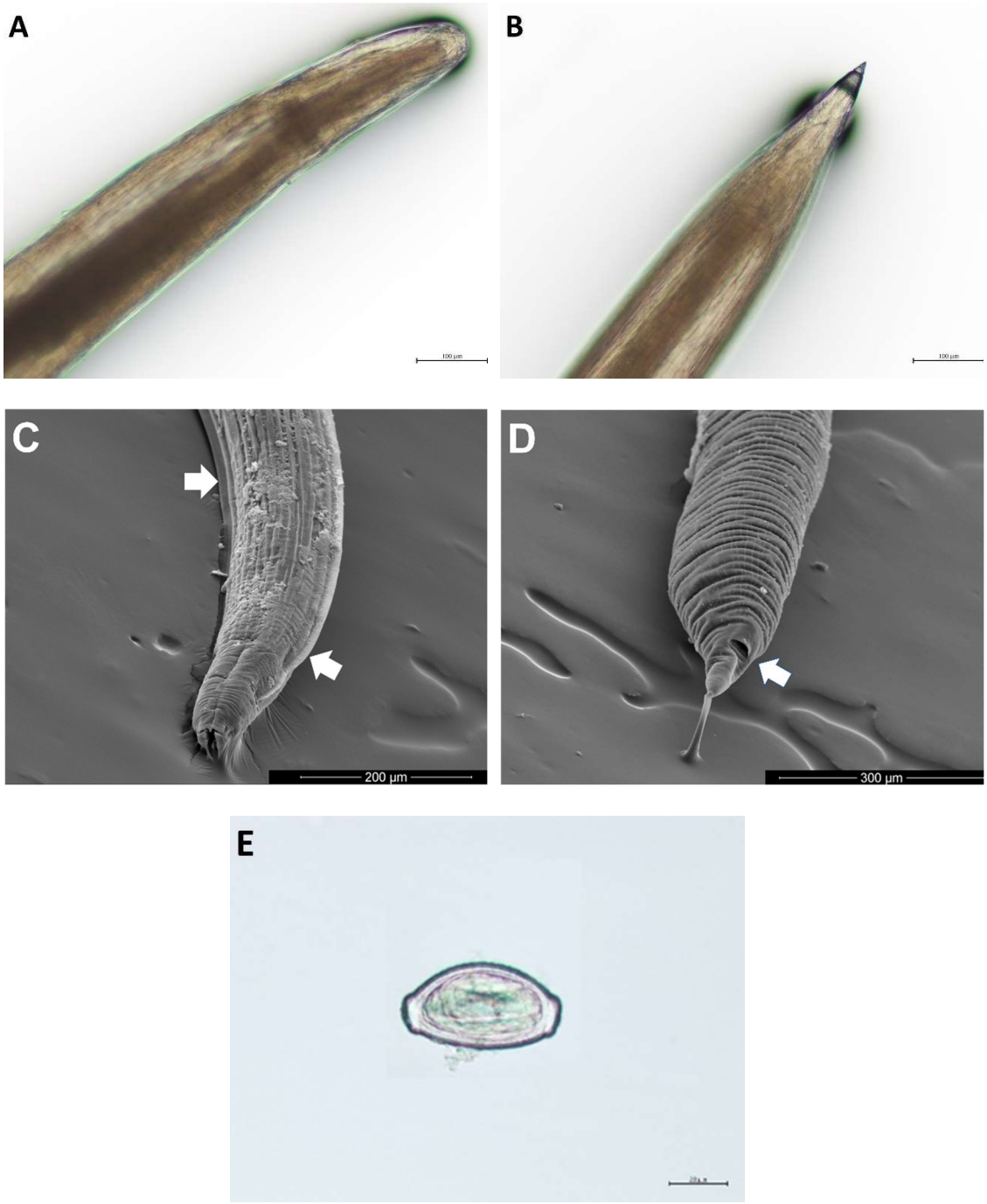

Figure 4.2. Adult female proventricular-type nematode. A: Head (Uncleared specimen, bar = 100 µm). B: Tail (Uncleared specimen, bar = 100 µm). C: Anterior body demonstrating low bilateral alae (arrows) – note that some adhesive artefact distorts the oral features (SEM, bar = 200 µm). D: Tail demonstrating subterminal orifice (arrow) – note adhesive artefact between tip of tail and surface (SEM, bar = 300 µm). E: Egg, as dissected from uterus (bar = 20µm).

including one suspected larval nematode that appeared coiled and embedded within the superficial mucosa, surrounded by a moderate lymphocytic inflammatory infiltrate (Figure 4.3A). Several separate cross-sections were also evident, considered consistent with larval

Table 4.2. Histological results including comparative presence of nematodes identified grossly versus in histological section (PV = proventriculus; G = gizzard; SI = small intestine); the identification of lesions within organs consistent with or suggestive of nematode *larva migrans*; and cause of death per the final necropsy report. X = not performed; - = negative result; + = positive result; ? = possible positive result (not conclusive); (+) = positive result but the only nematodes present were not morphologically typical for the location.

Kiwi #	GI nematodes (Gross/Histological)				*Larva migrans* (Histological)			Cause of death
	PV	G	SI	Caeca	Liver	Lung	Brain	
1	X/X	+/+	-/X	+/X	-	-	-	Avian malaria
2	-/-	-/+	-/-	+/+	-	-	-	Open
3	-/-	+/-	-/-	-/-	-	-	-	Drowning
4	-/-	+/(+)[a*]	-/+[b]	-/-	-	-	-	Asphyxiation
5	-/-	+/-	-/-	-/-	-	-	-	Open
6	-/-	+/-	-/-	-/-	-	-	-	Trauma
7	-/-	+/+	-/-	+/-	?	+	+	Predation
8	X/-	+/+	-/-	+/+	-	-	-	Trauma
9	-/-	+/+	+/-	-/?(+)	-	-	-	Predation
10	-/-	+/-	-/-	+/+	+*	-	-	Open
11	-/-	+/-	-/-	+/ova	-	-	X	Pneumonia
12	-/-	-/+	-/-	+/+	-	-	-	Perforated oviduct
13	-/-	+/+	-/-	+/-	-	-	X	Open
14	(+)/-	+/+	-/-	+/+	-	X	-	Predation
15	+/-	+/-	+/-	+/-	+	+	+	Predation
16	-/-	+/-	X/X	X/X	-	-	-	Trauma
17	-/-	+/+	-/X	-/-	-	-	X	Predation
18	-/-	-/-	-/X	-/X	+*	X	X	Predation
19	-/-	+/-	-/?+	+/+	-	-	-	Predation
20	-/-	-/-	-/-	+/-	-	-	-	Trauma
21	-/(+)	+/+	c/-	+/+	-	-	-	Avian malaria
22	-/-	+/+[a]	+/(+)[a*b]	+/(+)[a*]	+	?	-	Predation
23	-/-	+/+	+/-	+/+	+	+*	+	Predation
24	-/-	(+)/+	+/-[b]	+/-	+	-	-	Predation
25	-/-	+/+	+/-	+/-	+	-	-	Predation
26	-/-	-/-	-/-[b]	+/+	+	?	X	Pneumonia
27	-/X	+/X	X/X	X/X	X	X	X	Predation
28	-/-	-/-	-/-	-/-	-	-	-	Coccidiosis
29	-/-	+/+	c/-	-/?+	-	-	-	Trauma
30	-/-	+/-	(+)/X	+/X	-	X	X	Predation
31	-/-	-/-	-/-	+/-	-	-	-	Avian malaria
32	-/-	+/+	-/-	+/-	-	-	X	Avian malaria
33	-/-	+/-	-/-	-/-	-	-	X	Avian malaria
34	-/-	-/-	-/-	-/-	-	-	X	Avian malaria
35	-/-	+/+	-/-	+/+	-	-	-	Predation

[a]gastrointestinal mural granuloma(s) present histologically; [b]mural nodules in small intestine identified grossly; *larva identified within gastrointestinal mural or organ granuloma

Table 4.2. Continued.

Kiwi #	GI nematodes (Gross/Histological)				*Larva migrans* (Histological)			Cause of death
	PV	G	SI	Caeca	Liver	Lung	Brain	
36	+/+	+/+	+/-	+/+	+	+*	+*	Predation
37	+/-	+/+	+/+[b]	+/+	-	+	X	Predation
38	-/-	+/-	-/-	-/-	-	-	X	Trauma
39	-/(+)[a*]	+/+[a]	-/-	+/+	+	+	X	Predation
40	-/-	+/-	-/(+)[a*]	+/+[a]	+	?	?	Trauma
41	-/-	+/+	-/-	+/+	-	+	?	Drowning
42	-/-	+/+	-/-	-/-	-	-	-	Trauma
43	-/-	+/-	-/-	-/-	-	-	-	Predation
44	-/-	+/-	X/X	+/X	-	-	-	Predation
45	X	+/+	-/-	-/-	-	-	-	Drowning
46	-/-	+/+	-/-	+/+	-	-	-	Predation
47	+/-[a]	+/-	-/-	+/+	+	-	-	Predation
48	+/-	+/+	+/+[b]	+/+	+	-	-	Predation
49	-/-	+/-	-/-	-/-	-	-	-	Cardiac disease
50	(+)/-	+/-	+/+[b]	+/-	+	+*	-	Predation

[a]gastrointestinal mural granuloma(s) present histologically; [b]mural nodules in small intestine identified grossly; *larva identified within gastrointestinal mural or organ granuloma

forms, of relatively small diameter (~75-90 µm), with prominent lateral cords, coelomyarian musculature, no alae or other identifying cuticular features and no sex organs within the sections available for evaluation (Figure 4.3B).

Two further cases (#15 and #37) in which proventricular-type nematodes had been identified grossly did also include one or more focus of chronic-active inflammation surrounding an empty space within the superficial mucosa, raising the possibility of a localised response to the previous presence of a nematode, although some other cause e.g. a penetrating foreign body reaction could not be ruled out and deeper sectioning to look for parasitic elements was not performed; however, similar lesions were not evident in other proventricular sections (Figure 4.3C & D).

Of the other two kiwi in which nematodes were present in proventricular sections histologically, one case (#21) included only the single cross-section of a nematode for which the histomorphology was compatible with an adult male as was commonly identified in gizzard histology (see section 4.4.3.3); the nematode section was present above the mucosal surface of

the proventriculus and therefore could reflect physical displacement/contamination of the proventricular section at the time of or after sample collection.

In the third case (#39) the proventricular section contained multifocal discrete granulomas within the superficial and deep mucosa, consisting of central accumulation of eosinophilic, necrotic cellular debris surrounded by epithelioid macrophages, multinucleated giant cells, and other mononuclear inflammatory cells (Figure 4.3E). In one of the superficial mucosal granulomas, an oblique section of a small larval nematode (~11 µm diameter) was present (Figure 4.3F); no alae or other distinguishing histomorphological features were apparent. It was also noted in one further case (#47) that a focal similar granuloma was present within the deep proventricular mucosa but with no larvae identified.

Apart from these specific cases, some degree of mucosal inflammation was commonly present throughout the proventricular sections with no obvious distinction in its presence or severity between cases in which proventricular nematodes were identified grossly and those without. This was predominantly lymphocytic in nature and variably minimal to moderate and multifocal to regional within the superficial mucosa, and mild to moderate multifocally, or sometimes focally restricted to a single gland, within the deeper mucosa.

4.4.3. Gizzard

4.4.3.1. Prevalence

Gizzard contents were variable in amount and composition, typically including some combination of stones, remnants of chitinous insects, and plant material. In some cases, nematodes were visible grossly (or with use of a handheld magnifying glass) partially embedded within the koilin lining (Fig. 4.4). Nematodes harvested from both gizzard contents and mucosal scrapings were, in the vast majority of cases, morphologically similar under the dissecting microscope and so total counts are combined (Table 4.1). Subjectively, the numbers of nematodes present in gizzard contents appeared to increase in association with autolytic change, primarily the breakdown of the koilin layer of the mucosa which was often very friable,

partially detached, or virtually non-existent in the most poorly-preserved specimens, while in fresher specimens a deep scrape of the mucosa was required with careful teasing apart of the rubbery, intact koilin under the dissecting microscope to free tightly embedded worms.

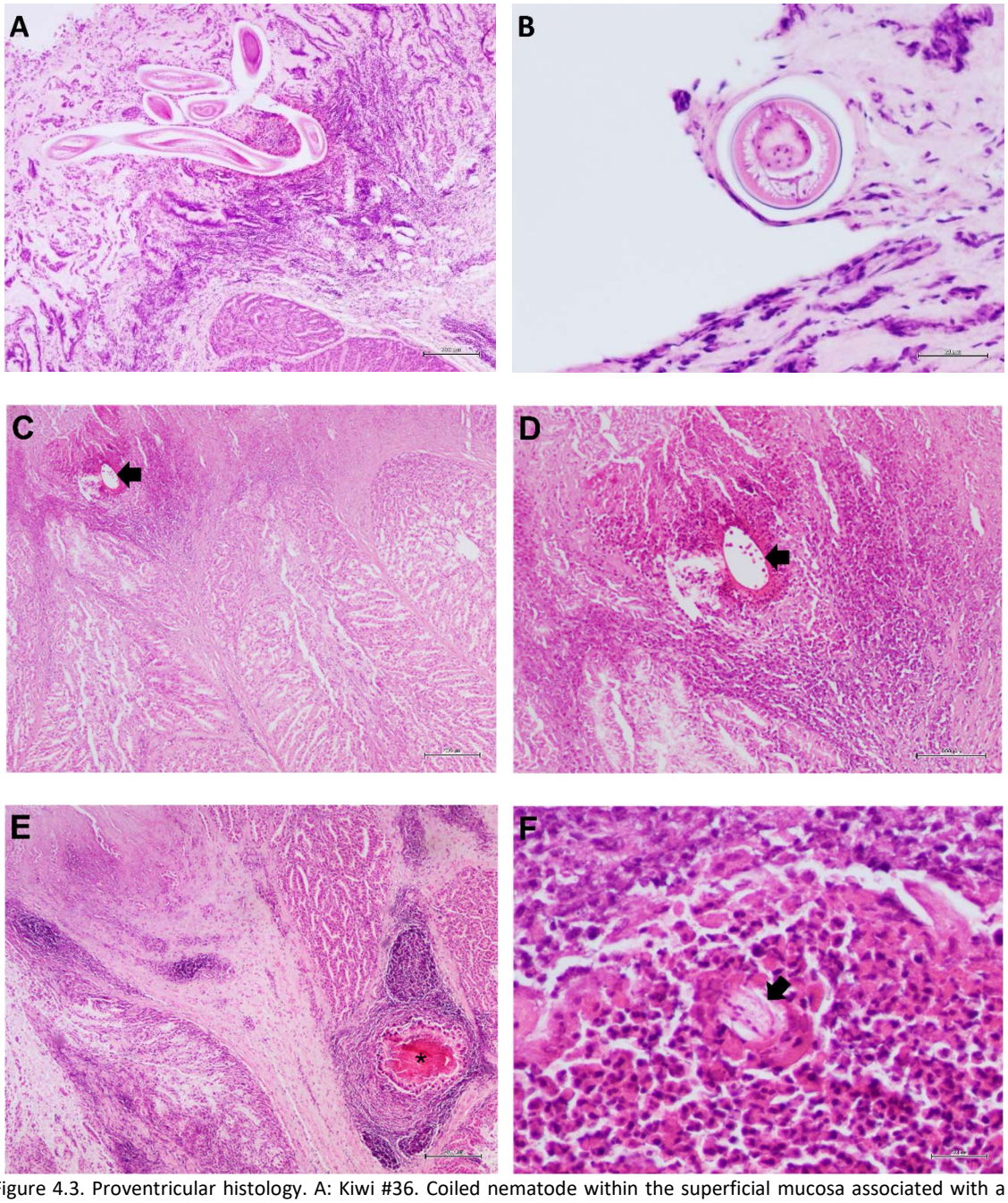

Figure 4.3. Proventricular histology. A: Kiwi #36. Coiled nematode within the superficial mucosa associated with a lymphocytic inflammatory infiltrate (H&E, bar = 200 µm). B: Kiwi #36. Cross-section of a larval nematode within superficial mucosa (H&E, bar = 50 µm). C: Kiwi #15. Mixed inflammatory reaction surrounding an empty space in the superficial mucosa (arrow) (H&E, bar = 200 µm). D: Kiwi #15. Higher magnification of C. (H&E, bar = 100 µm). E: Kiwi #39. Discrete granuloma within deep mucosa (asterisk) (H&E, bar = 200 µm). F: Kiwi #39. Granuloma within superficial mucosa containing a larval nematode (arrow) (H&E, bar = 20 µm).

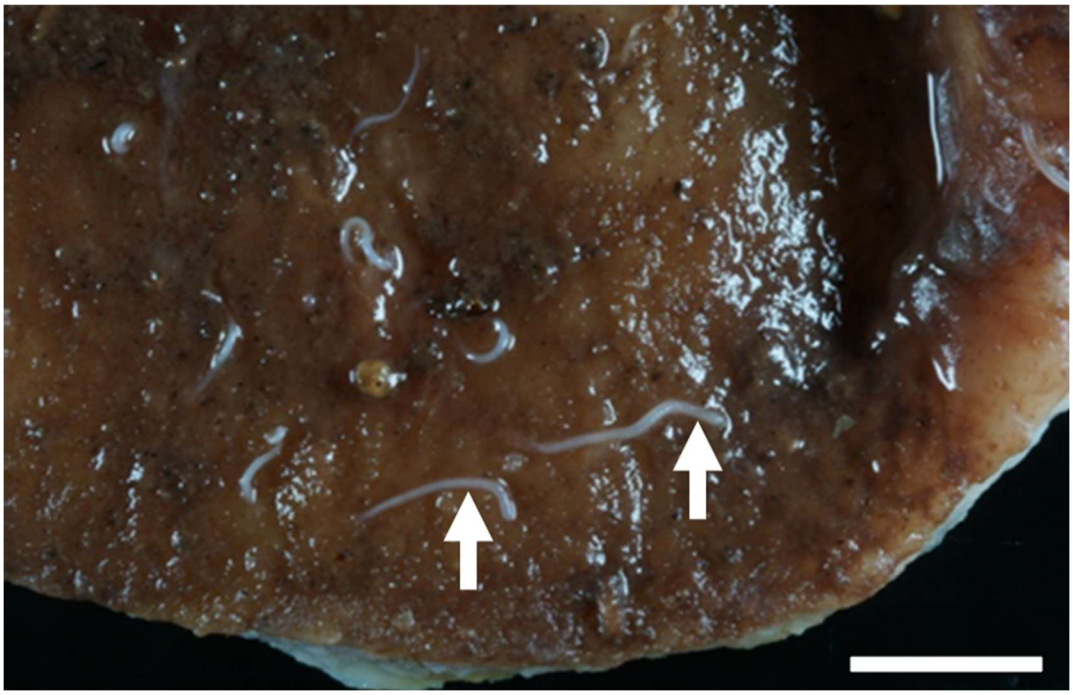

Figure 4.4. Gizzard of a kiwi in which several small nematodes can be seen partially embedded within the superficial koilin layer (arrows) (bar = 5 mm). Image taken by Stuart Hunter; reproduced with permission.

Out of the 50 kiwi examined, 42 (84%) had nematodes within the gizzard: 13 birds had few, 22 had moderate numbers, and seven had many; those with the highest burdens were all either juveniles or subadults. Gizzard nematodes were identified in 36 out of the 41 NIB kiwi (including one of the three birds originating from captive facilities in the South Island) and five of the seven LSK, while just a single gizzard nematode was found in the only rowi to be sampled and none were identified from the sole HT.

4.4.3.2. Morphology

The vast majority of nematodes located within the gizzard were of similar size and low-magnification morphology, considered compatible with the spirurid nematode *C. apterycis* as described by Harris (1975) or a similar, closely related species as suggested by Clark (1983a, b). Adult females measured approximately 4 to 6 mm in length and their body diameter increased from anterior (~70-100 µm) to posterior (~230-300 µm), ending in a bluntly rounded to conical tail (Figure 4.5A). On higher magnification, the rounded female tail typically included a short,

tapering tip that often terminated in a small ornamentation (described as a “mucron” by Harris, 1975) which was somewhat variable in its appearance as well as its presence (Figure 4.5B & C). Adult females contained large numbers of eggs visible in intact worms with or without lactophenol clearing; morphologically indistinguishable from those of the proventricular-type nematodes, the eggs measured ~50-55 x 25-30 µm, were larvated, and had the same asymmetrical ovoid shape with flattened bipolar caps. On microdissection, within the uterus the eggs were noted to progress from a smaller, more uniform oval shape (as depicted in Figure 5 of Harris, 1975) to development of the distinct asymmetrical shape with bipolar structure as they matured, increased in size, and became larvated (Figure 4.5D). Adult males appeared somewhat shorter and a little finer than females but true body length was difficult to estimate as they typically presented with a variably tightly coiled tail (Figure 4.5E & F, Figure 4.9A); this and the presence of well-developed caudal alae made further identifying features difficult to evaluate. Not always visible, especially in uncleared specimens, multiple examined males were confirmed to contain one short and one distinctly longer spicule, although the relative length of the longer spicule did appear variable between specimens (Figure 4.5E & F). On SEM images, oral conformation resembled that illustrated by Harris (1975, Plate I), with well-developed, bilobed dorsal and ventral lips and smaller lateral (or “pseudo-”) lips (Figure 4.6A & B), but comparative en face SEM images were not obtained.

The gizzard of one kiwi (#24) contained only two large (up to 4 cm in length) nematodes resembling those found in the small intestine of the same bird and others, which are discussed further along with the small intestinal samples (see section 4.4.4.2). Additionally, at least three of the five kiwi in which the proventricular-type nematodes were identified within the proventriculus (see section 4.4.2.1) were also noted to include a small number of similar, longer, proventricular-type nematodes admixed with the *Cyrnea*-type gizzard nematodes within the gizzard contents and/or mucosal scrapings.

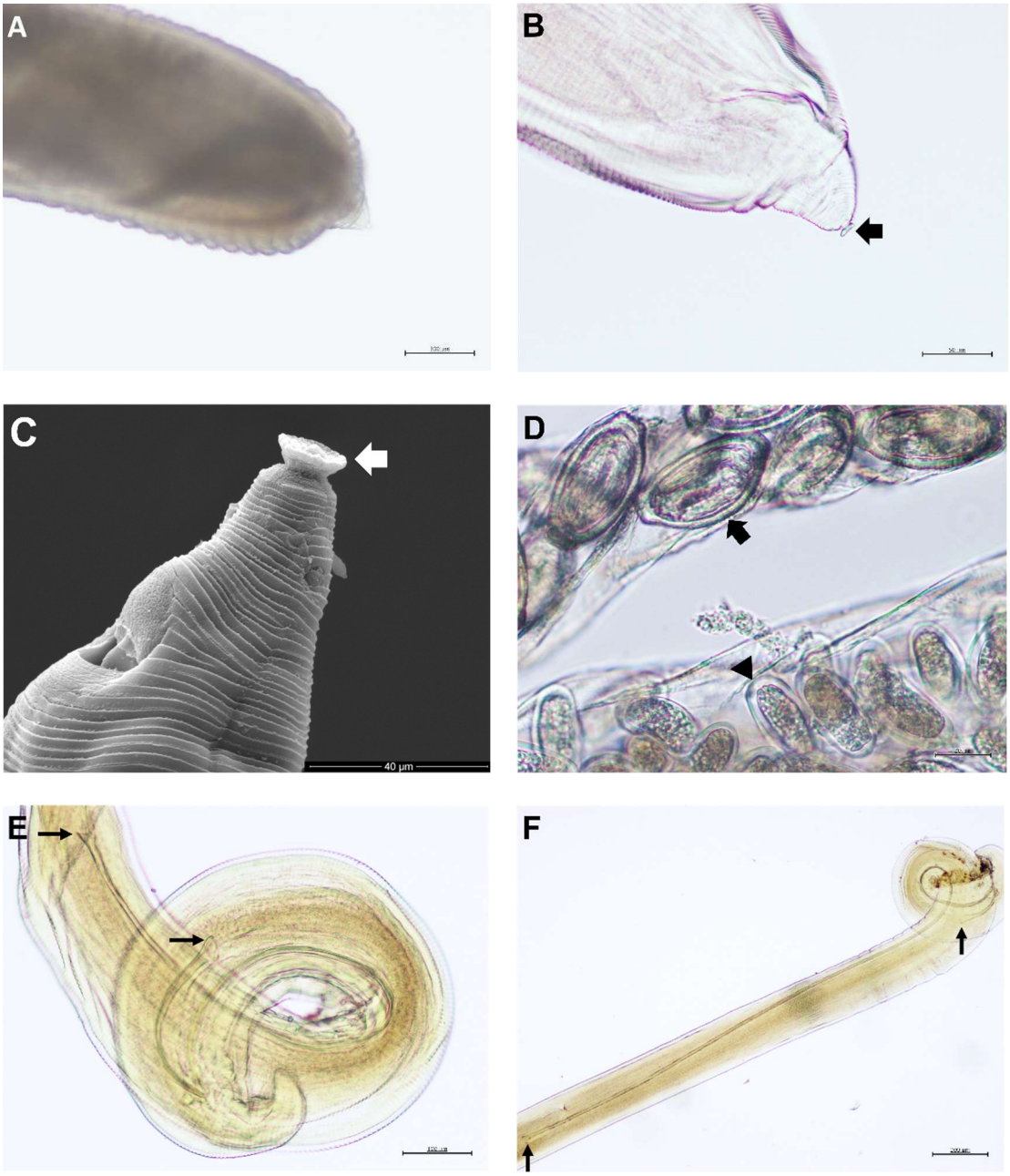

Figure 4.5. *Cyrnea*-type gizzard nematodes. A: Posterior female demonstrating bluntly rounded end (uncleared specimen, bar = 100 µm). B: Female tail demonstrating terminal "mucron" (arrow) (cleared specimen, bar = 50 µm). C: Female tail demonstrating terminal "mucron" (arrow) (SEM, bar = 40 µm). D: Microdissection of uterus showing progression of eggs from smaller uniform oval shape (arrowhead) to mature, larvated, assymetrical shape (arrow) (bar = 20 µm). E: Male tail demonstrating spicules of unequal length (arrows indicating heads of left and right spicules) (cleared specimen, bar = 100 µm). F: Male tail demonstrating spicules of unequal length (arrows indicating heads of left and right spicules) (cleared specimen, bar = 200 µm).

Three out of four kiwi (#'s 22, 23, and 25) originating from the same location in Northland, submitted together and necropsied on the same day, contained moderate to high numbers of

very long, thin helminths within the gizzard lumen, which presented grossly tangled together forming a “Gordian knot”-type configuration (Figure 4.7). While initially counted as potential nematodes, closer morphological examination and the distinctive gross presentation were considered more consistent with members of a different phylum, the Nematomorpha (aka “hairworms”) (Tobias et al., 2017). The precise identification of these organisms was not pursued further and their numbers were not included in the final nematode counts, as these are considered to be invertebrate parasites either consumed by the kiwi within their insect hosts or ingested as free-living adults from a water source.

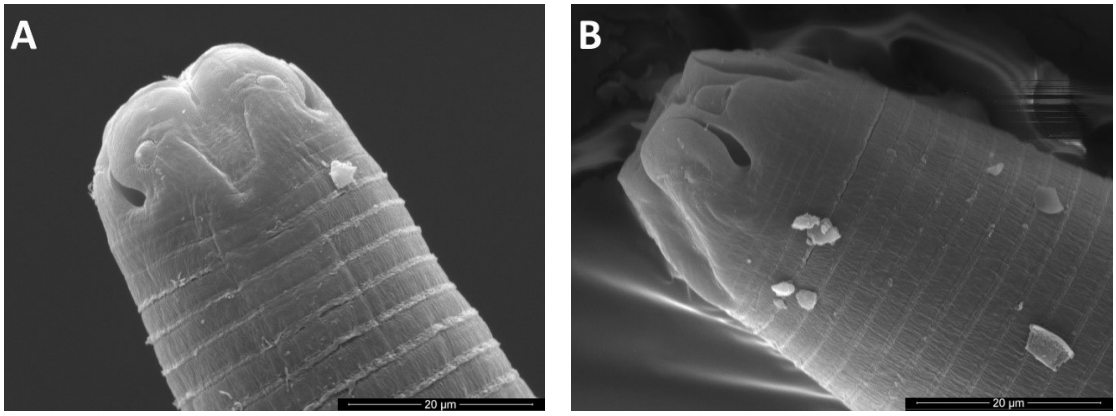

Figure 4.6. *Cyrnea*-type gizzard nematodes. A: Head of a female nematode (SEM, bar = 20 µm). B: Head of a male nematode (SEM, bar = 20 µm).

Figure 4.7. Gizzard containing numerous presumed Nematomorpha (bar = 10 mm). Image taken by Brett Gartrell.

4.4.3.3. Histology

Histological sections of gizzard exhibited variable mild to severe autolytic change, primarily affecting the mucosa, with very few cases completely covered by a well-preserved, intact koilin layer. In many cases the degree of autolysis varied even within the examined section(s), with the observation that regional autolysis was often more notable affecting the gizzard mucosa directly adjacent to the proventricular junction. Of 49 kiwi in which both gross and histological examination of gizzard were performed, 40 had *Cyrnea*-type gizzard nematodes identified grossly, and of these only 23 (57.5%) also had histological evidence of nematode infection (Table 4.2). Additionally, there were three cases in which histological evidence of gizzard infection was present in the absence of *Cyrnea*-type gizzard nematodes identified grossly (two with none found grossly, and one in which only the two large, unidentified intestinal-type nematodes had been found).

The number of nematodes present in histological section varied from one to many, which did not consistently reflect the gross burden. Adult nematodes were most commonly located within the koilin (or remnants of disrupted/autolytic koilin) or the superficial mucosa, with no apparent inflammatory reaction to their presence, and/or sections were evident or extended above the surface of the gizzard mucosa. In several cross-sections from the anterior region of probable adult nematodes (including presence of a tripartite oesophagus), low bilaterally symmetrical alae were evident corresponding to the sites of the internal lateral chords (Figure 4.8A). In many other cross-sections, some from the anterior body including oesophagus with or without gonadal tissue, most presumed adult males, and in a single section from a confirmed female including uterus/eggs, the cuticle exhibited only a single lateral ala (Figure 4.8B, C, & E). Confirmed adult females measured up to ~250 µm diameter in cross-sections including uterus and eggs, and with the rare exception described above, typically exhibited no external cuticular ornamentation (Figure 4.8D). Thick-shelled, larvated eggs were occasionally seen free within the koilin. Cross-sections of suspected or confirmed males were typically smaller, up to ~150 µm

diameter. Occasional cross-sections including sublateral, variably elongated alae and low ventral cuticular ridges were considered consistent with sectioning through the caudal alae in the coiled tail of adult males, supported by the presence in some sections of either one or two spicules evident internally (Figure 4.8F).

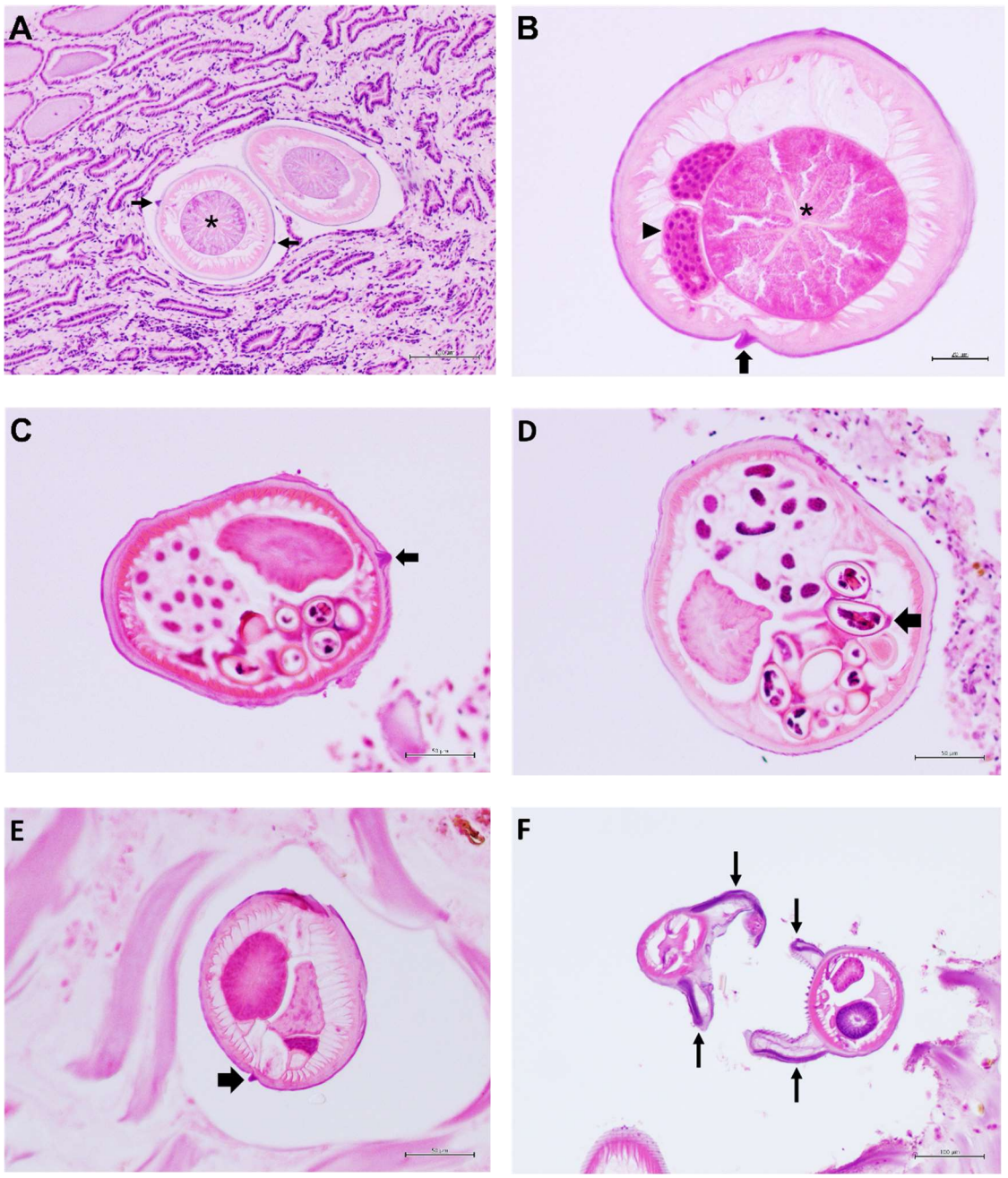

Figure 4.8. Histology of presumed *Cyrnea*-type nematodes within the gizzard. A: Cross-sections through anterior body including tripartite oesophagus (asterisk) and low bilateral alae (arrows), present within gizzard mucosa (H&E, bar = 100 µm). B: Cross-section of adult nematode with tripartite oesophagus (asterisk) and gonadal tissue (arrowhead), and only one distinct lateral ala (arrow) (H&E, bar = 20 µm). C: Cross-section of a female nematode demonstrating a single ala (arrow) (H&E, bar = 50 µm). D: Cross-section of a female nematode with no alae, including the longitudinal section of an egg with characteristic shape in utero (arrow) (H&E, bar = 50 µm). E: Cross-section of a male nematode demonstrating single ala (arrow) (H&E, bar = 50 µm). F: Cross-section through posterior/tail end of a male nematode with broad sublateral caudal alae (arrows) (H&E, bar = 100 µm).

In SEM images of several examples of male and female *Cyrnea*-type gizzard nematodes, low lateral alae were evident on the body, which did sometimes appear quite localised on female nematodes, but the extent and consistency in location of these structures was not documented (Figure 4.9).

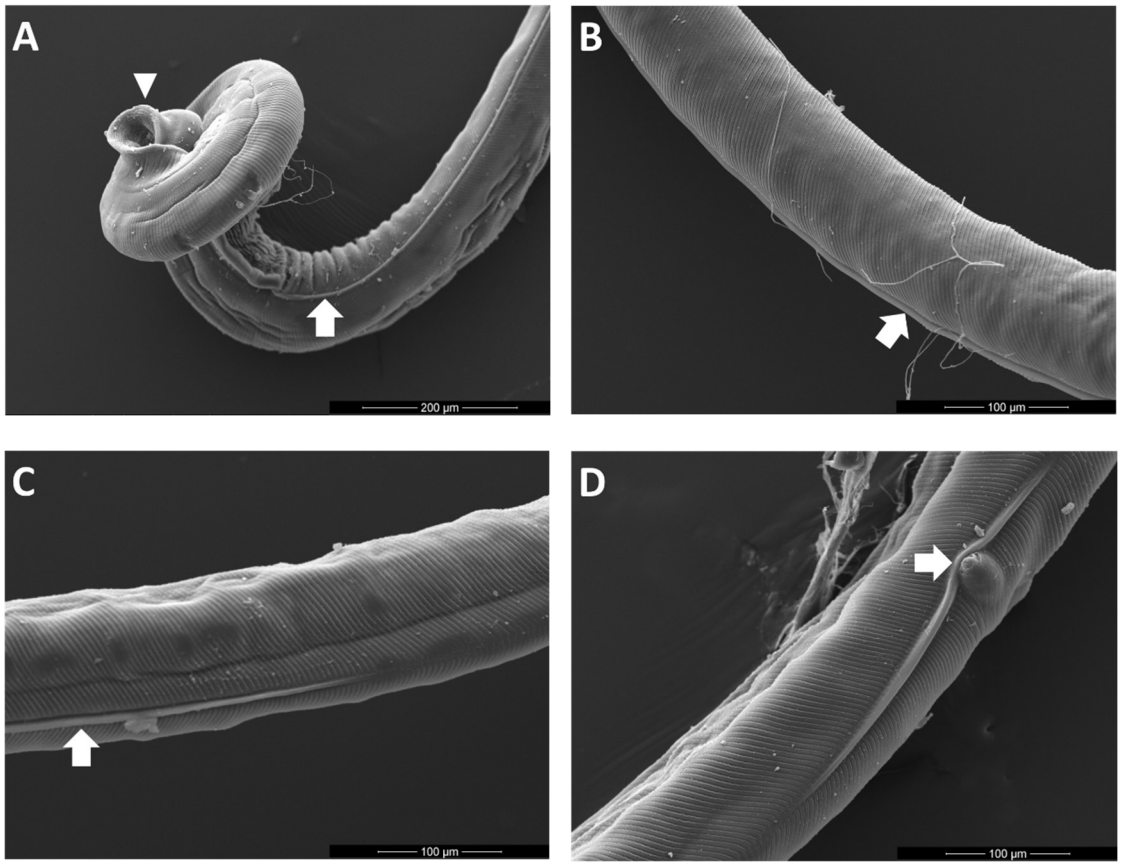

Figure 4.9. *Cyrnea*-type gizzard nematodes. A: Posterior male including low lateral ala (arrow) extending to tail, with typical coiled tail configuration and well-developed caudal alae (arrowhead) (SEM, bar = 200 µm), B: Body, uncertain sex, demonstrating lateral ala (arrow) (SEM, bar = 100 µm). C: Female body demonstrating localised lateral ala (arrow) (SEM, bar = 100 µm). D: Female body demonstrating presumed localised lateral ala (versus artefact) at the level of the vulva (arrow) (SEM, bar = 100 µm).

Variably sized, presumed larval nematodes (smaller diameter, no sex organs apparent) were identified at all levels of the mucosa but were often present within the deeper mucosa, some located within gastric glands showing no apparent inflammatory reaction, and some located within the lamina propria associated with a localised mild to moderate reaction consisting of mixed inflammatory cells with or without early fibroplasia. In the most severely affected gizzard histologically (#21), presence of multiple, relatively large presumed larval nematodes within the

deep mucosa was associated with loss of gastric glands, inflammation, fibroplasia, and an irregularly hyperplastic appearance to the mucosa. Larval forms had no alae or other distinguishing characteristic features identified in section (Figure 4.10).

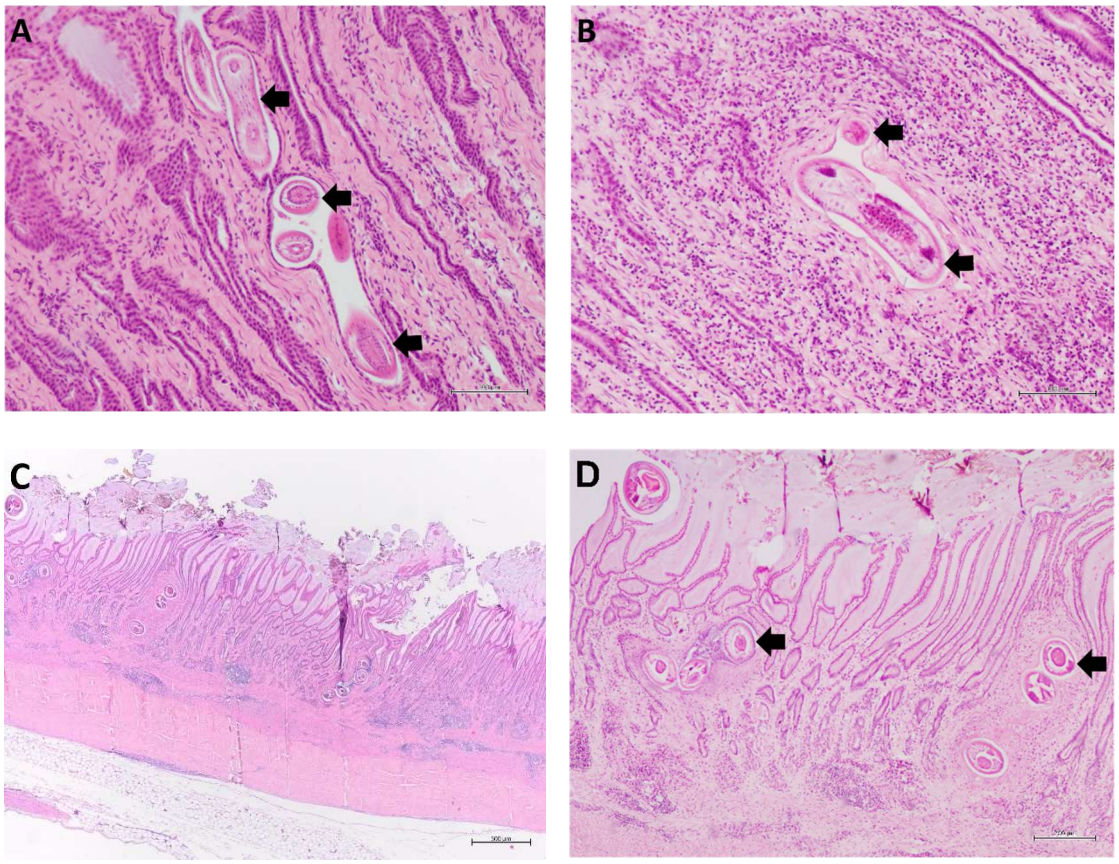

Figure 4.10. Histology of larval nematodes in gizzard mucosa. A: Larvae within mucosal glands (arrows), no inflammatory reaction (H&E bar = 100 µm). B: Larvae within the lamina propria of the deep mucosa (arrows), surrounded by an inflammatory reaction (H&E, bar = 100 µm). C: Kiwi #21. Irregularly hyperplastic and inflamed mucosa (H&E, bar = 500 µm). D: Kiwi #21. Higher magnification of C – multiple larval sections within lamina propria (arrows), with loss of gastric glands, inflammation, and fibroplasia (H&E, bar = 200 µm).

Some degree of lymphocytic inflammatory infiltration, varying from mild to marked, was noted in almost all the gizzard sections, including those in which no nematodes had been found grossly. This was generally in the form of small aggregates or larger follicular formations multifocally present along the base of the mucosa; while non-specific, it was in many cases very prominent in the presence of nematode infections. There were also multiple sections in which foci of more acute to mixed inflammation were noted in the deep mucosa, sometimes surrounding an empty space, suggesting possibly a site of previous larval infection, although potentially a result of

other focal penetrating foreign body-type reaction as this type of reaction was also noted in multiple sections centred on confirmed sections of foreign material. Of uncertain association to the presence of nematodes, in the gizzard of one kiwi (#29) there was also regionally extensive colonisation of the koilin layer, which appeared hypereosinophilic and fragmented, by small round fungal (yeast) bodies (Figure 4.11A & B), and in one other case (#37), a very localised infection of the glandular mucosa by protozoal structures consistent with coccidia was noted underlying the section of an adult nematode within the koilin (Figure 4.11C & D).

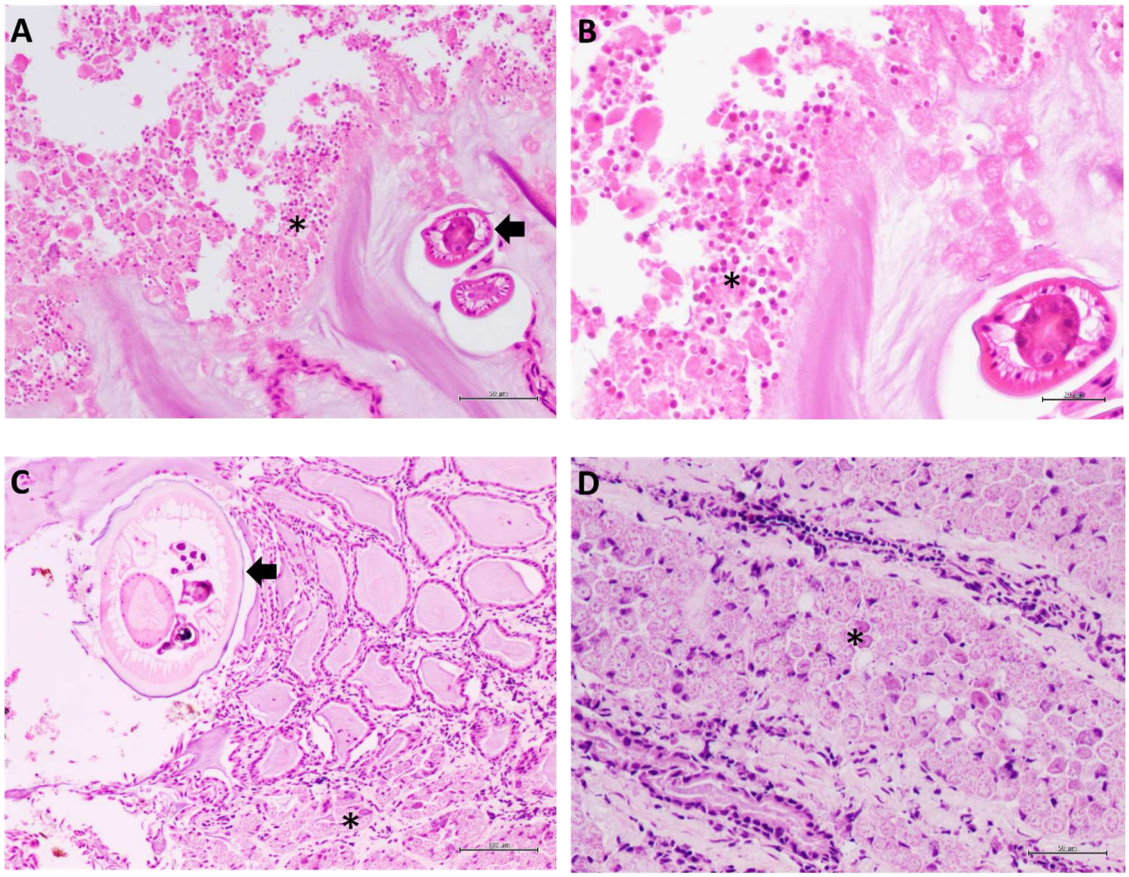

Figure 4.11. A: Kiwi #29. Disrupted koilin layer of the gizzard mucosa, containing large numbers of yeast organisms (asterisk) and two sections of larval nematodes (arrow) (H&E, bar = 50 µm). B: Kiwi #29. Higher magnification of A – disrupted koilin containing yeast (asterisk) (H&E, bar = 20 µm). C: Kiwi #37. Cross-section of an adult nematode within the superficial mucosa (arrow), overlying a focal infection by coccidia (asterisk) (H&E, bar = 200 µm). D: Kiwi #37. Localised coccidial infection in gizzard mucosa (asterisk) (H&E, bar = 50 µm).

In one case, in which very low numbers of *Cyrnea*-type adult gizzard nematodes had been found grossly (#4), the only nematodes seen on gizzard histology were few very small larval forms (~10-14 µm in diameter) present within a discrete granuloma characterised by central accumulation

of abundant eosinophilic necrotic cellular debris surrounded by macrophages, multinucleated giant cells, and other mononuclear inflammatory cells, extending from the deep submucosa into superficial muscularis (Figure 4.12A & B). In two other cases (#'s 22 and 39), in addition to sections of adult and larval nematodes within the gizzard mucosa, a focal discrete granuloma as described above was present within the submucosa or muscularis (Figure 4.12C & D) but no larval forms were evident within; in one of these cases (#39), multiple similar granulomas, one including larvae, had also been described within the proventriculus (see section 4.4.3.3.).

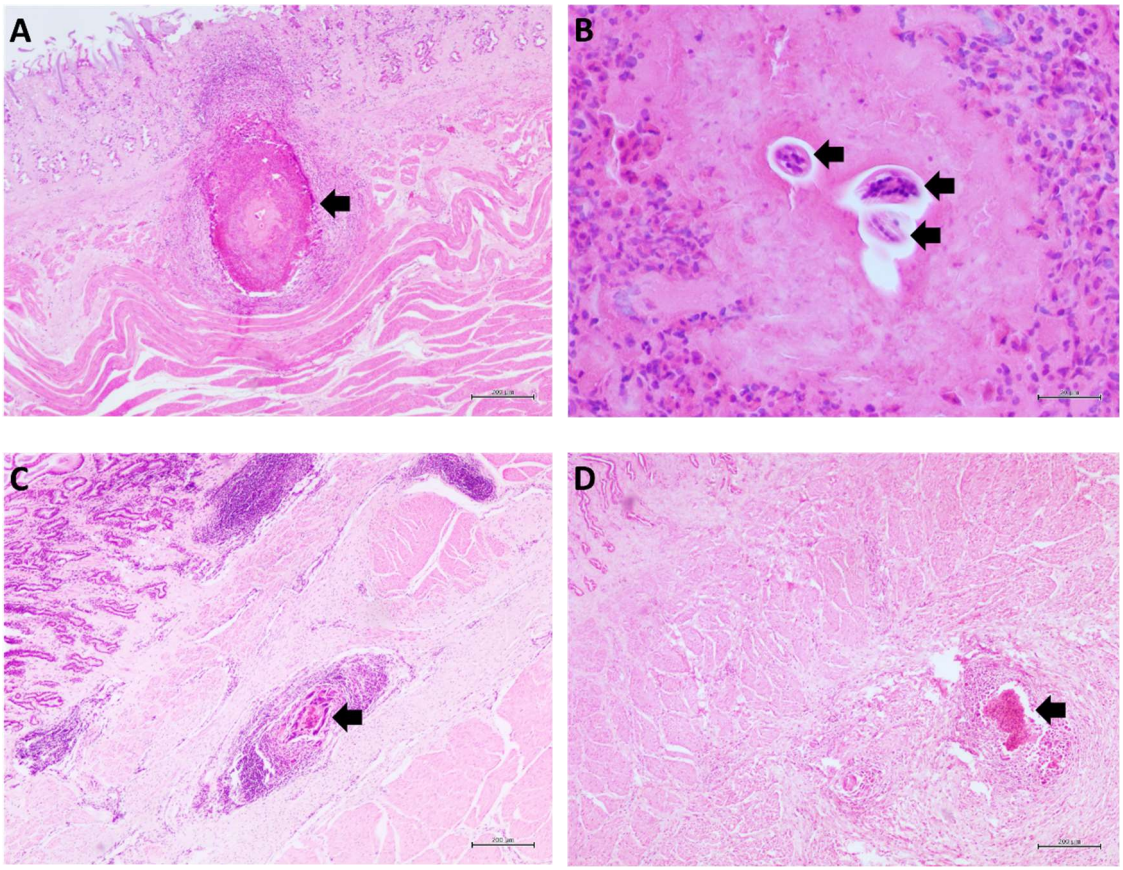

Figure 4.12. Histology of mural granulomas in the gizzard. A: Kiwi #4. Focal granuloma (arrow) extending from deep mucosa into muscularis (H&E, bar = 200 µm). B: Kiwi #4. Higher magnification of A - sections of larvae present within the centre of the mural granuloma (arrows) (H&E, bar = 20 µm). C: Kiwi #22. Focal granuloma within submucosa (arrow) (H&E, bar = 200 µm) D: Kiwi #39. Focal granuloma within muscularis (arrow) (H&E, bar = 200 µm).

In the two cases in which nematodes were identified histologically but not on gross parasitological examination, only one (#12) or two (#2), suspected larval organisms were present within the lamina propria of the deep mucosa with an associated inflammatory response, and so could potentially reflect a very early stage of infection. In the case in which only two of the

intestinal-type nematodes had been found in the gizzard (#34), sections from what appeared to be a single adult male gizzard-type nematode were focally present in the superficial mucosa/koilin of the gizzard section, suggesting a potentially low burden of infection that was missed at the time of collection of gross samples.

4.4.3.4. Additional case ~ "Juvenile kiwi A"

During the same time period as the survey was performed, but not included within the survey population, the body of a juvenile, ~three-month old male NIB kiwi chick from a captive facility was submitted for necropsy, which had died following a long-term history of eating soil and subsequent radiographic confirmation of a gizzard impacted with stones. The most significant finding histologically was an extensive area of deep ulceration within the gizzard with an overlying membrane of hypereosinophilic and pyknotic cellular debris including bacterial cocci and scattered fungal elements. Elsewhere in the gizzard the koilin was thickened, disorganised, and fragmented with irregular loss and/or hyperplasia of the underlying glands, a moderate granulocytic infiltrate, multifocal fibroplasia throughout the lamina propria, and multifocal lymphocytic aggregates in the deeper mucosa. Larval and adult nematodes were identified within the koilin and larvae deeper within the lamina propria, including within the area of ulceration (Figure 4.13). The diagnosis of ulcerative ventriculitis was made, with speculation as to whether the primary cause was mechanical irritation secondary to the gizzard impaction by stones, allowing the nematodes to take advantage of the damaged koilin versus being the primary cause of ulceration themselves. A full parasitological examination was not performed.

4.4.4. Small intestine

4.4.4.1. Prevalence

Small intestinal contents varied widely in amount (often voluminous), colour, and consistency, although variably severe autolysis was also present in many cases which may have altered the characteristics of digesta, and two kiwi had a majority of the small intestine and caeca missing due to post-mortem predation/scavenging. Nematodes were found in 19 out of 47 kiwi (~40%) in which small intestinal tract contents were examined and were always few in number (less

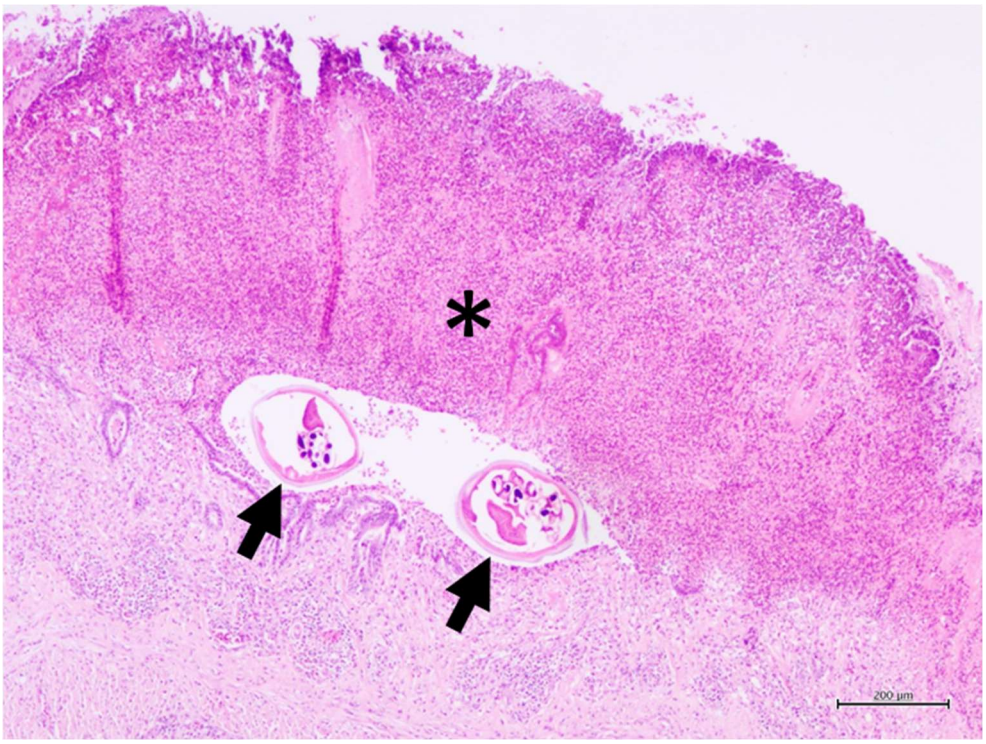

Figure 4.13. Juvenile kiwi A. Histology of the gizzard mucosa from a juvenile male NIB kiwi that died following radiographic diagnosis of gizzard impaction, showing regional ulceration with a superficial thick, fibrinonecrotic exudate (asterisk), and two sections of adult nematode centrally (arrows) (H&E, bar = 200 µm).

than 20 total) (Table 4.1). Of these, four consisted only of one to two nematodes present in the duodenum which were morphologically consistent with the *Cyrnea*-type nematodes of gizzard origin (see section 4.4.3.2), and two consisted of only single nematodes present in the ileum that were morphologically compatible with those found in the caeca (see section 4.4.5.2); these were all considered likely to represent displacement from the adjacent sites. One further kiwi (#49) contained only two small, probable larval nematodes in the anterior small intestinal tract which could not be identified further morphologically, and which were noted to be alive and motile at collection in contrast to all other nematodes found in that kiwi (which body had been frozen and thawed prior to necropsy), leading to speculation that they may reflect environmental organisms.

4.4.4.2. Large spirurids

Ten of the 47 kiwi (~21%) from which small intestine was examined contained low numbers (one to ~19) of large nematodes which were a distinct pink-red colour at time of collection (fading to

tan-white with alcohol fixation); all affected kiwi were identified as subadult or adult, and they originated predominantly (in eight out of 10 cases) from Northland with one each from the Auckland region and the Coromandel (Table 4.1). Four of the eight Northland birds originated from the same site and were submitted together and necropsied on the same day (#'s 22-25), as did/were another two (#'s 36 and 37). The large nematodes were variably located within all levels of the small intestine although more frequently within the jejunum and ileum. Two morphologically similar nematodes were identified in the gizzard of one kiwi (#24, see section 4.4.3.1), which also contained a single similar large nematode in the distal small intestine; the presence of these nematodes in the gizzard was thought possibly to reflect either post-mortem migration or artefactual displacement from the small intestine during necropsy.

On examination, the heads of the nematodes were often curled (Figure 4.14A), but narrow cervical alae were invariably evident in the larger, presumed adult examples (Figure 4.14C & D). Confirmed adult females measured up to ~4 cm in length with straight tails that tapered to a long point. Adult males were shorter, up ~3 cm in length and finer in body diameter, with what appeared to be narrow sublateral caudal alae and, per one cleared specimen, spicules of unequal length (Figure 4.14E). In other similar but shorter, suspected immature specimens (<2 cm in length), no alae (either cervical or caudal) were obvious under the dissecting microscope. On microdissection of two of the adult females, eggs were found that measured ~44-46 x 24-28 µm, were larvated, with a similar asymmetrical shape but less distinct bipolar structure than that seen in the eggs of the nematodes of proventricular and gizzard origin (possibly not reflective of fully mature eggs) (Figure 4.14F). The morphology of the eggs and SEM of the lip conformation of one of adult female nematodes, in which two pairs of lips were evident (Figure 4.14B), supported a nematode of spirurid origin. Further attempt at morphological identification was not undertaken.

At the time of necropsy, the small intestine of five of the 10 birds infected with the large spirurid-type nematodes were also noted to exhibit discrete mural nodules; in most cases there was only a single nodule, but at least two had multiple similar lesions, with upward of 15 nodules present in one case (kiwi #50) in which the lesions became more prevalent distally within the intestine. The nodules were typically around 0.5 cm in diameter and protruded from the serosal surface of the intestine. In one or two cases when the intestine was opened a large nematode could be seen partially protruding into the intestinal lumen from the mucosal aspect of a nodule. Similar, focal nodules were also present in two further birds in which no nematodes were identified grossly within the small intestine (Table 4.2).

4.4.4.3. Capillarids

In 2/47 kiwi (~4%), few very long, fine nematodes were found on examination of small intestinal samples under the dissecting microscope; in one case (#29) these originated from within the proximal to mid-small intestine (one each in duodenum and jejunum), in the other (#21) up to six (some not intact) were found in the distal small intestine (ileum). Intestinal contents and mucosal scrapings were not collected separately, and so the original location within the lumen versus embedded within the intestinal mucosa was not established. The nematodes measured approximately 1.5 cm in length with a very fine body diameter, and on light microscopic examination (without clearing) large cells with a central nucleus consistent with elongated stichocytes could be identified within the anterior/oesophageal region (Figure 4.15A), suggesting members of *Capillaria sensu lato*. Additionally, in one case (#49) the examined nematode was confirmed as an adult female containing typical capillarid-type eggs (barrel-shaped with parallel sides and distinct bipolar plugs), measuring approximately 54-57 x 20-23 µm, and appearing non-embryonated in utero (Figure 4.15B).

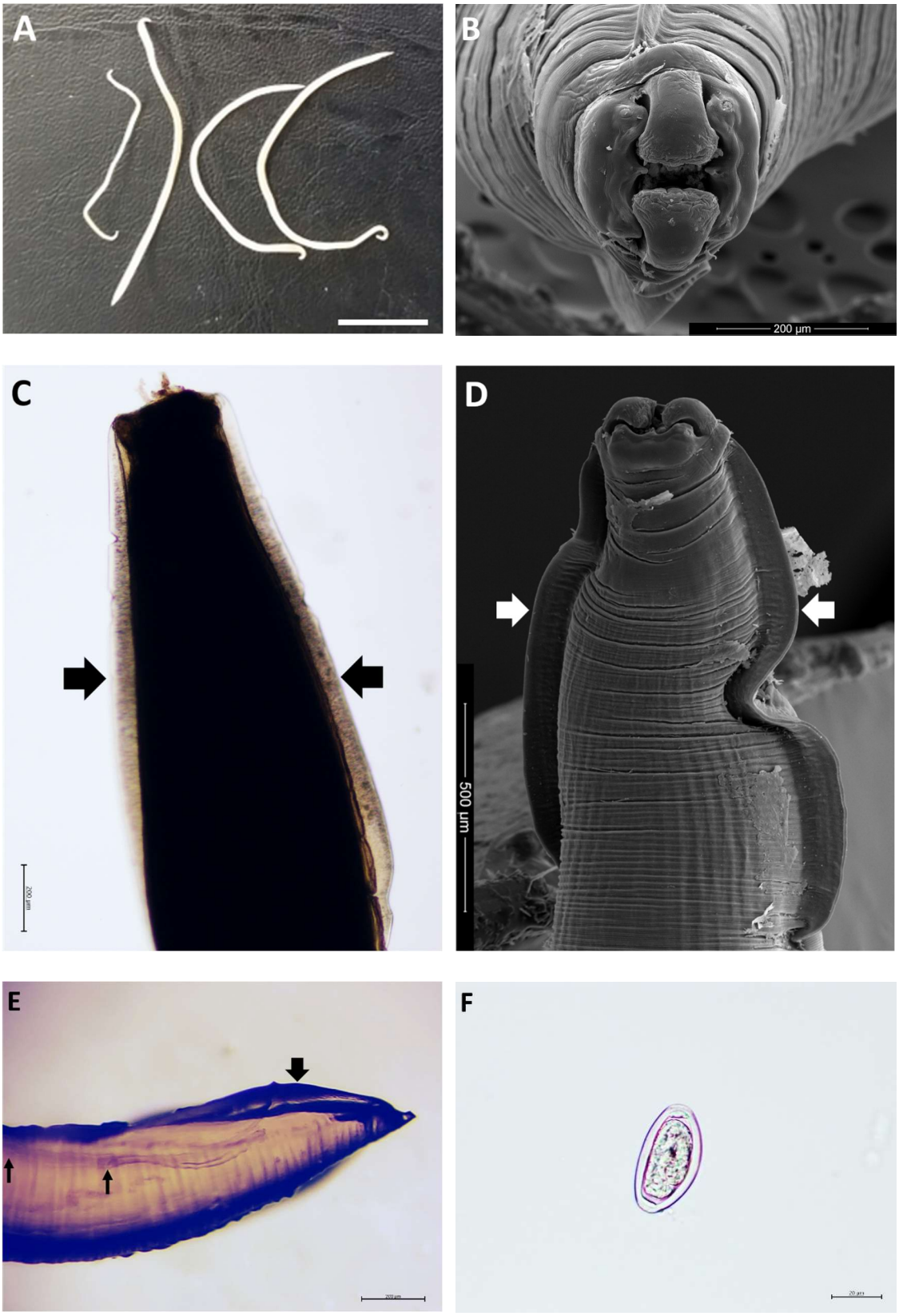

Figure 4.14. Intestinal-type spirurid nematodes. A: Gross appearance (alcohol-fixed, bar = 1 cm). B: Oral features of an adult female (SEM, bar = 200 µm). C: Head of adult female demonstrating bilateral narrow cervical alae (arrows) (uncleared specimen, bar = 200 µm). D: Head of adult female demonstrating cervical alae (arrows) (SEM, bar = 500 µm). E: Male tail with narrow, sublateral caudal alae (thick arrow) and unequal spicules (thin arrows) (cleared specimen, bar = 200 µm). F: Egg, as dissected from uterus (bar = 20 µm).

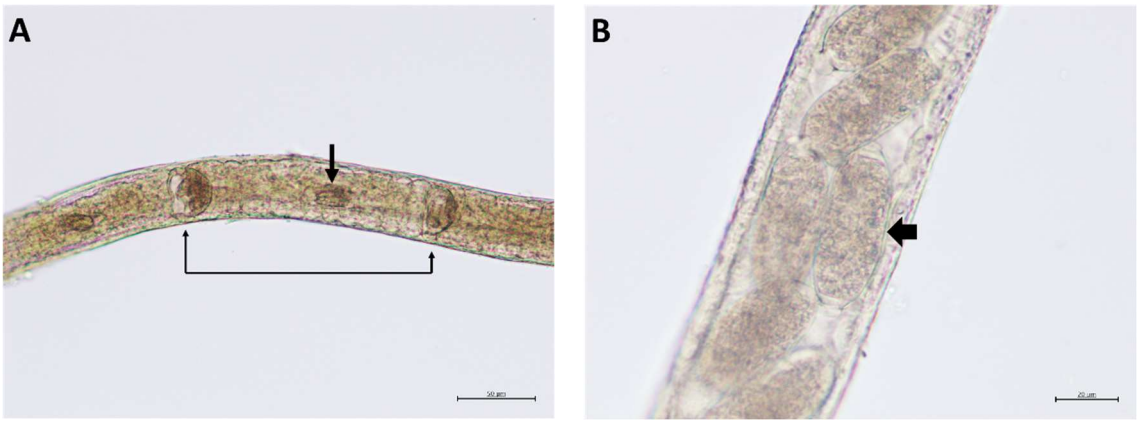

Figure 4.15. Capillarid-type nematodes from the small intestine. A: Oesophageal region, demonstrating elongated stichocytes (one cell delineated by thin arrows) with central nucleus (thick arrow) (uncleared specimen, bar = 50 µm). B: Body of adult female containing eggs (arrow) (uncleared specimen, bar = 20 µm).

4.4.4.4. Histology

Apart from those necropsies in which intestinal segments were absent, in several other cases histology was not attempted due to the severity of autolysis. Histologically, most of the examined cases exhibited some degree of mild (mucosal) to severe (transmural) autolysis, limiting meaningful comparative evaluation of any potential, more generalised inflammatory changes; histopathological interpretation has therefore been limited to the presence or absence of nematodes and/or grossly identified lesions (i.e. mural nodules).

Of 44 cases in which the small intestine was evaluated, only 7 (~16%) showed histological evidence of nematodes in the sections examined (Table 4.2). This included three cases (#'s 37, 48, and 50) which also had large nematodes identified grossly within the small intestine, and which also comprised three of the seven cases in which small intestinal mural nodules were present. In all three cases, the nematodes seen histologically were relatively large but appeared larval, measuring up to ~300 µm in cross-sectional diameter with prominent lateral cords, coelomyarian musculature, no alae and no sex organs evident in sections available for examination (Figure 4.16C). The nematode sections were present in the intestinal lumen in one case (#37), in the centre of a mural nodule as well as within MD in another (#48), and within multiple mural nodules as well as within as the intestinal lumen in the third (#50). A fourth case (#4) contained similar sections of immature nematodes present histologically within a mural

nodule that had been identified grossly, but no nematodes had been found in the small intestinal contents in this case. In two further cases in which both large spirurid-type nematodes and mural nodules had been identified grossly, no nematodes were identified histologically within these lesions.

The mural nodules themselves were largely present in the deep muscularis and protruded from the serosal aspect of the intestine, typically consisting of central accumulation of necrotic debris and inflammatory cells (+/- nematode larvae), surrounded by maturing fibroplasia (Figure 4.16A & B); a "tract" of necrotic tissue was sometimes evident communicating through the mucosa to the lumen of the small intestinal section (Figure 4.16D). Occasional nodules were predominantly comprised of fibrosis with minimal residual inflammation. In the final case (#26) in which a mural nodule had been found in the duodenum, no nematodes were identified either grossly or histologically and the nodule consisted of a more solid aggregation of lymphoid tissue and mature fibrosis.

The remaining three cases in which evidence of nematode infection was seen histologically in the small intestine included one bird (#19) in which sections of smaller, probable larval nematodes (~25x20 µm) were present within MD, associated with some acute inflammatory changes (Figure 4.17A & B); no large nematodes had been found grossly in this case (only a *Cyrnea*-type nematode in the duodenum), and the suspected nematode larvae within MD exhibited no distinguishing histomorphological features.

In the other two cases (#'s 22 and 40) sections of small larvae were present focally within discrete mural granulomas, similar to those previously described in proventriculus and gizzard, in the sections of terminal ileum. The larvae measured ~23 µm diameter in #22 (which also had a similar granuloma but no larvae present within the submucosa of the gizzard, see section 4.4.3.3) (Figure 4.17C & D) and ~10-14 µm diameter in #40 (Figure 4.17E & F). A single oblique

section of a larger, probable larval nematode with no identifying histomorphological features was also noted in the lumen of the duodenal section of #40.

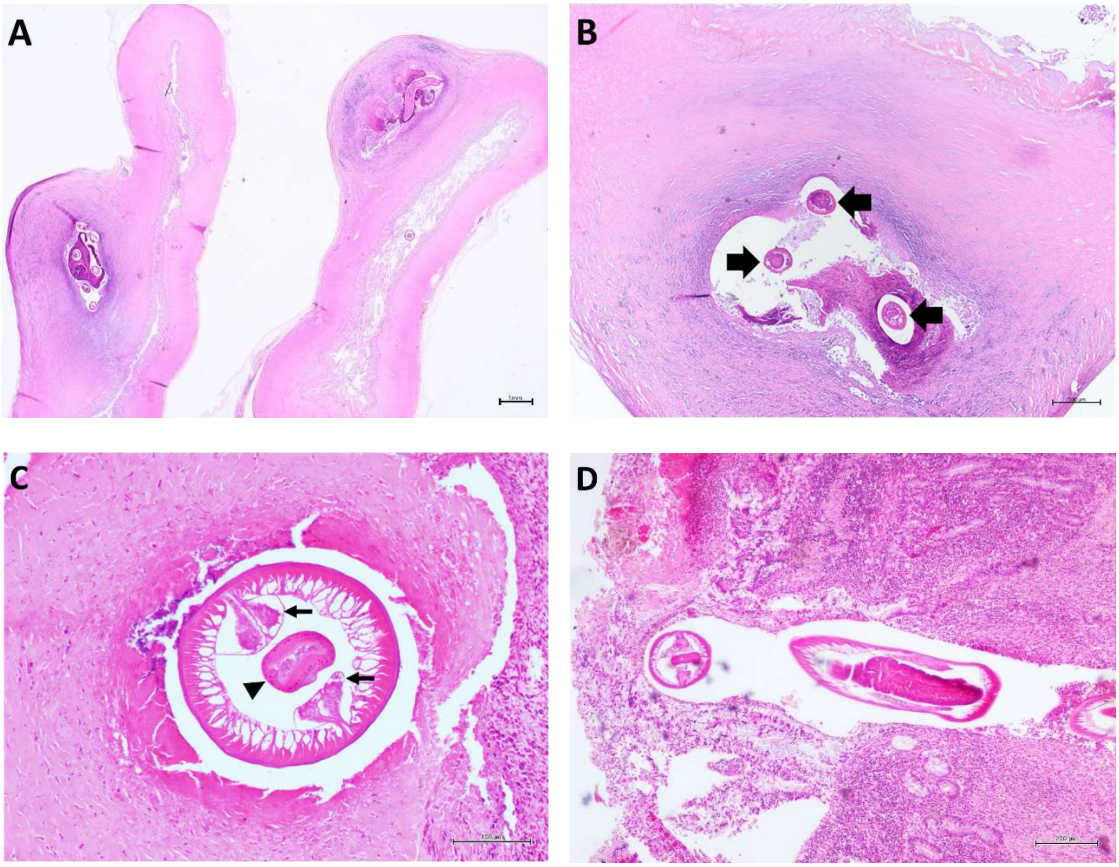

Figure 4.16. Histology of small intestinal mural nodules. A: Two sections of intestine with nodules protruding from the serosal aspect of the intestinal wall (H&E, bar = 1mm). B: Nodule containing nematode larvae (arrows) and necrotic cellular debris centrally (H&E, bar = 500 µm). C: Cross-section of a larval nematode demonstrating well-developed lateral cords (arrows) and intestinal tract (arrowhead) (H&E, bar = 100 µm). D: Larval nematode extending through intestinal mucosa from the centre of a mural nodule (H&E, bar = 200 µm).

No nematodes morphologically consistent with capillarid origin were identified histologically in any of the intestinal sections.

4.4.5. Caeca

4.4.5.1. Prevalence

Caecal contents varied widely in amount and also in consistency, from liquid to inspissated, and were almost always a very dark brown-black colour; nematodes were not grossly obvious at time of collection. Of 48 kiwi in which caecal contents were examined, 32 (~67%) contained nematodes: eight (~17%) had few, five (~10%) had moderate numbers, and 19 (~40%) had many, in some cases estimated to be >500-1000; high burdens were identified in all age categories

(Table 4.1). Caecal nematodes were present in 29/39 NIB kiwi (including all three birds within South Island captive facilities), two out of seven LSK, and the single HT but not the sole rowi representative. In many cases the caecal nematodes appeared more poorly preserved than those found at other sites in the same kiwi.

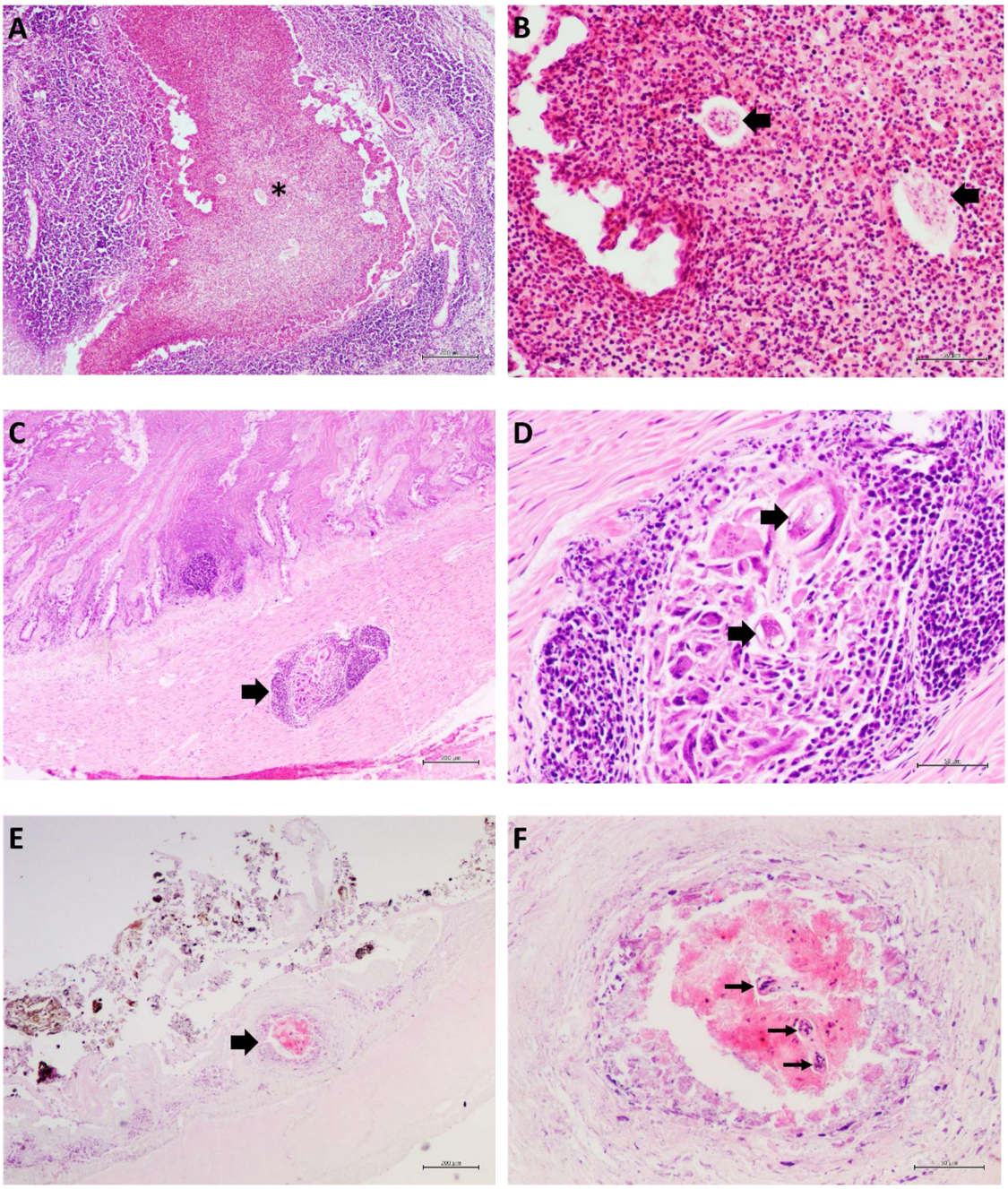

Figure 4.17. A: Kiwi #19. Meckel's diverticulum containing necrotic and inflammatory debris (asterisk) (H&E, bar = 200 µm). B: Kiwi #19. Higher magnification of A – suspected nematode larvae within inflamed Meckel's diverticulum (arrows) (H&E, bar = 50 µm). C: Kiwi #22. Focal granuloma within muscularis of distal ileum (arrow) (H&E, bar = 200 µm). D. Kiwi #22. Higher magnification of C – nematode larva within mural granuloma (arrows) (H&E, bar = 50 µm). E: Kiwi #40. Focal granuloma within submucosa of distal ileum (arrow) (H&E, bar = 200µm). F: Kiwi #40. Higher magnification of E – nematode larvae within submucosal granuloma (arrows) (H&E, bar = 50 µm).

4.4.5.2. Morphology

Under the dissecting microscope the caecal nematodes superficially resembled *Heterakis* (syn. *Kiwinema*) *gracilicauda* as described by Harris (1975) and re-described by Inglis and Harris (1990), or a related heterakoid species as suggested by Clark (1983a, b), primarily taking into account their small size (adult females up to 7.5mm in length) and the variably elongated, thin tail of both males and females (Figure 4.18B & C). Spicules, where visible, appeared slightly unequal but close in length and sometimes disparate in thickness and shape, while other structures, such as a ventral sucker, were inconsistently identifiable especially in uncleared male specimens. The females contained many eggs visible in both cleared and uncleared specimens which on microdissection were confirmed to be ovoid, measuring ~64-74 x 34-39 µm, with a rough coat (Figure 4.18D).

Figure 4.18. Heterakoid nematodes from the caeca. A: Head (uncleared specimen, bar = 100 µm). B: Caudal male demonstrating spicules (arrow heads) and elongated, thin tail (arrow) (uncleared specimen, bar = 100 µm). C: Caudal female demonstrating elongated, thin tail (arrow) (uncleared specimen, bar = 100 µm). D: Typical kiwi heterakoid egg with rough coat, from faecal flotation (bar = 20 µm).

4.4.5.3. Histology

Of 44 cases from which both gross and histological examination of the caeca were performed, 29 had caecal nematodes confirmed grossly and of these 19 (~66%) also showed histological evidence of infection, in most cases consisting of sections of nematodes but in one case reflected only by a few eggs present within the caecal lumen (Table 4.2). In only one case (#29) were a few oblique sections of possible larval nematodes seen histologically within the caecal lumen in the absence of grossly identified caecal organisms.

Nematodes were almost exclusively present within the lumen of the caeca (Figure 4.19D), although in one case (#23) they were only noted free within the adjacent mesenteric fat, reflecting displacement of caecal contents and contamination of the tissue sample at or post-collection. A varying degree (mild to severe) of autolysis was common, particularly in sections from the distal caeca even where the proximal sections were relatively well-preserved. Prominent mucosa-associated lymphoid tissue was also common, including sometimes large, nodular aggregates of lymphoid follicles that had occasionally been noted grossly also as small white nodules multifocally visible from the serosal surface of the caeca. Numbers of nematodes seen histologically varied from single organisms to large numbers with no consistent correlation to gross counts; the nematodes themselves were also variably well-preserved in section.

The cross-sectional diameter of adult females ranged widely from ~120 up to ~ 210 µm in diameter, although smaller sizes were more common (Figure 4.19B); this range overlapped with that of adult males, which were less commonly identifiable histologically but where present appeared to be on the smaller side or less than the female size range (Figure 4.19C). Sections of both males and females included distinct bilateral alae. Musculature was variably high coelomyarian and lateral chords were typically low and somewhat indistinct.

The two cases (#s 22 and 40) in which mural granulomas had been found in the sections of distal ileum (see section 4.4.4.4) also contained similar focal (#22) or multifocal (#40) granulomas in

the sections from distal caeca. That of #22 was present in the submucosa and contained small larval cross-sections ~25 µm diameter (Figure 4.20A & B). In the case of #40 several granulomas were present within the deep mucosa/submucosa but larvae were not identified in any of these lesions (Figure 4.20C). In one final case (#9), a very small longitudinal section (~11 µm across) of a possible larval nematode was identified within a follicle of markedly hyperplastic submucosal lymphoid tissue, with an associated acute reaction to its presence (Figure 4.20D) – this case had no caecal nematodes identified on gross evaluation.

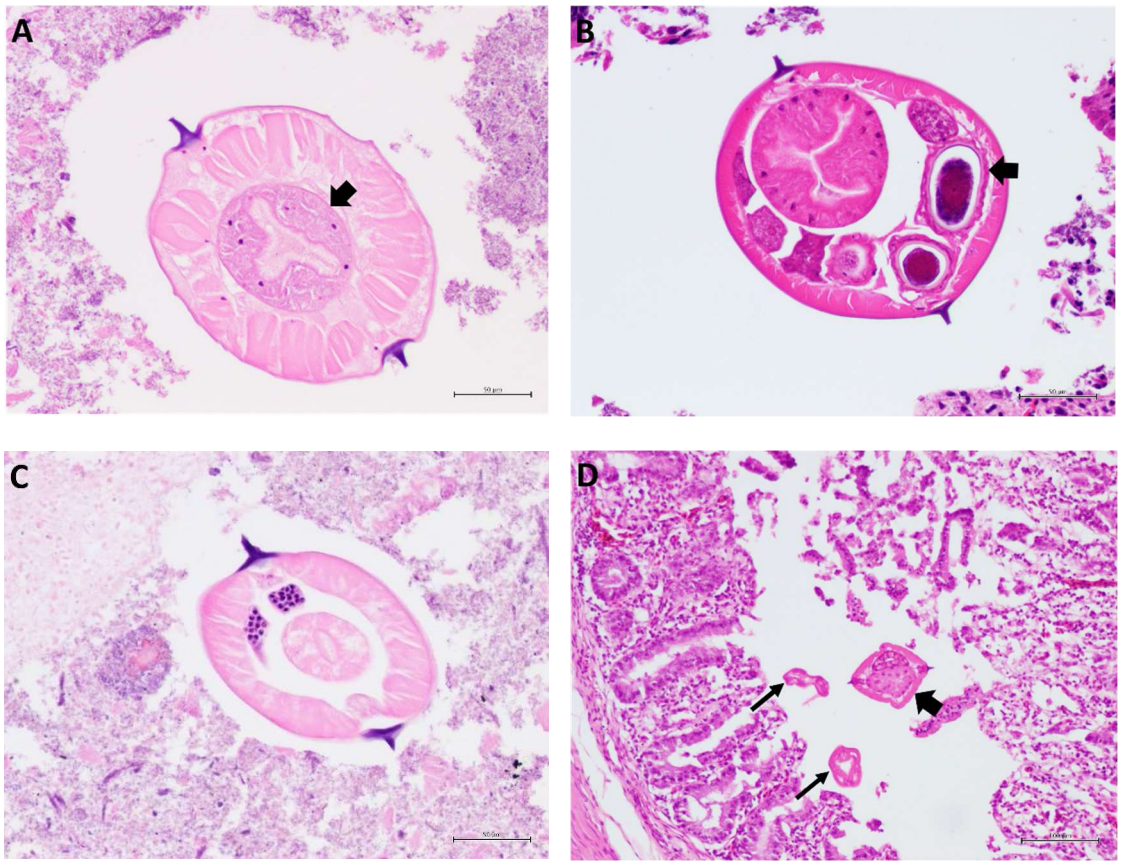

Figure 4.19. Histology of presumed heterakoid nematodes within the caecal lumen. A: Presumed anterior body containing tripartite oesophagus (arrow) (H&E, bar 20x bar = 50um). B: Adult female containing eggs (arrow) (H&E, bar 20x bar = 50um). C: Presumed adult male (H&E, bar 20x bar = 50um). D. Adult (thick arrow) and two larvae (thin arrows) above the caecal mucosa (H&E, bar 20x bar = 100um).

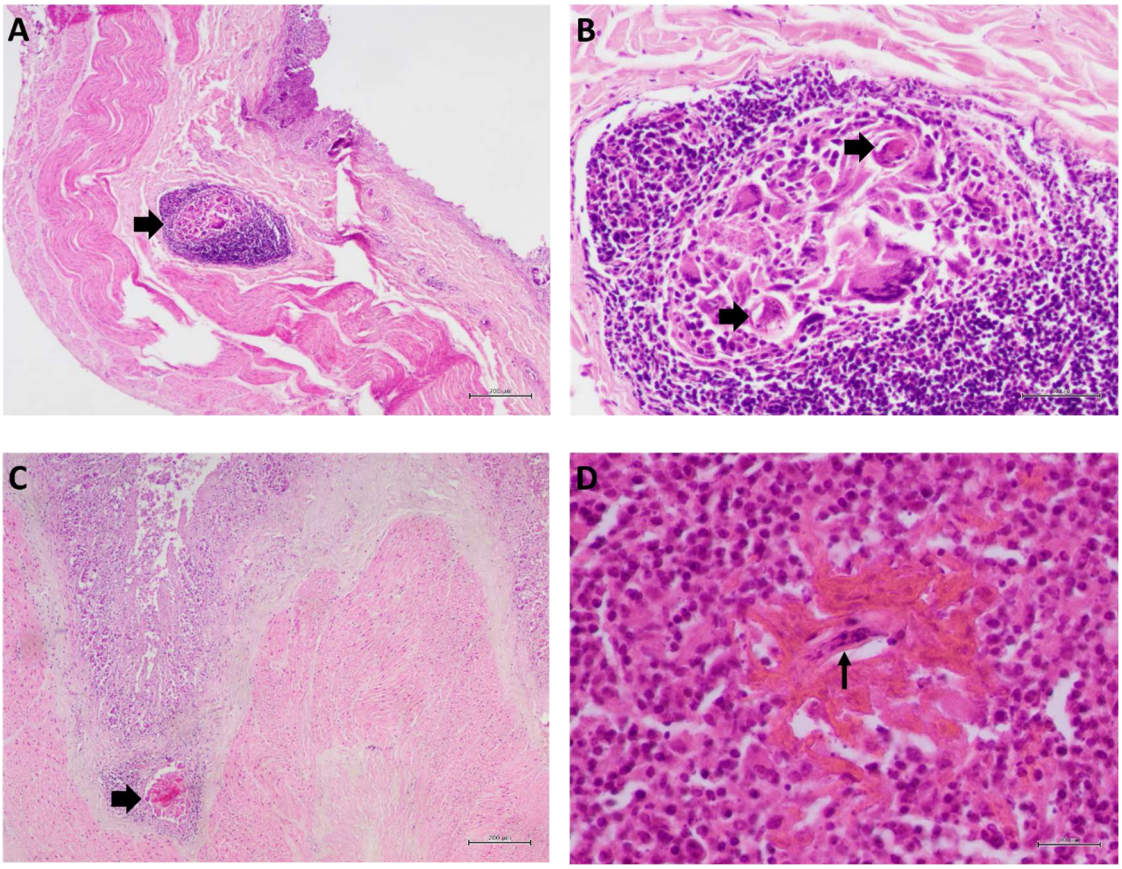

Figure 4.20. A: Kiwi #22. Submucosal granuloma (arrow) in distal caecum (H&E, bar = 200 µm). B: Kiwi #22. Higher magnification of A – sections of a nematode larva (arrow) within distal caecal submucosal granuloma (H&E, bar = 50 µm). C: Kiwi #40. Submucosal granuloma (arrow) in distal caecum (H&E, bar = 200 µm). D: Kiwi #9. Suspect tiny nematode larva (arrow) within hyperplastic submucosal lymphoid tissue in the caecum (H&E, bar = 20 µm).

4.4.6. Organ histology

Gross lesions that may have suggested the presence of nematode LM were not specifically recorded in any case. Out of 49 kiwi in which organ histology was performed, typical histological lesions suggestive of nematode larval migration (as previously described by French et al, 2020b) were identified in a total of 17 kiwi (34.7%), although in only five cases were nematode larvae present in at least one section to definitively confirm the diagnosis (Table 4.2). All affected kiwi were NIB, with the majority (12/17, ~71%) originating from Northland; four of the 12 affected Northland birds originated from the same site and were submitted together and necropsied on the same day (#'s 22-25), as did/were two other sets of two birds (#'s 36 and 37, and #'s 47 and 48).

Liver histology was examined and/or diagnostic histology reports including liver were reviewed in 49 kiwi, with lung and brain histology likewise in 47 and 36 kiwi respectively. Lesions consistent with nematode *larva migrans* (LM) were identified in the liver of 14 (28.6%), the lung of eight (17.0%), and the brain of four (11.1%) kiwi from which these organs were examined; brain lesions were never identified without concurrent liver or lung lesions, and lesions were identified within two or more sites in at least 6 of the cases (Table 4.2). In several of the affected (and other) kiwi non-specific inflammatory changes were sometimes noted in organs that could potentially have been associated with presence of larval migration, but only the "classic" eosinophilic granuloma lesion was counted as evidence of probable LM unless a larval organism was also present.

"Classic" LM-type inflammatory granulomas were as previously described (French et al, 2020b; Chapter 2), with central accumulation of brightly eosinophilic, necrotic cellular and pyknotic nuclear debris and a peripheral rim of epithelioid macrophages and multinucleated giant cells. In some cases with confirmed larvae, the response was either more acute or granulomatous (Figure 4.21A & B respectively). Larvae ranged in diameter from 11.5-17.5 µm; in only one case (#36) did the histological section include a larval cross-section in which distinct bilateral alae could be confirmed (Figure 4.21B).

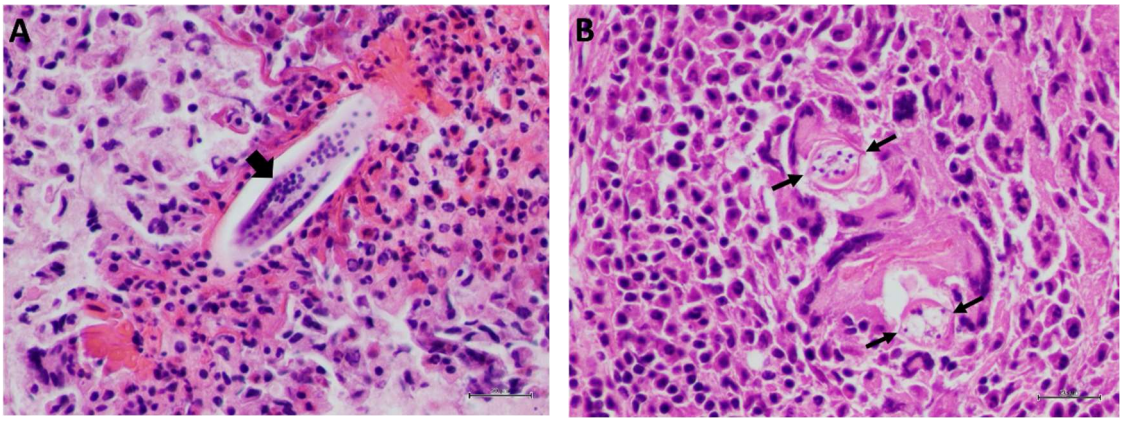

Figure 4.21. A: Kiwi #23. Focus of acute inflammation in the lung surrounding an oblique section of larval nematode (arrow) (H&E, bar = 20 µm). B: Kiwi #36. Focal granulomatous inflammation in the lung surrounding cross-sections of nematode larvae demonstrating small bilateral alae (arrows) (H&E, bar = 20 µm).

One of the affected kiwi (#18) was one of only three kiwi in which no nematodes had been identified on gross parasitological evaluation of the gastrointestinal tract. Among the 16 other kiwi exhibiting visceral lesions suggestive of nematode LM were included nine out of the 11 kiwi that had histologically or grossly confirmed large spirurid-type nematodes within the small intestine.

4.4.7. Faecal flotation

4.4.7.1. Survey results

In 39 of the 50 necropsy cases faecal samples (either rectal or cloacal contents, or a mixture of both) were able to be collected for routine faecal flotation (Table 4.1). In two of these samples, results were subsequently excluded due to obvious soil contamination. Of 27 kiwi in which caecal heterakids were identified and from which faecal flotation was performed, only 10 (~37%) had "*Heterakis*" eggs reported; one of these samples also contained suspected heterakid larvae, and one additional case had suspected heterakid larvae reported but no eggs. In two cases, "*Heterakis*" eggs were identified in the faecal sample in the absence of any caecal nematodes being found on parasitological examination.

Eight of the 37 faecal samples (~22%) contained capillarid-type eggs; however, the only two birds with capillarid nematodes identified in the small intestine (#21 and #29) were negative for any nematode eggs. Other parasitic nematode egg types reported included "ascarid" (in some cases specified as "not *Toxocara*") (4/37, ~11%), "pinworm" (1/37, ~3%), and "strongylid" (1/37, ~3%). In none of the 37 faecal samples examined, whether positive or negative for proventricular, gizzard, or intestinal spirurid-type nematodes, were any spirurid-type eggs reported.

4.4.7.2. Additional investigation ~ "Juvenile kiwi B"

Subsequent to the survey results, a live juvenile male NIB kiwi was admitted to the Wildbase Hospital (Massey University, Palmerston North, New Zealand) from a North Island captive facility. Initial treatment targeted previously diagnosed coccidial infection and a gizzard

impacted with stones, but after a period of minimal clinical response including persistent anaemia, gizzard endoscopy was performed which identified fibrinoulcerative ventriculitis and the presence of numerous small nematodes partially embedded within the koilin lining (Figure 4.22). Clinical improvement, including resolution of anaemia, followed the addition of fenbendazole to the treatment regime.

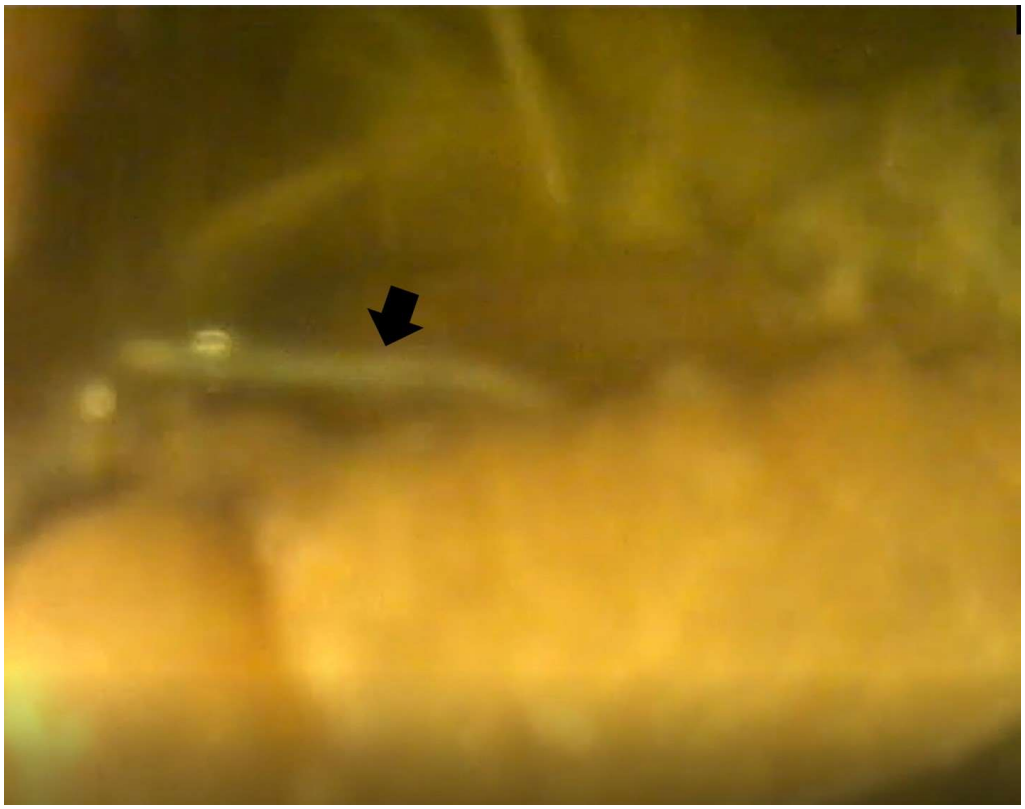

Figure 4.22. Juvenile kiwi B. Still image from a video taken of gizzard endoscopy that identified the presence of nematodes (arrow) partially embedded within the koilin lining. Video taken by Dr. Megan Jolly; image reproduced with permission.

Multiple faecal samples submitted during the time of hospitalisation to monitor the coccidiosis had all been negative for nematode eggs using routine 33% zinc sulphate flotation. Further investigations were performed on a pre-treatment faecal sample (10 days old at the time of investigation, refrigerated following submission and initial diagnostic flotation). Nematode eggs were not identified on repeat routine 33% zinc sulphate flotation (specific gravity ~1.2), nor were any identified using a saturated sugar flotation (specific gravity ~1.24), but eggs morphologically typical for those identified from *Cyrnea*-type gizzard nematodes were confirmed to be present

on examination of the sediment of both of these solutions post-centrifugation (Figure 4.23). The eggs were subsequently identified by flotation using higher concentrations of zinc sulphate (60% and specific gravity ~1.3, and above).

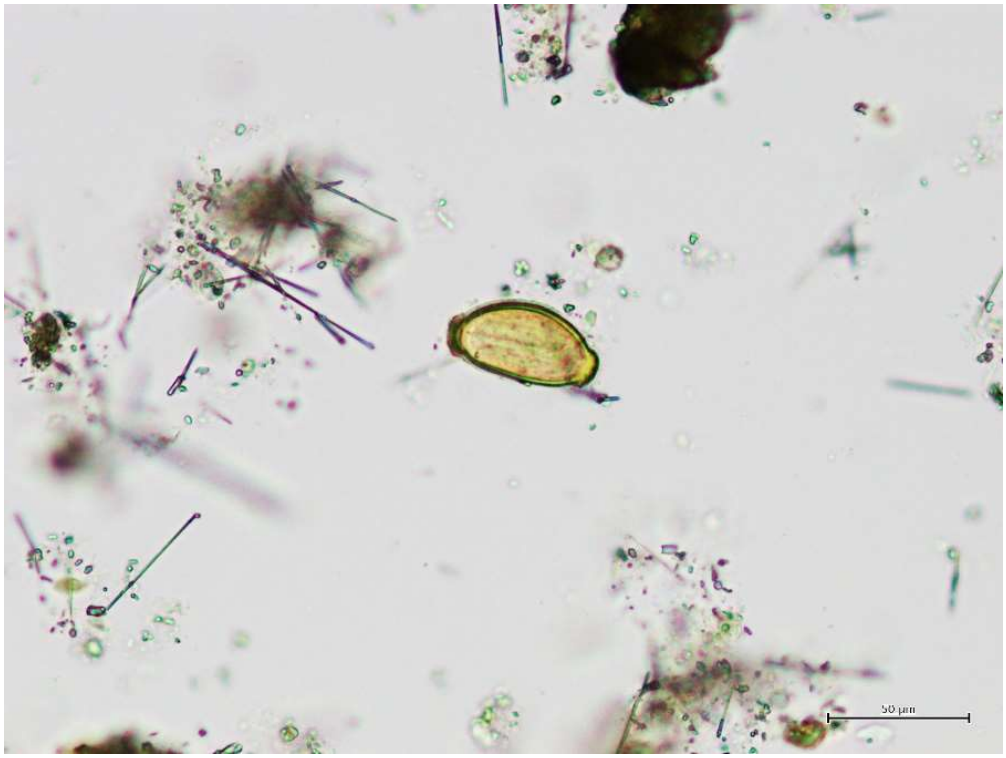

Figure 4.23. Spirurid-type egg found on faecal flotation using 60% Zinc Sulphate solution (bar = 50µm).

4.5. Discussion

This opportunistic survey of gastrointestinal nematodes in kiwi was performed in cooperation with the Wildbase pathology service (Massey University, Palmerston North, New Zealand), which performs necropsies on native species on behalf of the New Zealand Department of Conservation for disease surveillance purposes as well as in the investigation of disease outbreaks. As found in the previous retrospective study by Van Zyl (2014), the study population of 50 kiwi necropsied over an 18-month period showed a strong bias toward North Island brown kiwi (82%), which is considered to be largely due to proximity of the submitters to the facility, being located in the central North Island. Among this cohort, there was a further bias (17/41, 41.5%) towards those submitted from the Northland region. The relative lack of representation

from other kiwi species renders meaningful comparative commentary difficult and, unless otherwise specified, comments are therefore generalised to the whole survey population.

Prevalence of gastrointestinal nematodiasis

The results of this survey confirmed that gastrointestinal nematode infections in kiwi are common, as 94% of the kiwi examined had some degree of nematode burden, and at least five morphologically distinct nematode types were identified with varying prevalence (summarised in Table 4.3). The most common sites parasitised were the gizzard, with 44 out of 50 kiwi (88%) showing some evidence of infection based on gross and/or histological findings, and the caeca, in which infection was confirmed in at least 32 of 48 kiwi (~67%). Co-infections with both gizzard and caecal nematodes were present in around half of the examined kiwi. Additionally, capillarid nematodes were identified from the small intestine of two out of 47 kiwi (~4%), while infection with two different, previously undescribed spirurid-type nematodes were found in the proventriculus of five of 47 kiwi (~11%) and confirmed grossly and/or histologically within the small intestine of 11 of 47 kiwi (~23%).

The survey findings also support the supposition that the previous retrospective prevalence study based on histological diagnosis and reporting of gastrointestinal nematode infection (van Zyl, 2014) significantly underestimated how common nematodiasis is in kiwi. For example, among the NIB kiwi examined in the course of this survey, gross confirmation of some degree of gizzard nematode burden was found in 36 out of 41 birds (~88%), compared with 60 of 228 (~26%) from the retrospective study (van Zyl, 2014). The confirmed burden in many cases was relatively low which may go some way towards explaining a lower prevalence of nematodiasis based on histology alone, when only a small, variably representative section of the gizzard is processed for histological analysis. Even within this survey, when the gross presence or absence of nematodiasis was often already known at the time of histological processing (thereby introducing a potential unconscious or even conscious bias in the section of gizzard selected and

Table 4.3. Summary of origin, presumptive identification, morphological features, and gross prevalence of gastrointestinal nematodes identified from the survey population of 50 kiwi.

Primary location	Order	Genus	Length	Subgross features	Eggs	Gross prevalence
Proventriculus	Spirurida	Unknown	up to 1.2 cm (F)	Tail tapering to a point (F)	50-55x25-30 µm, larvated, asymmetric with flattened bipolar caps	5/47 (~11%)
Gizzard	Spirurida	Presumptive *Cyrnea*[a,b]	4-6 mm (F)	Bluntly rounded tail (F); tightly coiled tail with caudal alae (M)	50-55x25-30 µm, larvated, asymmetric with flattened bipolar caps	41/40 (82%)
Small intestine	Spirurida	Unknown	up to 4 cm (F); up to 3 cm (M)	Narrow cervical alae (F&M); Tail tapering to a point (F&M); Sublateral caudal alae (M)	44-46x24-28 µm (possibly immature), larvated, asymmetric with indistinct bipolar caps	10/47 (~21%)
Small intestine	Enoplida	*Capillaria sensu lato*	~1.5 cm (F)	Very fine body diameter	54-57x20-23 µm, non-larvated, barrel-shaped with distinct bipolar plugs	2/47 (~4%)
Caeca	Ascaridida	Presumptive *Heterakis* (syn. *Kiwinema*)[a,c] or novel genera[b]	up to 7.5 mm (F)	Elongated thin tail (F&M)	64-74x34-39 µm, non-larvated, ovoid and blunt-ended with a rough coat	32/48 (~67%)

[a](Harris, 1975), [b](Clark, 1983a, b), [c](Inglis and Harris, 1990)

processed for histology), infection was confirmed histologically in only ~58% (24/41) of total cases for which gizzard nematodiasis had been grossly confirmed.

Similarly, from the caeca, gross evidence of nematode infection in the NIB kiwi of this survey was found in 29 out of 39 birds (~74%) compared to 36 out of 217 (~17%) described by van Zyl (2014), with the added caveat that histological evidence of small intestinal and caecal nematodiasis was combined under "intestinal" origin in the retrospective analysis (although most likely to predominantly reflect caecal infection). While the burden of caecal nematodiasis was often significantly higher than that of the gizzard nematodes, the localisation almost exclusively within the lumen of the caeca means that loss of caecal contents at the time of or post-collection of the samples could greatly reduce the probability of nematodes being caught in histological section. In this survey, even where there was potentially a greater conscious effort to preserve caecal contents during collection of fixed samples, in only 19 of 29 (~66%) total birds where caecal nematodiasis was confirmed grossly and histology performed was infection also confirmed histologically.

It was also observed that caecal nematodes often appeared more degenerate, both grossly and in histological section, than other nematodes harvested from different anatomic sites in the same bird, which could be a reflection of the fermentative environment of the caeca (Potter et al., 2006) having a detrimental effect on post-mortem nematode preservation. It is possible that this may have contributed to some extent to an underestimation of observed nematode burdens, and even potentially a complete loss of identifiable organisms from the caeca of some kiwi in which longer post-mortem intervals had passed prior to examination.

Gizzard spirurids

The only formally identified spirurid nematode of kiwi, originating from the gizzard, was designated *Cyrnea (Cyrnea) apterycis* and placed within the family Spiruridae by Harris (1975). However, as the other subgenera of the genus *Cyrnea* (i.e. *Procyrnea* and *Metacyrnea*) have both since been raised to the level of genus (Chabaud, 2009), the name *Cyrnea apterycis* for the

kiwi nematode is therefore probably now adequate, and the related genera now reside within the family Habronematidae (Chabaud, 2009). Clark (1983a, b) subsequently suggested the existence also of one or possibly two further, novel *Cyrnea* species although the only published information, in the form of two conference abstracts, does not provide any detail as to the precise anatomic origin of the novel organisms nor of the specific morphological features that differentiate them from the species identified by Harris (1975). Clark further suggested the existence of another novel spirurid, from the genus *Tetrameres* (Clark, 1983b), while the only other spirurid genus that has been described (in brief) from kiwi was identified histologically within the gizzard and thought most likely to be *Acuaria* sp. as may be commonly found in finches and other ground-feeding birds (Orr, 1995). Members of the Acuariidae family have also previously been listed among those identified from kiwi (Reid and Williams, 1975); however, in neither case were included any details regarding how this identification was reached, and it could be speculated that on the finding of spirurid-type nematodes parasitising the gizzard lining of kiwi, some extrapolation from the common species of spirurids infecting more well-known avian hosts may have been applied.

Based on limited light microscopic and SEM evaluations, the most common nematodes identified from the gizzard of kiwi in this survey resembled *C. apterycis* as described by Harris (1975), or a closely related species as suggested by Clark (1983a, b); whether such morphological features as the notable difference in spicule length ratio observed within some males could fall within the bounds of intra-species variation is uncertain. One interesting potential discrepancy was the morphological appearance of the eggs. In the formal written description, Harris (1975) gives only a size range for the eggs of *C. apterycis* and they are illustrated only within a line drawing that includes the uterus toward the posterior end of the female nematode, in which they are depicted as a simple ovoid shape. In contrast, the eggs examined from multiple female gizzard nematodes originating from multiple different kiwi hosts in this survey all presented with a very distinctive, asymmetrical appearance with bipolar, flattened “caps” – the exact

terminology for (or purpose of) which is uncertain. Examining the descriptions of closely related nematodes does not clarify matters. Illustration and description of the eggs of *Procyrnea kea* are depicted in a line drawing and described in text, strongly resembling those found in the gizzard nematodes of this survey: "…shape unusual in having one side of the egg markedly more convex than the other, and in bearing a subterminal flange at each end of the egg" (Clark, 1978; p. 326). The eggs of *Procyrnea uncinipenis*, a parasite of rhea (*Rhea americana*), are described as "bioperculated" (Ederli and de Oliveira, 2019; p. 739), and images of *Procyrnea*-type eggs found on faecal flotation from rhea in another study appear to illustrate this appearance (Gallo et al., 2018). However, in the first description of *Procyrnea choique*, also originating from rhea (*Rhea pennata*), eggs are described simply as "ellipsoid, thick-shelled, embryonated" (Bagnato et al., 2018; p. 505). Similarly, line illustrations of the eggs from *Cyrnea (Cyrnea) eurycerca* (Seureau and Quentin, 1983) and *Cyrnea (Procyrnea) mansioni* (Quentin et al., 1983) are depicted as a simple ovoid shape, as they are in illustrations from three further formal descriptions of new *Procyrnea* species (where they are also described in text as "ellipsoid") (Zhang et al., 2004).

It was noted on microdissection that, within the uterus of the kiwi *Cyrnea*-type gizzard nematode, as the eggs matured they altered in morphology from a smaller, simple ovoid shape to the larger, asymmetric and ornamented appearance. While the depiction only of immature forms of the egg could potentially explain the discrepancy between the observed egg morphology in this survey and that illustrated in utero by Harris (1975), it seems less likely that this should be the case from multiple formal descriptions of *Cyrnea* and *Procyrnea* species, and so the potential for differing egg morphologies between and within these closely related genera remains. Alternatively, it may be that detailed egg morphology is simply considered to be of minor diagnostic importance in formal nematode description. From a limited number of reports reviewed, eggs from species of *Acuaria* appear to be predominantly described and illustrated as a simple ovoid shape (e.g. Mutafchiev et al., 2013; Mutafchiev et al., 2017).

Many habronematids of avian origin do parasitise the gizzard (Mawson, 1968), where they may be found within or beneath the koilin lining; however, despite this superficially invasive behaviour, published reports of clinically significant disease in association with their presence appear to be infrequent. Clinical disease and potential mortality attributed to high burdens of infection with *Sicarius uncinipenis* (syn. *Procyrnea uncinipenis*, Ederli and de Oliveira, 2019) have been described in young, captive-bred rhea, presenting with ulceration, necrosis, and haemorrhage of the ventricular mucosa (Zettermann et al., 2005; Avelar et al., 2014; Ederli and de Oliveira, 2014). Morbidity and mortality has also been described in association with *Procyrnea* sp. infection of passerines within a zoo aviary (Niemuth et al., 2013). In these cases, histology showed fragmentation of the koilin with exudation of fibrin, inflammatory cells, and necrotic cellular debris, secondary bacterial and/or fungal infections, and occasional mucosal ulceration. The affected birds comprised several different passerine species, and it was unclear whether the apparently atypically severe clinicopathological response among this group was attributed to an increased susceptibility in passerines in general, or high exposure levels or other management aspects within the captive institution.

The *Cyrnea*-type gizzard nematodes in kiwi found in the current survey did commonly invade or partially invade the superficial koilin and deeper mucosa, although in most cases similar nematodes were found within luminal contents as well as embedded within the lining. It was observed that the koilin layer quickly lost integrity as a result of autolysis, and so it is difficult to comment on how much of this variation in localisation may be secondary to post-mortem change. This autolytic change also made histological observations on the true effect of parasitism to the superficial koilin difficult. Histologically, of the nematodes that were embedded within the gizzard lining, adults appeared predominantly restricted to the koilin or very superficial glandular mucosa where they typically did not incite a significant inflammatory response, while larval forms could be present at all levels of the mucosa. Variably sized larval forms within the lamina propria were associated with a localised mixed inflammatory response,

and in at least one case with a particularly high burden of gizzard nematodiasis, there was evidence of regionally extensive loss of gastric glands and fibroplasia in association with their presence.

As the majority of the kiwi necropsied as part of this survey originated from the wild and were found dead, antemortem clinical information is limited to non-existent, but based on both gross and histological evaluation of the gizzard no significant acute, ulcerative or necrotizing response to the presence of nematodes was observed among the survey population. However, in two separate additional cases that were brought to the attention of this investigation from outside of the survey population, the question of potentially significant pathology or clinical disease in conjunction with gizzard nematodiasis was raised. Both cases had similar histories, being juvenile male kiwi originating from intensive management settings, and both had a history of eating soil and/or stones leading to radiographic diagnosis of gizzard impaction. In one case, the kiwi died before further diagnostics or treatment could be performed and histology found regionally extensive ventricular ulceration and inflammation along with the presence of many gizzard nematodes. In the second case, the kiwi was treated for the gizzard impaction and other medical issues including coccidiosis and a beak injury; however, clinical improvement was only recorded after the endoscopic diagnosis of, and additional treatment targeting, an erosive ventriculitis and heavy burden of gizzard nematodes.

In both of these cases there is no definitive proof of cause and effect between the histologic or endoscopic evidence of ulcerative ventriculitis and the presence of nematodes. Ulceration and inflammation may have occurred secondary to mechanical damage from the gizzard impaction with stones and/or other pathophysiological factors, e.g. a non-specific stress response, and in the second case especially the diagnosis and treatment of concurrent coccidiosis and other medical issues has also to be taken into account. However, these cases raise speculation that in some circumstances – for example, immunologically naïve juvenile birds being exposed to a

magnified parasite burden in an intensive management setting – a normally well-tolerated nematode infection could develop into or contribute towards clinically significant disease. Although statistical analysis was not performed, it was observed in this survey that higher burdens of gizzard nematodes were more commonly found in juvenile and subadult kiwi, and the greatest evidence of histological gizzard pathology within the survey population was from a wild juvenile kiwi. The potential that more severe inflammatory responses may be associated with infection by a different species of gizzard nematode may also require consideration but was not explored further in this study.

Basic histomorphological features of the nematodes within gizzard sections appear typical for that of spirurids, including the presence of large lateral chords and larvated eggs in utero (Gardiner and Poynton, 1999). The distinct finding of a single, low lateral ala (or longitudinal ridge) in many cross-sections, especially those of confirmed and presumed adult male nematodes, is also described in histological sections of *Habronema microstoma*, an equine habronematid (Chitwood and Lichtenfels, 1972). However, the precise diagnostic significance of this is uncertain as the presence and extent of lateral alae appeared to be somewhat variable among the few examples of gizzard nematodes (of both sex) examined by SEM, and more extensive imaging to document this further would be interesting. Specific histological studies in which typical gizzard nematodes were individually embedded and step sections taken to evaluate the cuticular ornamentations may also help to further elucidate the histomorphology of these parasites.

Proventricular spirurids

Low numbers of morphologically distinct nematodes were identified in the proventriculus of just five kiwi. These did not correspond with the description of *C. apterycis* although the egg morphology by light microscopy was indistinguishable from the *Cyrnea*-type egg morphology, supportive of another member of the spirurid order. Adult female proventricular-type nematodes, confirmed by presence of eggs on microdissection, were approximately twice the

length of the *Cyrnea*-type female gizzard nematodes, with a more slender tail conformation that tapered to a point. Small numbers of similar, longer and more slender nematodes were also found admixed among the more typical *Cyrnea*-type nematodes from within the gizzard of three of the same five birds suggesting that these proventricular-type nematodes may not be as specifically restricted to the proventriculus as the *Cyrnea*-type nematodes appear to be to the gizzard. Morphologically distinct males were not specifically identified from the proventriculus, but it may be speculated that if the males of this nematode species present with a similar, tightly coiled tail as do the male *Cyrnea*-type gizzard spirurids, these would be more difficult to differentiate by such low magnification features as body length or tail conformation, and so more frequent mixed infections within the gizzard could not be ruled out without detailed evaluation of sometimes large numbers of nematodes. A precise morphological identification of the proventricular-type spirurids was not attempted. However, they do not appear consistent with *Tetrameres* – the existence of a novel species of which infecting kiwi was suggested by Clark (1983a, b), and which do parasitise the proventriculus of avian hosts (Chabaud, 2009) – as female tetramerids are generally described as having coiled or globular bodies (Chabaud, 2009).

Histological confirmation of proventricular nematodiasis was rare, with only one case found in which both the proventricular-type nematodes were identified grossly and larval nematodes were evident histologically embedded within the superficial proventricular mucosa. The larvae within this section exhibited no specific histomorphological features to distinguish them from larvae within the gizzard sections, and so spillover infection from the gizzard could not be ruled out. However, given the high prevalence of gizzard nematode infection, this single histological example of proventricular mucosal invasion tends to suggest that immature forms of the proventricular-type parasites may also invade the mucosa at this site.

Intestinal spirurids

No nematodes corresponding to the large spirurid-types found in the small intestine have been described from kiwi previously. Although the female nematodes did, on initial superficial

examination, resemble ascarids (e.g. *Toxocara* or *Porrocaecum* spp.) in features including their size and presence of narrow cervical alae, evaluation via SEM ruled out the typical ascarid tri-lobed lip conformation, and the presence of larvated eggs was supportive of another member of the spirurid order. No further attempt at a specific morphological identification of these organisms was made.

There was a clear association between the presence of these nematodes within the small intestinal lumen and the gross finding of focal or multifocal mural nodules, with histological confirmation of larval nematodes present within the centre of many of the mural nodules suggesting they may enter the intestinal wall to mature as part of their life cycle. However, the potential clinicopathological significance of such infections to the kiwi host remains uncertain. In none of the cases did there appear to be any breach of the integrity of the intestinal wall in association with the presence of the mural lesions.

Intestinal capillarids

Two NIB kiwi were found to contain a very few nematodes morphologically consistent with capillarid origin, identified from the small intestine. The capillarid group have a complicated and contentious taxonomy, at different times consolidated into the single genus *Capillaria* or differentiated into up to 27 proposed genera (Gibbons, 2010). Of these, up to 10 genera have to date been identified in avian species (Yabsley, 2008; Tamaru et al., 2015), all infecting sites within the gastrointestinal tract where adult nematodes most commonly burrow within the mucosal lining (Yabsley, 2008; Anderson and Bain, 2009). As mucosal scrapings and intestinal contents were not collected separately in this survey, and no sections of nematodes suggestive of capillarid origin were identified histologically, the precise site of infection in kiwi remains uncertain. Assuming mucosal localisation, the apparent low prevalence of infection in this survey may be an artefact of inconsistent or incomplete small intestinal mucosal scrapings, as in many cases the degree of autolysis was considered too great for scraping to be warranted or effective. Additionally, the very fine nature of the nematodes means that in low numbers they

could easily have been missed in cases where the small intestinal contents were particularly thick/dense or intensely coloured.

Clark (1983a, b) previously suggested the existence of one or two new species of *Capillaria* present in kiwi that were yet to be formally identified, and also made the statement that these were "notable for their scarcity" as well as for their small size, with males found to be only 5mm in length (Clark, 1983b; p. 93). No further detail as to morphology or precise anatomic origin was given. The capillarid nematodes found in this survey were around 1.5cm in length with at least one of these confirmed by the presence of eggs to be an adult female; again, it is possible that if these do reflect the same as those previously encountered by Clark, the much smaller males were simply missed on gross evaluation. In any case, the apparent low incidence of infection and low parasitic burdens (whether an underestimation or not), along with a lack of histological evidence of infection identified in any of the examined cases, does suggest that the potential pathogenicity of small intestinal capillarid infections in kiwi is likely to be negligible.

Ventral dermatitis associated with the migration of capillarid nematodes within the epidermis around the vent has been described in juvenile rowi being raised on an island sanctuary (crèche) (French et al., 2020a; Chapter 3). Similar lesions have not been recorded in kiwi of other species or geographic origin, and were not apparent among any of the kiwi evaluated in this survey. Based on molecular evaluation, the capillarids associated with the rowi skin lesions aligned among other sequences identified as from *Eucoleus* species, which in birds are most commonly reported as parasitising the upper gastrointestinal tract (oral cavity, oesophagus, crop, or more rarely, gizzard) (Yabsley, 2008). While kiwi do not have a crop, evaluation of the oral cavity or oesophagus for any nematodes or lesions suggestive of their presence was not specifically undertaken as part of this survey, and so potential infection in these sites (not previously reported in kiwi) may have been overlooked. Any association between the intestinal capillarid infections of kiwi and those causing skin disease in juvenile rowi remains unknown.

Caecal heterakoids

The only formally identified nematode originating from the caeca of kiwi was originally designated *Heterakis gracilicauda*, from the family Heterakidae, by Harris (1975). Subsequent to this identification, Clark (1983a, b) suggested also the existence of at least five novel nematodes from the superfamily Heterakoidea, two belonging within the genus *Heterakis* and three requiring the additional creation of new genera near *Spinacauda*, *Hatterianema*, and/or *Lauroia*. However, once again, no detail as to their precise anatomic origin or the specific morphological features differentiating them from *H. gracilicauda* was provided, with the exception of a mention that *Lauroia* were the only members of the Heterakoidea lacking a pre-cloacal sucker (implying, perhaps, that at least one of the novel species presented similarly) (Clark, 1983b). Indeed, while Clark acknowledged the existence of *H. gracilicauda*, it was not entirely clear from the available, brief, published conference abstracts whether he included this organism among the heterakoids he had personally identified from kiwi. This question arises because *H. gracilicauda* was subsequently re-evaluated and, finding morphological affinity to *Hatterianema hollandei* – the only member of this genus and a parasite of another ancient New Zealand species, the tuatara (*Sphenodon punctatus*) – the creation of a new genus *Kiwinema* for the kiwi nematode was proposed (Inglis and Harris, 1990). A new family, Kiwinematidae, was also created to incorporate both of these native New Zealand parasites, considered to reflect some of the most basal members of the heterakoid superfamily (Inglis and Harris, 1990).

Formal description of the sometimes-innumerable nematodes harvested from the caeca of kiwi in this survey was not attempted, but they were, on the whole, considered to be morphologically consistent with *Heterakis* (syn. *Kiwinema*) *gracilicauda* (Harris, 1975; Inglis and Harris, 1990) or related heterakoid nematodes as suggested by Clark (1983a, b), based primarily on size, egg morphology, and variably elongated, slender tails. The identification of *Heterakis* spp. relies largely on anatomic features of the male, particularly the size and shape of the spicules (Cupo and Beckstead, 2019). While the females of *Heterakis* species are described as “generically

identical" (Cupo and Beckstead, 2019; p. 382), the eggs of the females evaluated in the course of this survey exhibited a distinct rough coat, a detail which was not included in the original description of *H. gracilicauda* (Harris, 1975), but which was emphasised in its re-description (Inglis and Harris, 1990) as a feature more in common with eggs of *H. hollandei* than those of other avian *Heterakis* spp. which typically have a smooth surface (e.g. Cupo and Beckstead, 2019).

Based on histology, the caecal nematode infections in kiwi in this survey were almost exclusively luminal in location, with (taking into account limitations in histological evaluation due to degrees of autolysis) no indication of a significant active inflammatory response either in relation to or incidental to their presence. Typhlitis in association with a kiwi heterakid infection has been described in one previous case report (Alley and Gartrell, 2006); in this bird, which also had concurrent nematode LM, the colonic mucosa appeared diffusely sloughed histologically, with little remaining viable glandular tissue, haemorrhagic and necrotic debris present over the exposed mucosal surface, and multiple sections of nematodes within the lumen morphologically consistent with heterakid species. No similar changes were identified in any of the survey kiwi, and whether or not, perhaps in rare cases, caecal nematode infection may be associated with clinically significant inflammatory disease remains unresolved.

Heterakid nematodes infect the caeca of many ground-feeding avian species (Bobrek et al., 2019; Cupo and Beckstead, 2019). In the majority of cases infections are considered to be of limited clinicopathological significance, with the notable exception of *Heterakis gallinarum* which may be an indirect cause for significant morbidity and mortality in poultry and game bird species as the only known vector for the protozoal organism *Histomonas meleagridis* (the causative agent of avian histomoniasis or "blackhead") (Cupo and Beckstead, 2019). However, various reports do suggest there is a difference in host susceptibility to development of potential pathological lesions directly related to the nematode; there are, for example, reports of nodular

typhlitis and the formation of stromal neoplasms associated with infections of *Heterakis isolonche* and/or *H. gallinarum* in pheasants, while similar infections are considered largely benign in other species (Griner et al., 1977; Menezes et al., 2003). Heavy burdens of *H. gallinarum* may be related to ill-thrift in poultry (Cupo and Beckstead, 2019) but while high burdens of infection – often numbering in the many hundreds of organisms – were commonly found, the majority of kiwi in this survey were presented dead from the wild with no prior clinical data available, and whether any potential similar relationship between heavy infections and detrimental effects on body condition could occur in kiwi again remains uncertain.

Gastrointestinal mural granulomas

Distinctive, focal granulomas, histologically differing from either the more typical inflammatory response to the presence of larval nematodes found within the lamina propria of the proventriculus and gizzard or the larger, grossly evident mural nodules in the small intestine, were identified in the wall (deep mucosa, submucosa, and/or muscularis) of five kiwi, sometimes containing sections of very small larval nematodes. These were identified focally in the gizzard (#4) or proventriculus (#47), or multifocally in gizzard, distal ileum, and distal caecum (#22), proventriculus and gizzard (#39), or distal ileum and distal caecum (#40), and histologically resembled the lesions described in viscera and brain caused by the aberrant migration of larval *T. cati* (French et al., 2020b; Chapter 2). The specific cause of these lesions in the gastrointestinal tract remains uncertain, however, as even where larvae could be identified they included no distinguishing histomorphological features.

It seems likely that these lesions are caused by the early invasion and migration of an acquired or non-host-adapted nematode infection rather than the local tissue invasion by more typical, host and/or site-adapted nematodes as part of their normal life cycle. While larval forms of *Cyrnea*-type spirurids do appear to commonly invade and inhabit the deep gizzard mucosa for a time, the relative scarcity of this distinctive, deeper mural inflammatory response in contrast to the high prevalence of gizzard infection suggest these organisms are unlikely to be the cause of

these more rare, discrete mural granulomas at the proventricular and ventricular sites. Similarly, the infective larvae of *H. gallinarum* are reported to undergo a brief tissue phase, entering and remaining within the caecal mucosa for several days (Cupo and Beckstead, 2019); whether this phase occurs in all heterakoid species, and whether or not tissue migration may in some cases extend beyond the mucosa producing a localised granulomatous inflammatory response within the caecal wall is uncertain, but mucosal localisation by larval nematodes was not otherwise evident among the many kiwi in this survey with confirmed caecal nematode infections, suggesting that this explanation is also an unlikely one.

For such lesions within the small intestine, an early-stage infection by the intestinal-type spirurid nematodes – which larvae do appear to invade and mature deep within the muscularis – would be a consideration, but one that does not necessarily explain the lesions at other sites. It is, however, worthy of note that four out of the five affected kiwi did have similar granulomas present within the liver and lung consistent with visceral LM, raising the possibility that the gastrointestinal lesions could reflect an early migration stage of infective *T. cati* larvae; based on the life cycle of *T. canis* in its definitive host, infective *Toxocara* larvae typically hatch in the duodenum and penetrate the intestinal wall at this site (Schnieder et al., 2011), but in an aberrant host the site of hatching and initial migration may be less tightly controlled. Arguing against this hypothesis though, in one of these cases (#22) the cross-sectional diameter of the intralesional larvae exceeded that of the infective larval form of *T. cati* (Nichols, 1956a).

Possibly, other aberrant, dead-end infections by nematode larvae may occur in kiwi, given their ground-feeding lifestyle and potential exposure to a variety of infective eggs originating from the faeces of other animals, and the occasional gastrointestinal mural granulomas at different levels of the gastrointestinal tract may be a non-specific response to organisms that are able to hatch and invade locally, but which are subsequently destroyed by the inflammatory response to their presence. Molecular evaluation of sections containing such lesions, where larvae are

evident, may help to elucidate the cause(s) but care would have to be taken to avoid the overwhelming amplification of DNA from the more typical nematodes that may also be present in gastrointestinal sections.

Nematode larva migrans

Cases of visceral and neural LM were identified only in NIB kiwi in this survey. Lesions suggestive of (i.e. no larvae present) or confirmed (i.e. larvae present) nematode LM were diagnosed in the viscera of 17 out of 40 (42.5%) NIB kiwi in which liver (n = 40) and/or lung (n = 37) were evaluated histologically in this survey, compared to only 15 of 333 (4.5%) NIB kiwi necropsied over the course of ~11 years in which visceral organs were evaluated histologically, as reported in the retrospective study by van Zyl (2014). Some of this discrepancy may be attributed to the deliberate histological examination of internal organs in almost every case included in this prospective survey, including many cases where a gross diagnosis (or the post-mortem condition) would normally have led to a decision not to proceed with histology, but this does support a significant under-estimation of the prevalence of such lesions based only on the retrospective data. Even within this study there may be some degree of underestimation, as the diagnosis relies on the identification of what are often localised granulomas that are randomly distributed within the organ parenchyma. Conversely, it is also worth noting that for the majority of the cases the diagnosis was presumptive, based solely on the presence of inflammatory lesions which are characteristic of but not pathognomonic for the disease, and other potential causes for such lesions could not be entirely ruled out.

Neural LM, considered likely to be the most clinically significant form of disease (as discussed in French et al., 2020b, Chapter 2), was diagnosed with less frequency than visceral disease in this survey despite performing brain histology on many cases which would not ordinarily have had this evaluation done routinely; brain lesions were found in only 4 out of 32 (12.5%) NIB kiwi for which sections of brain were examined histologically, which was still increased comparative to the retrospective study in which 6 out of 158 (3.8%) cases of neural LM were reported (van Zyl,

2014). Brain lesions were never identified in the absence of lesions also in the liver and/or lung, which is consistent with the migration pattern of *Toxocara* spp. via the liver to the lungs, prior to more widespread dissemination (Strube et al., 2013). While it has been previously shown that the distribution of *Toxocara* larvae within aberrant or paratenic hosts differs between species (Beaver, 1969; Strube et al., 2013), the evaluation of other potentially significant sites of infection, e.g. skeletal muscle, eye, or spinal cord, was not attempted as part of this study.

It has been previously established through molecular evaluation that a primary cause for visceral and neural LM in NIB kiwi is the aberrant migration of *T. cati* larvae (French et al., 2020b; Chapter 2). Despite the single report of a kiwi presenting in the role of a definitive host of *T. cati* (Clark and McKenzie, 1982), no adult ascarid nematodes consistent with *Toxocara* species were identified in any of the kiwi examined. However, it was notable that among the 17 cases of suspected and confirmed LM were included nine out of the 11 kiwi in which the intestinal-type spirurid nematodes were identified grossly or histologically, raising the question as to whether larval migration beyond the intestinal wall could occur in some of these cases. There is some precedent for this within the spirurid order: lesions of nematode LM associated with the aberrant migration of larvae from the genus *Gnathostoma* are described in humans and the larvae may also migrate within the liver and abdomen of their definitive host before maturation to adults within nodules in the gastric wall (Herman and Chiodini, 2009). The morphology of the kiwi intestinal-type spirurids in no way resembles that of gnathostomes, though, which have distinct cephalic hooks and variably extensive cuticular spines (Anderson et al., 2009). There have also been occasional reports of aberrant neurological localisation of avian spirurid nematodes that normally parasitise the air sacs of their hosts e.g. *Serratospiculum* or *Serratospiculoides* spp. (Hawkins et al., 2001; Van Wettere et al., 2018), but these cases were not restricted to larval migration, including often the confirmed presence of adults and/or eggs at the aberrant site, and so the histological presentation of such infections would not be typical for LM.

Nevertheless, molecular evaluation to confirm the cause for LM in these kiwi cases would be interesting. The overlap between concurrent presence of LM and intestinal spirurids could simply be coincidental, or a result of geographical or environmental factors that increase potential for soil contamination and/or survival of the infective eggs of both spirurid and ascarid nematodes. The latter possibility is supported to some extent by not only the prevalence of cases from Northland (which could simply reflect the overall bias toward submissions from this area) but also several small geographic clusters of such dual infections found in groups of two to four birds originating from the same site and within the same time frame.

Faecal flotation

For kiwi samples submitted to the SoVS parasitology laboratory (Massey University, Palmerston North, New Zealand), faecal flotation using a 33% $ZnS0_4$ solution (specific gravity 1.18-1.2) is routinely performed. The primary purpose for this is largely the diagnosis and monitoring of coccidiosis, a disease of emerging importance particularly in crèche populations (Morgan *et al.* 2012); however, given that the diversity and significance of gastrointestinal nematode infections in kiwi is currently quite poorly understood, the presence of any nematode eggs reported from a faecal sample is often interpreted by the clinician as evidence of patent infection.

Heterakid infections are relatively commonly identified though routine faecal flotation in diagnostic samples from kiwi, although this does not appear to be a particularly sensitive method of detecting infection, at least based on these post-mortem samples in which only 37% of kiwi with confirmed caecal heterakids and concurrent faecal flotations had positive results. This may be misleading; delays between death and necropsy could potentially lead to the continued maturation of larvae within the eggs, increasing the required specific gravity for successful flotation, or even the hatching of the larvae within the faecal contents (Greiner and Ritchie, 1994), although these same confounding factors could equally apply secondary to delays due to transit in samples sent to an external laboratory for examination. Additionally, based on study of *H. gallinarum*, there may be a diurnal fluctuation in ova production (Daş and Gauly,

2014) which could contribute to the variable presence of eggs within the faeces of live birds. Therefore, a negative faecal flotation result cannot be taken as proof of non-infection, while (as is often the case with avian nematode infections (Greiner and Ritchie, 1994)) for a positive result the number of eggs seen per gram of faeces does not appear to have any direct correlation with the degree of caecal nematode burden. The presence of heterakid eggs in the faecal flotation of two cases in this survey where nematodes were not identified on gross evaluation could potentially be explained by a low nematode burden combined with incomplete collection of caecal contents, and/or the complete degeneration of caecal nematodes due to a prolonged post-mortem interval.

Eggs morphologically consistent with strongylid and oxyurid (aka "pinworm") origin were identified from a single kiwi each in this survey, and are sporadically encountered in the routine faecal examination of faeces from live kiwi (B. Adlington, personal communication). No nematodes from these orders have yet been described in kiwi, and these findings are considered most likely to reflect ingestion of eggs in soil or of earthworm origin (e.g. strongylid eggs) and/or coprophagia of other species' faecal material (e.g. pinworm eggs). The identification in four cases of "ascarid" eggs where no ascarids other than the heterakoids were found are similarly likely to have been the result of ingestion of eggs within soil or ingestion of other species' faecal material; coprophagy has been established in the dog to be a potential cause both for over-estimation of patent infections as well as for the presence on faecal examination of the eggs and oocysts of parasites for which dogs are not a definitive host (Nijsse et al., 2014).

Both kiwi in which intestinal capillarid nematodes were found had faecal flotations performed, with no nematode eggs identified in either case; however, eight other kiwi did have *Capillaria*-type eggs reported. It remains uncertain whether these could reflect true infection (with the nematodes missed at gross evaluation) or the ingestion and passing of eggs originating from other species. Given also the unusual case of cutaneous capillariasis in juvenile rowi (French et

al., 2020a; Chapter 3), future investigations may benefit from a careful and thorough examination of both the oesophagus and the small intestinal tract for potential capillarid infections in cases with a positive faecal flotation result. PCR and comparative sequencing of faecal samples carrying capillarid eggs may also allow further elucidation of the true incidence and origin of capillarid infections in kiwi.

Of greatest interest and potential concern is the lack of any spirurid-type eggs identified from kiwi, despite spirurid infections being very common in gizzard, and confirmed at a lesser prevalence also in small intestine and proventriculus. It is repeatedly acknowledged that spirurid eggs in general are difficult to float by routine methods (e.g. Niemuth et al., 2013; Bagnato et al., 2018), but successful flotation has been reported, most commonly using a solution of saturated sucrose (e.g. Mejia-Fava et al., 2013; Gallo et al., 2018) which has a higher specific gravity than 33% zinc sulphate, up to 1.27 or greater.

The mean specific gravity of selected nematode species' eggs has been previously reported as ranging from 1.0559 for *Toxascaris leonina* and *Ancylostoma caninum*, an ascarid and a strongylid (hookworm) respectively, to 1.2376 for *Physeloptera* sp., a spirurid agent (David and Lindquist, 1982); in this study it was discussed that although the spirurid eggs were relatively smaller, their mass per unit volume was higher than that of ascarid or hookworm eggs because they include essentially no perivitellus space. The necessity to use a flotation solution with a higher specific gravity in order to identify spirurid infections in kiwi was supported by an additional investigation using the faecal sample from a juvenile kiwi with an endoscopically confirmed gizzard nematode infection. In this case even the use of a saturated sugar solution with a specific gravity of around 1.24-1.25 did not allow flotation, but zinc sulphate solutions with concentrations of 60% or greater (specific gravity of equal to and greater than 1.3) did confirm the presence of the characteristic, asymmetrical, larvated spirurid eggs within the sample.

These results raise the question as to whether a different approach, using a higher specific gravity flotation solution, should be routinely applied to kiwi faecal samples submitted for diagnostic flotation. Such a decision requires further consideration and investigation into how this could impact the identification and/or quantification of coccidia and other nematode eggs, as higher specific gravities would not only allow the flotation of greater amounts of background faecal debris which may obscure the structures of interest, but the increased osmotic pressure could distort or even destroy some parasitic elements. Given the suggestion that under some circumstances the presence of gizzard nematodiasis may be associated with significant clinical disease, a reliable method of antemortem confirmation of infection would have definite clinical value, and this is certainly an area that demands further research.

4.6. Conclusion

Gastrointestinal nematode infections of kiwi, particularly those of *Cyrnea*-type spirurid infections within the gizzard and heterakoid infections within the caeca, are very common but confirmation of significant gastrointestinal pathology in association with their presence was not consistently found. One possible exception arising from this study was the suggestion that in some cases a heavy burden of spirurid nematodes within the gizzard of juvenile kiwi may have an association with the development or exacerbation of a clinically significant ulcerative ventriculitis. The current routine method of faecal flotation is inadequate for the identification of spirurid eggs, indicating that further investigation into a reliable method for antemortem diagnosis of such infections is required. Also of concern is the relatively high prevalence of *larva migrans* within this survey population. While low burdens of visceral migratory lesions may be of little to no clinical significance to the kiwi host, infections involving the nervous system could have fatal consequences either directly or through predisposition to death by predation or misadventure. These results emphasise that while it may be speculated that host-adapted gastrointestinal parasites of kiwi are well-tolerated on the whole, abnormally high burdens of gizzard nematodes, such as may be encountered in young birds being raised in intensive

management settings, or exposure to more recently introduced, acquired nematode infections such as *Toxocara cati*, are more likely to be associated with significant clinical disease.

Chapter 5: Preliminary molecular characterisation of selected gastrointestinal nematodes from kiwi (*Apteryx* spp.)

5.1. Abstract

A prospective survey of gastrointestinal nematodes infecting kiwi (*Apteryx* spp.) identified at least five morphologically distinct nematode types including three spirurid agents, infecting proventriculus, gizzard, and small intestine; heterakoid ascarids in the caeca; and rare capillarids in the small intestine. PCR, sequencing, and BLAST analysis of regions of the 18S gene of nuclear ribosomal DNA and the COI gene of mitochondrial DNA were performed on a small selection of these nematodes in an attempt to investigate further their identification and diversity. No species-level identification could be obtained for any of the organisms tested; however, results support the presence of multiple heterakoid nematode species infecting the caeca of kiwi, as has been previously suggested based on morphology. Sequence comparison indicated that the intestinal capillarid is a different species to that which has been previously identified causing skin lesions in juvenile rowi kiwi. In addition to the analysis of gastrointestinal nematodes, PCR performed on three cases of visceral and neural nematode *larva migrans* produced results that support the causative agent as *Toxocara cati*, although some issues with probable tissue contamination by DNA originating from the gastrointestinal nematodes was encountered – thus highlighting a need for stricter protocols in sample collection in cases where future molecular evaluation may be indicated.

5.2. Introduction

In a prospective survey evaluating the prevalence of gastrointestinal nematode infections of kiwi (*Apteryx* spp.), the most common sites parasitised were found to be the gizzard, (44 out of 50 kiwi, 88%) and the caeca (32 out of 48 kiwi, ~67%) (Chapter 4). Based on superficial morphological examination, the majority of nematodes present at these two anatomical sites resembled the two kiwi nematodes formally described by Harris (1975), *Cyrnea apterycis* in the gizzard and *Heterakis gracilicauda* in the caeca. However, subsequent to these formal identifications, Clark (1983a, b) presented findings that suggested that kiwi may host more than one species of *Cyrnea* and up to five further heterakoid nematodes yet to be formally described, as well as potential novel species of *Capillaria*, *Primasubulura*, and *Tetrameres*.

Little progress has since been made in evaluation of the diversity of nematodes carried in the gastrointestinal tract of kiwi. However, the original specimens of *H. gracilicauda* were subsequently re-evaluated by Inglis and Harris (1990) and re-classified into a proposed new family, Kiwinematidae, with the kiwi caecal nematode re-named as the type-species of a proposed new genus, *Kiwinema*. This re-description was based on the recognition of morphological features more in common with *Hatterianema hollandei* – the only member of this genus and an intestinal parasite of another ancient New Zealand species, the tuatara (*Sphenodon punctatus*) – than with other *Heterakis* species of avian origin (Inglis and Harris, 1990). Interestingly, and prior to this publication, Clark also proposed that of the novel heterakoid species of kiwi he had examined were two that would require placement into a new genus close to either *Spinicauda* or *Hatterianema*, both of which genera contain nematodes that primarily parasitise reptiles (Clark, 1983a, b).

In addition to the high prevalence and, as yet, unresolved diversity of gizzard and caecal nematodes, the prospective survey (Chapter 4) found low numbers of capillarid nematodes present within the small intestine of two out of 47 kiwi (~4%) (Chapter 4). Clark (1983b) had proposed the existence of one or two novel capillarid species parasitising kiwi, and it has also

been found that a capillarid species, identified as a probable member of the genus *Eucoleus*, is the cause of crusting ventral dermatitis in a population of juvenile rowi kiwi (*Apteryx rowi*) (French et al., 2020a; Chapter 3). Given the geographically localised nature of this unusual skin disease, any potential relationship between this organism and a novel kiwi gastrointestinal capillarid is uncertain.

The prospective survey (Chapter 4) also encountered two previously undescribed nematodes parasitising the proventriculus (present in five of 47 kiwi, ~11%) and the small intestine (present in 11 of 47 kiwi, ~23%) (Chapter 4). Both were considered morphologically consistent with members of the nematode order Spirurida, and it was not determined whether these may reflect novel (undescribed) agents specific to kiwi, or infections acquired from other native or introduced species. There was also observed to be an overlap (nine of 17, ~53%) between kiwi carrying the intestinal-type spirurid nematode and those in which visceral and/or neurological lesions consistent with nematode *larva migrans* (LM) were diagnosed histologically. Previous investigation of LM in North Island brown (NIB) kiwi (*Apteryx mantelli*) implicated larvae of the ascarid nematode *Toxocara cati* as the primary cause (French et al., 2020b; Chapter 2), and so it seems most likely that this loose association is coincidental or due to environmental factors favouring the presence and/or persistence of infective ova of both ascarid and spirurid origin within the kiwi habitat. However, the possibility that agents other than *T. cati* may be involved in this disease could not be entirely discounted.

Accurate morphological identification of parasites encountered in novel hosts, and those that have potentially not been previously encountered or formally described at all, relies heavily on the experience and expertise of the examiner and, of concern, there has been a declining trend worldwide in the number of nematologists skilled in traditional morphological taxonomy (Ferris, 1994). The purpose of this study was to investigate whether the identification and/or species diversity among the gastrointestinal nematodes commonly encountered in kiwi could be further

evaluated by molecular methods, and to assess for any potential involvement of these organisms in the syndrome of nematode LM.

5.3. Methods

5.3.1. Selection of specimens

5.3.1.1. Nematodes

During the course of the prospective necropsy survey, nematodes found within the gastrointestinal tract of 47 kiwi were collected into 70% ethanol, and these were stored at room temperature for up to three years prior to DNA extraction and analysis. For the most common and numerous of the nematodes, those originating from the gizzard and caeca, individual kiwi hosts were chosen to represent at least one of each of the species included in the survey (NIB, little spotted kiwi (LSK), Haast tokoeka (HT) and/or rowi) as well as, among the NIB kiwi, one of each from the broad geographic divisions of this species within the North Island (Northland, Western, Eastern, and Coromandel). Where there were more than one kiwi with specimens available from each species or location, the final choice was somewhat arbitrary, but took into consideration the number of nematodes available from the host/anatomic site and the post-mortem condition of the body as reported at necropsy. While caecal nematodes were often the greatest in numbers, they were also often the most degenerate in morphology even compared to those collected from other anatomic sites in the same bird, and so selection and extractions were performed on these primarily, and then where possible the same host source was sampled for the gizzard nematodes. At least two individual nematodes from each anatomic site (if available) were selected, generally one female and one male.

For the nematodes that had both lower prevalence and burdens, comprising the proventricular- and intestinal-type spirurids and the intestinal capillarids, individual examples representing several different hosts and differing geographical origins were selected for molecular analysis. For molecular comparison with the caecal nematodes of kiwi, the remains of several faecal samples from tuatara submitted to the School of Veterinary Science (SoVS) parasitology

laboratory (Massey University, Palmerston North, New Zealand) for routine faecal flotation were examined under a dissecting microscope, and any nematodes found were collected into 70% ethanol.

5.3.1.2. Formalin-fixed, paraffin-embedded tissue sections

Following the survey results including organ (liver, lung, and/or brain) histology, cases were selected in which both intestinal-type spirurids had been identified grossly and lesions consistent with visceral LM had been identified histologically, including the histological confirmation of larvae in at least one of the organ sections from each host. From these cases "sandwich" sections were cut from the formalin-fixed, paraffin-embedded (FFPE) tissue block, consisting of a 4 µm section mounted on a slide and stained with Haematoxylin and Eosin (H&E), followed by a 10 µm tissue scroll for molecular analysis, then a further 4 µm section mounted and stained with H&E.

5.3.2. Molecular evaluation

5.3.2.1. DNA extraction

For small nematodes (up to 1.5 cm in length), selected individuals were removed from the 70% ethanol, examined under light microscopy (uncleared) to confirm morphology, then either the entire nematode or a segment from the body of the nematode was placed into a 0.2 ml microcentrifuge tube and either extraction was performed immediately or the specimen was frozen until required. A mixture of 200 µL DirectPCR Lysis Reagent (Tail) (Viagen Biotech Inc., USA) and 5µL Proteinase K (20 mg/ml, Ambion, CA, USA) was prepared and 10 µL of this solution added to each PCR tube. Samples were incubated in an Applied Biosystems GeneAmp PCR system 2400 thermocycler (Thermofisher, USA) for 16 hours at 55 °C followed by 1 hour at 90 °C, after which the tubes were checked under a dissecting microscope to confirm successful lysis.

DNA was extracted from larger nematodes (greater than 1.5 cm in length) using a commercial kit (NucleoSpin Tissue kit, Macherey-Nagel, Germany) per the manufacturer's instructions. DNA was extracted from FFPE tissue scrolls using a commercial kit (NucleoSpin DNA FFPE XS kit,

Macherey-Nagel, Germany), per the manufacturer's instructions with the exception of the lysis step which was performed in a 56 °C water bath overnight.

5.3.2.2. PCR

PCR on nematode specimens was performed using primer sets targeting the 18S unit of nuclear ribosomal DNA (rDNA) and the cytochrome oxidase *c* subunit I (COI) gene of mitochondrial DNA (mtDNA), while for FFPE tissue specimens, 18S primer sets and an additional species-specific primer set targeting the rDNA internal transcribed spacer (ITS)-2 region of *Toxocara cati* were applied (Table 5.1). For all primer sets, each PCR contained 1X HOT FIREPol Blend Master Mix (10 mM $MgCl_2$, Solis Biodyne, Estonia), 300 nM each of forward and reverse primers (Integrated DNA Technologies, IA, USA), and 1 µl of template DNA, made to a total of 20 µl with nuclease free water. A negative (blank) control containing 1 µl of nuclease free water in place of template DNA was run simultaneously, as was a positive control consisting of either DNA extracted from an adult *Toxocara canis* (as previously described by French et al., 2020b; Chapter 2), or DNA extracted from an unidentified capillarid nematode originating from the proventriculus of a red-billed gull (*Chroicocephalus novaehollandiae scopulinus*) (as previously described by French et al., 2020a; Chapter 3).

PCR was performed on a Labcycler (SensoQuest, Germany) using a touchdown PCR protocol. For the ITS-2 and 18S primer sets, the following conditions were used: initial activation of 15 minutes at 95 °C, 12 cycles of 95 °C for 30 seconds, annealing for 30 seconds (starting at 60 °C , reducing by 0.5 °C per cycle), and 72 °C for 30 seconds, followed by a further 35 cycles of 95 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 30 seconds, and final elongation at 72 °C for 7 minutes. For the COI primer sets a similar touchdown PCR protocol was used but with lower annealing temperatures, starting at 51 °C and reducing by 0.5 °C per cycle for 12 cycles, then 35 cycles at the final annealing temperature of 45 °C.

The PCR product was separated by electrophoresis on a 1% w/v agarose gel (Bioline, UK) using RedSafe (iNtRON Biotechnology, South Korea) to stain the DNA, and visualized with a gel image

Table 5.1. Primers used in this study, including one set designed for the specific identification of the internal transcribed spacer (ITS)-2 region of nuclear ribosomal DNA of *Toxocara cati*, and several more universal primer sets targeting the 18S gene of nuclear ribosomal DNA and the cytochrome oxidase *c* subunit I (COI) gene of mitochondrial DNA of nematodes. All primers were sourced from Integrated DNA Technologies (IA, USA).

Primer name	Primer sequence (5' to 3')	Target	Approximate amplicon size (bp)	Reference
Tcat1	GGAGAAGTAAGATCGTGGCACGCGT	ITS-2 region (*Toxocara cati*)	400	Jacobs et al., 1997
NC2	TTAGTTTCTTTTCCTCCGCT			
18SF	CCATGCATGTCTAAGTTCAA	18S gene (Nematode)	325	Dangoudoubiyam et al., 2009
18SR	TTATTCTCCGTTACCCGTTA			
Nemo 18S F	GGCTAAGCCATGCATGTC	18S gene (Ascaridoid)	265	Pinelli et al., 2013
Nemo 18S R	ACTTGATAGACACGTCGCC			
Kt875351.1	CCCTAGTTGCGACTTTAAACGA	18S gene (Capillarid)	290	Fischer et al., 2018
Capillaria 18S1R	TCCACCAACTAAGAACGGCC			
COIF	TTTTTTGGTCATCCTGAGGTTTAT	COI gene (Nematode)	385	Guo et al. 2019
COIR	ACATAATGAAAATGACTAACAAC			
JB3	TTTTTTGGGCATCCTGAGGTTTAT	COI gene (Nematode)	450	Zhu et al., 2000
JB4.5	TAAAGAAAGAACATAATGAAAATG			
Eu COI F1-B	GGTCCWYTAGGWATAATYTATGC	COI gene (Capillarid/*Eucoleus* sp.)	290	French et al., 2020a
Eu COI R1	ARATCTAAAGATGCATTRGAAAG			

system (MultiDoc-It Imaging System, UVP, CA, USA). Size of PCR products was estimated in comparison to a HyperLadder™ 100 bp molecular ladder (Bioline, UK).

5.3.2.3. Sequencing and BLAST

Amplicons of the appropriate size were cut from the gel, eluted overnight in elution buffer (10 mM Tris-HCl, pH 8.0), and the eluate submitted to the Massey Genome Service (Massey University, Palmerston North, New Zealand) for bi-directional Sanger sequencing. The resultant forward and reverse sequences were aligned using Geneious v. 10.2.3 (Kearse et al., 2012) and the low-quality ends of sequence alignments were manually trimmed. The resultant sequences were subjected to BLAST (Basic Local Alignment Search Tool) analysis (Altschul, 2014) through the GenBank® database (https://www.ncbi.nlm.nih.gov).

5.3.2.4. Phylogenetic analysis

To further compare the relationships between sequences obtained from this study and comparative sequences as indicated by BLAST analysis through the GenBank database, phylogenetic trees were created for five separate groups of nematode sequences: (a) proventricular, gizzard, and intestinal spirurid 18S; (b) proventricular, gizzard, and intestinal spirurid COI; (c) capillarid 18S including sequences obtained from previous study (French et al., 2020a; Chapter 3); (d) kiwi caecal and tuatara faecal ascarid 18S; and (e) kiwi caecal and tuatara faecal ascarid COI. The top 100 discontiguous megaBLAST results were combined and then filtered to include only sequences identified as from the order Spirurida (groups (a) and (b); members of *Capillaria sensu lato* (group (c)); or the order Ascaridida (groups (d) and (e)). An outgroup sequence was chosen for each individual tree.

Sequences were aligned using MAFFT (Katoh and Standley, 2013), employing the “auto” algorithm setting, in Geneious v. 10.2.6 (Kearse et al., 2012). Maximum likelihood analysis was performed on the alignments using PhyML version 3.0 (Guindon et al., 2010), available on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/phyml/). Phylogenetic trees were inferred employing Subtree Pruning and Regrafting (SPR) branch-swapping and nucleotide

substitution models determined by Smart Model Selection (SMS) (Lefort et al., 2017). Branch support was assessed using an approximate likelihood ratio test (aLRT) with the Shimodaira–Hasegawa-like procedure. Tree visualization was performed with Interactive Tree Of Life (https://itol.embl.de) (Letunic and Bork, 2019).

5.4. Results

5.4.1. Proventricular spirurids

Individual nematodes from three of the five survey NIB kiwi in which proventricular-type nematodes had been identified on gross examination, one from each specific geographic location, were selected for DNA extraction and PCR (Table 5.2). These included an adult female (confirmed by microdissection and identification of eggs) from each of #'s 15 and 47, and a nematode from #36 which was of shorter length (~0.6 cm) but otherwise similar morphology, suspected to be an immature female. Good-quality 18S sequences were obtained from the two confirmed adult females (#15-PV and #47-PV) using the primer set 18SF-18SR, but the third sample (#36-PV) amplified a product which on sequencing and BLAST analysis indicated only a fungal contaminant. Trimmed 18S sequences from the two adult female nematodes were both 326 bases in length and 100% identical with each other on alignment. On BLAST analysis, their consensus sequence aligned among spirurid nematodes with no exact match, the closest being to a sequence identified as *Cyrnea mansioni* (AY702701) with 100% cover and 97.9% pairwise identity (Table 5.3).

Using the COI primer set JB3-JB4.5, only one of the adult female proventricular-type nematode extractions (#15-PV) produced an amplicon. The resultant trimmed sequence was 331 bases in length and on BLAST analysis again aligned among spirurid nematodes with the closest match, to a specimen identified as *Mastophorus muris* (MK867480), showing only 92.3% pairwise identity (Table 5.3). No meaningful results were obtained from any sample using the alternative COI primer set COIF-COIR.

Table 5.2. PCR results using primer sets targeting the 18S region of nuclear ribosomal DNA and the COI gene of mitochondrial DNA from selected gastrointestinal nematodes, including the host information (ID = host identification – refer to Table 4.1, Chapter 4 for further details); species (NIB = North Island brown, HT = Haast tokoeka, LSK = little spotted kiwi); and region of origin within New Zealand (BOP = Bay of Plenty, SI = South Island); anatomic site of origin of the analysed nematodes; and results of PCR (Seq ID = sequence identifier for individual nematodes; 18S = 18S gene of nuclear ribosomal DNA; COI = cytochrome oxidase *c* subunit I; + = produced an amplicon of the appropriate size with nematode origin supported by sequencing and BLAST; - = no amplification or nematode origin not supported by sequencing).

	Host		Proventricular			Gizzard			Intestine-spirurids			Intestine-capillarids			Caecal/Faceal		
ID	Species	Origin	Seq ID	18S	COI	Seq ID	18S	COI	Seq ID	18S	COI	Seq ID	18S	COI	Seq ID	18S	COI
9	NIB	Auckland							#9-SI	+	+						
11	NIB	BOP				#11-G(M)	+	-							#11-C(F)	+	+
						#11-G(F)	+	-							#11-C(M)	+	+
15	NIB	Coromandel	#15-PV	+	+	#15-G(M)	+	-	#15-SI	+	+				#15-C(F1)	-	
						#15-G(F)	+	+							#15-C(F2)	-	
															#15-C(M1)	-	
															#15-C(M2)	-	
20	HT	SI													#20-C(F1)	+	+
															#20-C(F2)	+	+
															#20-C(M1)	+	+
															#20-C(M2)	+	-
21	NIB	Taupo										#21-Cp	+	-			
22	NIB	Northland							#22-SI	+	+						
29	NIB	Northland										#29-Cp	-	-			
32	LSK	Auckland				#32-G(M)	+	-							#32-C(M)	-	-
						#32-G(F)	+	-							#32-C(F)	+	+
35	NIB	Northland				#35-G(M)	+	-							#35-C(M1)	+	+
						#35-G(F)	+	-							#35-C(M2)	+	+
36	NIB	Northland	#36-PV	-	-				#36-SI	+	+						
38	Rowi	SI				#38-G(F)	+	-									
41	NIB	Taranaki				#41-G(M)	+	-							#41-C(F)	+	+
						#41-G(F)	+	+							#41-C(M)	+	+
47	NIB	Northland	#47-PV	+	-												
48	NIB	Northland							#48-SI	+	-						
TuataraA															TuataraA	+	+
TuataraB															TuataraB	+	-

Table 5.3. Top discontiguous megaBLAST results for sequences obtained in this study, including the consensus or individual nematode sequence identifier; the primer set applied to obtain the sequences and the region of DNA targeted (18S gene of nuclear ribosomal DNA or cytochrome oxidase *c* subunit I (COI)); the trimmed lengths of the obtained study sequences; GenBank accession number and the name of the organism as identified in the GenBack submission; and relevant match data (PI = pairwise identity).

Sequence ID	Primers (Target)	Sequence length (bp)	GenBank #	Organism name	Cover	PI	Bit-score	E-value
Proventricular 18S consensus[a]	18SF-18SR (18S)	326	AY702701	*Cyrnea mansioni*	100%	97.9%	577.626	2.53^{e-154}
Gizzard 18S consensus[b]	18SF-18SR (18S)	326	AY702701	*Cyrnea mansioni*	100%	97.5%	553.117	3.09^{e-153}
Intestinal 18S consensus[c]	18SF-18SR (18S)	325	DQ094175	*Onchocerca cervicalis*	100%	97.9%	552.215	1.07^{e-152}
NIB/LSK caecal 18S consensus[d]	18SF-18SR (18S)	326	e.g. LC133190[g]	*Strongyluris calotis*	100%	96.9%	538.690	7.01^{e-149}
HT caecal 18S "main" consensus[e]	18SF-18SR (18S)	326	e.g. LC133190[g]	*Strongyluris calotis*	100%	97.1%	540.494	2.01^{e-149}
HT caecal 18S "outlier" (#20-C(F1))	18SF-18SR (18S)	319	AF083005	*Paraspidodera* sp.	100%	96.9%	530.575	3.54^{e-146}
Tuatara 18S consensus[f]	18SF-18SR (18S)	323	EF180070	*Aspidodera* sp.	100%	98.0%	554.921	9.02^{e-154}
#21-Cp	Kt875351.1-Capillaria 18S1R (18S)	292	e.g. LC052348[h]	*Capillaria madseni*	100%	99.0%	514.345	2.48^{e-141}
#15-PV	JB3-JB4.5 (COI)	331	MK867480	*Mastophorus muris*	98.5%	92.3%	464.629	1.31^{e-126}
#15-G(F)	JB3-JB4.5 (COI)	367	KY284626	*Setaria digitata*	100%	91.6%	505.256	8.69^{e-139}
#41-G(F)	JB3-JB4.5 (COI)	387	NC_044071	*Setaria labiatopapillosa*	98.2%	90.0%	492.329	7.15^{e-135}
#9-SI	JB3-JB4.5 (COI)	351	NC_044071	*Setaria labiatopapillosa*	100%	90.3%	460.936	1.82^{e-125}
#15-SI	JB3-JB4.5 (COI)	392	NC_044071	*Setaria labiatopapillosa*	98.7%	90.7%	516.336	4.30^{e-142}
#22-SI	JB3-JB4.5 (COI)	434	NC_044071	*Setaria labiatopapillosa*	97.5%	90.8%	566.195	4.71^{e-157}
#36-SI	JB3-JB4.5 (COI)	435	NC_044071	*Setaria labiatopapillosa*	98.6%	90.7%	594.595	1.56^{e-165}
#11-C(F)	COIF-COIR (COI)	364	LC533903	*Strongyloides stercoralis*	94.5%	89.2%	454.834	2.52^{e-123}
#11-C(M)	COIF-COIR (COI)	385	KT613893	*Ascaridia galli*	100%	87.8%	483.687	5.53^{e-132}
#20-C(F1)	COIF-COIR (COI)	379	MN729572	*Falcaustra* sp.	100%	90.8%	526.968	5.09^{e-145}
#20-C(F2)	COIF-COIR (COI)	369	FJ009625	*Heterakis isolonche*	99.5%	90.8%	509.836	3.80^{e-140}
#20-C(M1)	COIF-COIR (COI)	391	KU529972	*Heterakis beramporia*	100%	89.5%	521.558	2.24^{e-143}
#41-C(F)	COIF-COIR (COI)	416	KP308361	*Heterakis gallinarum*	100%	87.7%	521.558	2.40^{e-143}
#32-C(F)	COIF-COIR (COI)	384	KT613893	*Ascaridia galli*	100%	87.5%	477.376	2.35^{e-130}
#41-C(M)	COIF-COIR (COI)	378	KT613893	*Ascaridia galli*	100%	88.6%	489.098	1.27^{e-133}
#35-C(M1)	COIF-COIR (COI)	416	KT613893	*Ascaridia galli*	100%	88.0%	526.067	5.64^{e-145}
#35-C(M2)	COIF-COIR (COI)	416	MH571871	*Heterakis spumosa*	98.8%	90.0%	557.626	3.33^{e-154}
TuataraA	JB3-JB4.5 (COI)	434	MH795149	*Baylisascaris procyonis*	98.9%	86.7%	517.951	3.06^{e-142}

[a]consensus of 18S sequences from #15-PV and #47-PV; [b]consensus of all gizzard spirurid 18S sequences; [c]consensus of all intestinal-type spirurid 18S sequences; [d]consensus of 18S sequences from #11-C(F), #11-C(M), #32-C(F), #35-C(M1), #35-C(M2), #41-C(F), and #41-C(M); [e]consensus of 18S sequences from #20-C(F2), #20-C(M1), and #20-C(M2); [f]consensus of 18S sequences from TuataraA and TuataraB; [g]representative of identical alignments with five sequences identified as *Strongyluris calotis* (LC133186 to LC133190); [h]representative of identical alignment with four sequences identified as *Capillaria madseni* (LC052344, LC052346, LC052347, and LC052348).

5.4.2. Gizzard spirurids

One male and one female nematode of the *Cyrnea*-type nematodes found within the gizzard were selected for DNA extraction and PCR from each of five survey kiwi including one LSK (#32), and four NIB kiwi from different regions (#11 – Bay of Plenty; #15 – Coromandel; #35 – Northland; and #41 – Taranaki); the single (female) gizzard nematode found in the sole rowi (#38) representative was also included in the molecular analysis (Table 5.2). Good-quality 18S sequences were obtained from all the samples using the 18SF-18SR primer set. Trimmed sequences ranged from 272 to 326 bases in length, and on alignment with each other eight of the 11 sequences were 100% identical apart from a single ambiguous base in the sequence from #11-G(F); two of the remaining sequences (#15-G(F) and #41-G(F)) exhibited the same, individual base difference from the majority, while the sole rowi representative (#38-G(F)) also contained a single base difference at a different locus. On BLAST analysis, the consensus sequence from all 11 samples aligned among spirurid nematodes with the nearest match again to the sequence identified as *Cyrnea mansioni* (AY702701), showing 100% cover and 97.5% pairwise identity (Table 5.3). Alignment of the 18S consensus from the proventricular-type nematodes and that of the *Cyrnea*-type gizzard nematodes was 98.5% identical (aligned 18S sequences from proventricular and gizzard spirurids are presented in appendix G).

Only two of the 11 gizzard nematode extractions (#15-G(F) and #41-G(F)) produced an amplicon and spirurid COI sequence using the JB3-JB4.5 primer set; no samples amplified with the alternate COIF-COIR primer set. The trimmed sequences were 367 and 387 bases in length and showed 96.5% pairwise identity with each other on alignment. On BLAST analysis, both aligned similarly among spirurid nematodes but with no match closer than 90-91.6% pairwise identity (Table 5.3). Alignment of the COI sequence from the proventricular-type nematode (#15-PV) with those from the two *Cyrnea*-type gizzard nematodes similarly showed only 91.2-92.6% pairwise identity (aligned COI sequences from proventricular- and gizzard-type spirurids are presented in appendix H).

5.4.3. Intestinal spirurids

Individual examples of the intestinal-type spirurid nematodes from five of the 10 grossly affected survey kiwi were selected for DNA extraction and molecular analysis. All affected kiwi were NIB, and selected hosts reflected differing specific geographic locations (Table 5.2). Selected nematodes included three adult females (#9-SI, #15-SI, and #36-SI), one probable adult male (#22-SI), and one suspected immature form (#48-SI). 18S sequences were obtained from all five samples using the primer set 18SF-18SR, with trimmed lengths ranging from 277 to 325 bases; on alignment, the five sequences were identical apart from a single base difference in the sequence from #22-SI (confirmed on repeat PCR) (aligned 18S sequences from intestinal-type spirurids are presented in appendix G). The consensus of the five sequences showed 98.2% and 98.5% identity with the proventricular 18S consensus and gizzard 18S consensus sequences respectively; BLAST analysis of the intestinal 18S consensus sequence aligned among spirurid nematodes with the closest match being to a sequence from *Onchocerca cervicalis* (DQ094175) with 100% cover and 97.9% pairwise identity (Table 5.3).

COI sequences were obtained from four of the five extractions using the primer set JB3-JB4.5, with no amplification from #48-SI using either this or the alternate COI primer set COIF-COIR. Trimmed sequence lengths ranged from 351 to 435 bases. On alignment with each other, the sequences from #9-SI and #15-SI were 100% identical, but there were multiple single base differences between these sequences and the other two, with only 91.6% pairwise identity between the sequences from #15-SI and #22-SI, 96.9% identity between #15-SI and #36-SI, and 93.0% identity between #22-SI and #36-SI (aligned COI sequences from intestinal-type spirurids are presented in appendix H). Despite this, BLAST results from all four sequences aligned nearest the same spirurid sequence, identified as *Setaria labiatopapillosa* (NC_044071), with variable 98.5 to 100% cover but only ~90.3 to 90.8% pairwise identity (Table 5.3). Comparison between the individual intestinal-type spirurid COI sequences with the individual proventricular- and gizzard-type COI sequences ranged from 87.9 to 90.6%.

The top 100 discontiguous megaBLAST results from the 18S consensus sequences of each of the proventricular-, gizzard-, and intestinal-type spirurids included a mixture of nematode sequences from both Spirurida and Ascaridida as well as a few from Oxyurida. The results were combined and filtered to include only the spirurid sequences, leaving a total of 77 GenBank sequences. A sequence identified as from *Aorurus agile* (MN190720), from order Oxyurida, was chosen as the outgroup, and the final alignment was 351 characters in length. The tree was produced using the general time-reversible model with invariable sites and gamma distribution (GTR+G[0.568]+I[0.617]). Within the resultant tree (Figure 5.1), none of the study sequences showed a clear affiliation to any specific superfamily, with proventricular and gizzard sequences both clustering separately among members of Spiruroidea, Habronematoidea, and Acuaroidea. While the closest BLAST match to the consensus intestinal-type spirurid sequence was the filarioid nematode *Onchocerca cervicalis*, within the tree the study sequences clustered distinctly separate from GenBank sequences originating from this and other filarioid nematodes (a list of all GenBank accessions included in the spirurid 18S tree is presented in appendix L).

The top 100 discontiguous megaBLAST results from the individual COI sequences of proventricular-, gizzard-, and intestinal-type spirurids were exclusively from the order Spirurida; combined results gave a total of 141 GenBank sequences. A sequence from *Abbreviata kazachstanica* (MK578751), from the spirurid superfamily Physelopteroidea (not otherwise represented in the BLAST results), was selected as an outgroup and the final alignment was 440 characters in length. The tree was produced using the general time-reversible model with invariable sites and gamma distribution (GTR+G[0.293]+I[0.309]). In the resultant tree (Figure 5.2) the study sequences from all anatomic sites clustered together with no close matches, appearing nearest to members of the superfamily Spiruroidea although comparative sequences from the superfamilies Habronematoidea and Acuaroidea were respectively limited or not represented at all. Again, despite the nearest BLAST results for the gizzard- and intestinal-type sequences being to *Setaria* spp. from the filarioid superfamily, within the tree all the study

sequences clustered distinctly separate from these and other filarioid nematode sequences (a list of all GenBank accessions included in the spirurid COI tree is presented in appendix M).

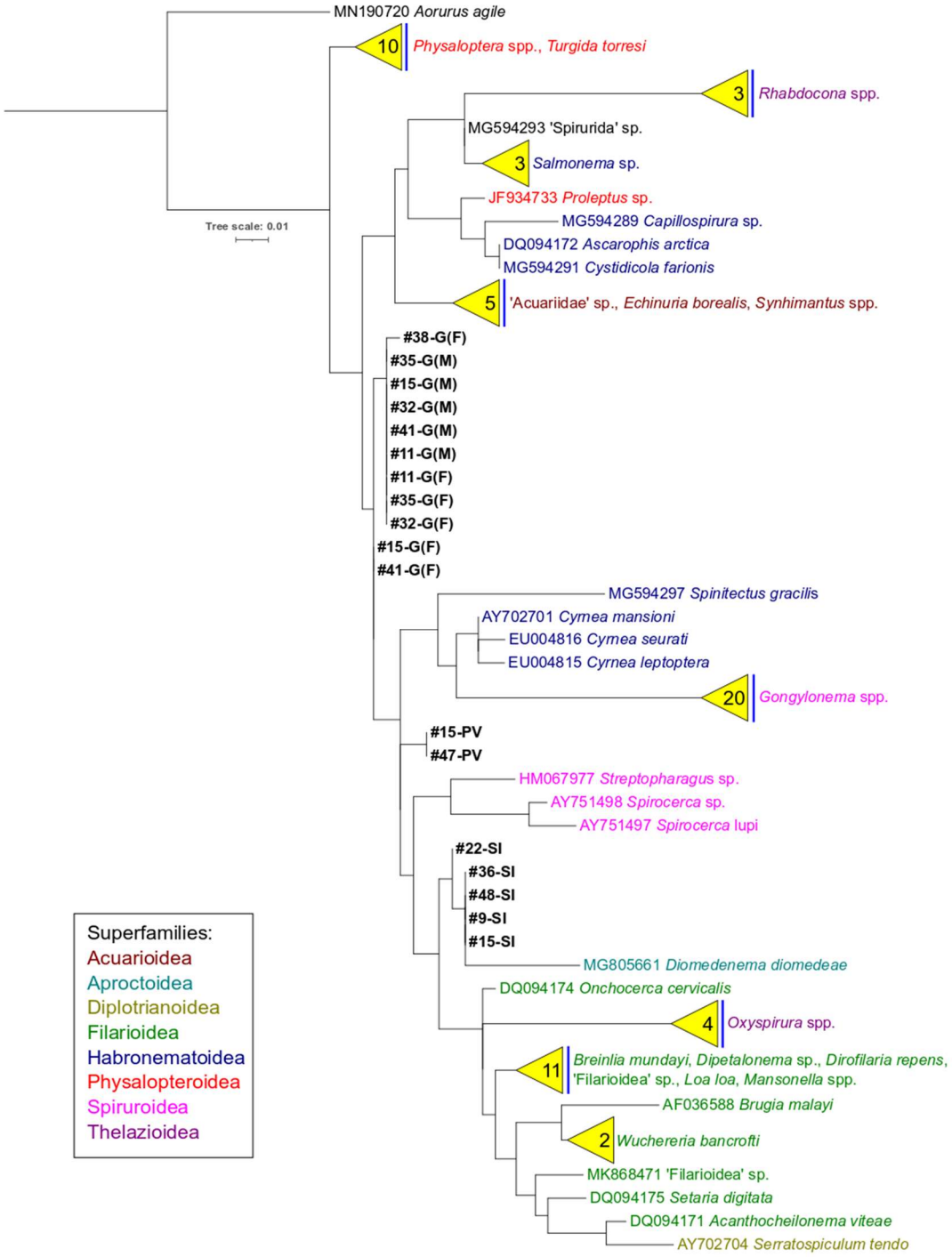

Figure 5.1. Maximum likelihood 18S phylogenetic tree of 18S sequences from spirurid taxa including the sequences produced from proventricular, gizzard, and small intestinal nematodes from this study and GenBank representatives derived from discontiguous megaBLAST analysis results. Yellow triangles represent collapsed nodes containing multiple closely related sequences, the number of which is represented by the number in the triangle, and blue vertical lines are used to partition multiple taxa present within the collapsed nodes (all GenBank accession numbers included within the uncollapsed tree are presented in appendix L). A sequence from *Aurorus agile* (order Oxyurida) was used as outgroup taxon. Taxonomic division into superfamilies is extrapolated from Anderson et al. (2009) and/or from NCBI submission data.

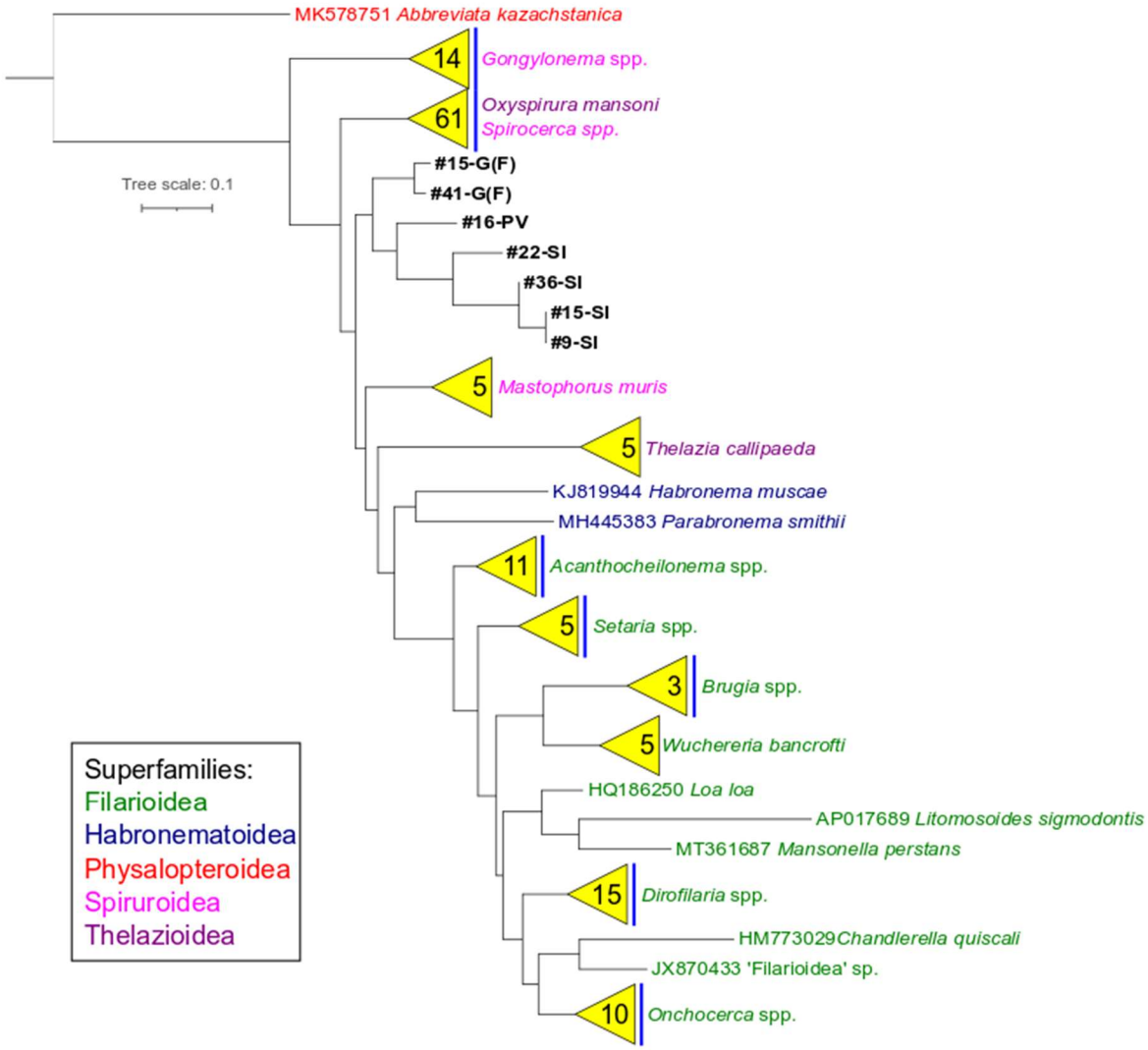

Figure 5.2. Maximum likelihood phylogenetic tree of COI sequences from spirurid taxa including the sequences produced from proventricular, gizzard, and small intestinal nematodes from this study along with GenBank representatives as selected from discontiguous megaBLAST analysis results. Yellow triangles represent collapsed nodes containing multiple closely related sequences, the number of which is represented by the number in the triangle, and blue vertical lines are used to partition multiple taxa present within the collapsed nodes (all GenBank accession numbers included within the uncollapsed tree are presented in appendix M). An *Abbreviata kazachstanica* COI sequence (order Spirurida) was used as outgroup taxon. Taxonomic division into superfamilies is extrapolated from Anderson et al. (2009) and/or from NCBI submission data.

5.4.4. Intestinal capillarids

One specimen of the capillarid-type nematodes from each affected bird (#'s 21 and 29) was selected for molecular analysis (Table 5.2). Only one of the two (#21-Cp) produced a good-quality 18S sequence using the primer set Kt875351.1-Capillaria 18S1R, with a trimmed length of 292 bases. On BLAST analysis the closest alignment was to four sequences identified as *Capillaria madseni* (LC052344, LC052346, LC052347, and LC052348), with 100% cover and 99.0%

pairwise identity (Table 5.3), although several other *Capillaria* sequences of avian origin also aligned relatively closely, e.g. *C. anatis*, *C. spinulosa*, and *C. pudendotecta*, with 100% cover and 97.6-98.6% pairwise identity. On alignment with the 18S consensus sequence previously obtained from the capillarid nematode causing ventral dermatitis in rowi kiwi (French et al., 2020a; Chapter 3), only 93.7% pairwise identity was found (aligned capillarid 18S sequences are presented in appendix I).

The top 100 megaBLAST results from the capillarid 18S sequence were filtered to include only members of *Capillaria sensu lato*, giving a total of 88 GenBank sequences; the three sequences previously obtained from capillarids originating from rowi skin lesions, oesophageal/crop infections of kahu (Australasian harrier hawk, *Circus approximans*), and the proventriculus of a red-billed gull (French et al., 2020a; Chapter 3) were also incorporated, and a sequence from *Enoplus brevis* (EBU88336) was used as outgroup. The tree was produced using the Kimura 80 model with invariable sites and gamma distribution (K80+G[0.783]+I[0.523]).

The resultant tree (Figure 5.3) confirmed that the small intestinal capillarid from this survey (#21-Cp) aligned among *Capillaria* spp. of avian origin, while the capillarid species originating from rowi skin, kahu crop and oesophagus, and red-billed gull proventriculus aligned among *Eucoleus* spp. as previously described (French et al., 2020a; Chapter 3); all were distinct from several other capillarid genera (e.g. *Aonchotheca* spp., *Baruscapillaria* spp., *Calodium* spp., and *Pearsonema* spp.) (a list of all GenBank accessions included in the capillarid 18S tree is presented in appendix N).

A capillarid COI sequence could not be obtained from either specimen using any of the primer sets COIF-COIR, JB3-JB4.5, or Eu COI F1B-Eu COI R1; the positive control, the putative *Eucoleus* species from a red-billed gull, did amplify appropriately using Eu COI F1B-Eu COI R1, but not with JB3-JB4.5, and only produced a very faint amplicon using the COIF-COIR primer pair (not sequenced).

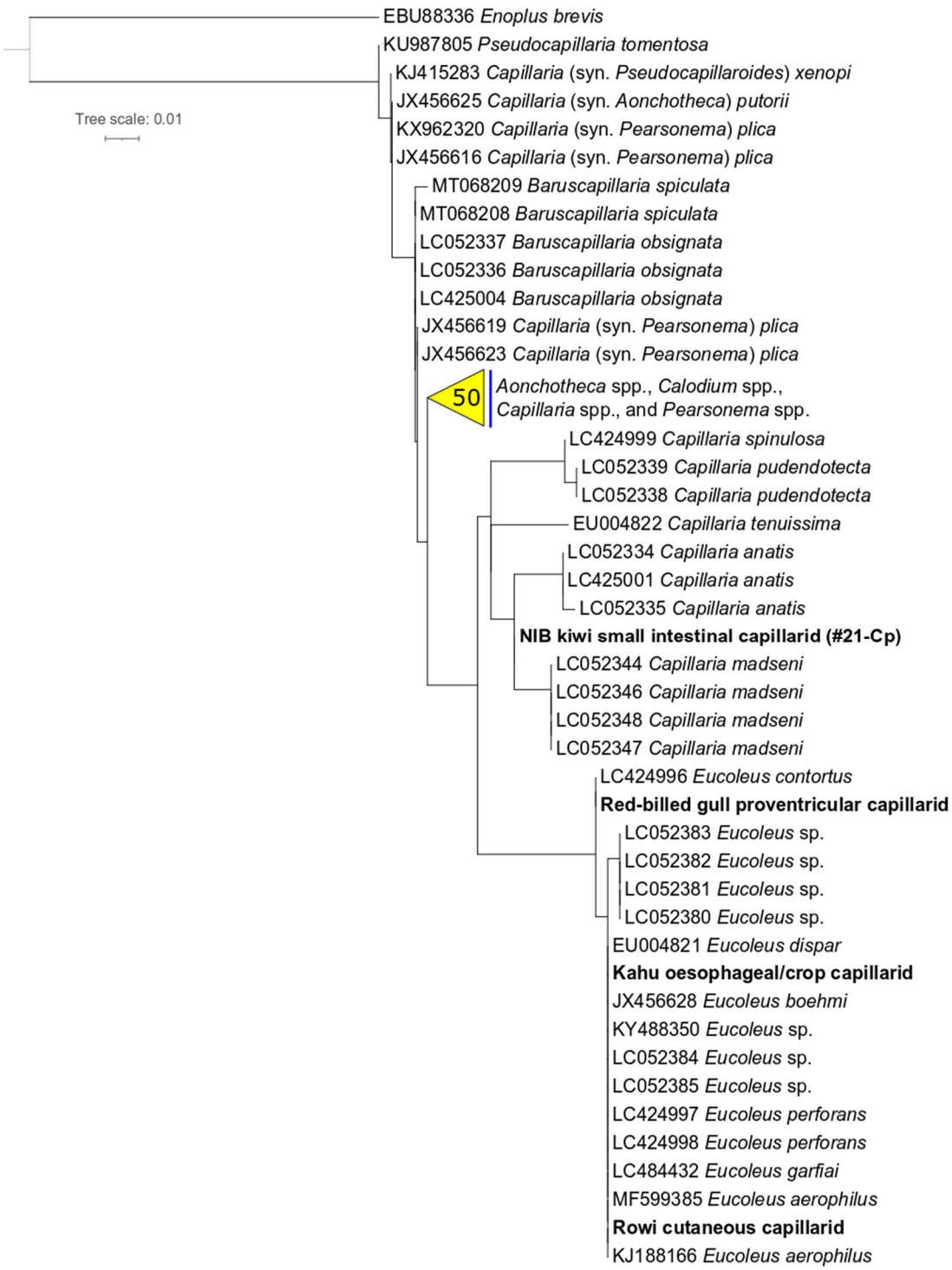

Figure 5.3. Maximum likelihood 18S phylogenetic tree of capillarid taxa including the sequence produced from this study (Kiwi #21 small intestinal capillarid) and those obtained from New Zealand avian species in the previous study of rowi cutaneous capillariasis (rowi, kahu, and red-billed gull capillarids) (French et al., 2020a) with GenBank representatives. The yellow triangle is a collapsed node containing multiple closely related sequences represented by the number in the triangle, and the blue vertical line partitions multiple different taxa within the collapsed node (all GenBank accession numbers included within the uncollapsed tree are presented in appendix N). An *Enoplus brevis* (order Enoplida) 18S sequence was used as outgroup taxon. Taxonomic names are as they appear in Genbank, with synonyms for genera (where considered relevant) added by author.

5.4.5. Caecal heterakoids

At least two individual nematodes were selected from the caecal samples of six of the survey kiwi, which comprised one LSK (#32), four NIB kiwi from different regions (#11 – Bay of Plenty; #15 – Coromandel; #35 – Northland; and #41 – Taranaki), and the sole representative of HT (#20) (Table 5.2). No amplification could be obtained using the 18SF-18SR primer set on a total of four separate nematode extractions from #15 (the only kiwi in the survey originating from the Coromandel), and no further attempts at molecular characterisation from the caecal organisms of this host were made. However, at least one (#32-C(F)) and generally two or more nematode sequences were obtained with this primer set from all other hosts. Because of notable differences in the initial sequences, a total of four individual nematodes from the HT (#20) were evaluated.

The trimmed 18S sequences originating from remaining NIB kiwi (#s 11, 35, and 41), and the single sequence originating from the LSK (#32-G(F)), ranged from 231 to 326 bases in length and aligned almost 100% with each other, apart from a single base difference near the beginning of the sequence from #35-C(M2) and few (one to three) ambiguous bases present in that and two other sequences (#35-C(M1) and (#11-C(M)). On BLAST analysis, the consensus of these seven sequences (aka NIB/LSK 18S consensus) aligned closest to sequences from *Strongyluris calotis* (LC133186 to LC133190) with 100% cover and 96.9% pairwise identity (Table 5.3).

18S sequences were also obtained from four individual nematodes (two males and two females) originating from the caeca of the HT (#20) using 18SF-18SR. Trimmed lengths were 319 to 326 bases and on alignment two (#20-C(F2) and #20-C(M1)) were near identical (98.9%) apart from several ambiguous bases, while the third (#20-C(M2)) also aligned closely (97.7-97.8%) but with two to three individual base differences apart from the ambiguous bases, as well as one additional base (within in a homopolymer run of Ts – possibly reflecting a sequencing error). However, the fourth sequence, from a female nematode (#20-C(F1)), was notably different, showing only 91.5 to 92.3% pairwise identity to the other three. Alignment of the consensus of

the three most similar sequences (#20-C(F2), #20-C(M1), and #20-C(M2); aka "main" HT 18S consensus) with the NIB/LSK 18S consensus showed 98.4% pairwise identity, and BLAST results for this "main" HT 18S consensus sequence were very similar to the NIB/LSK 18S consensus BLAST results, aligning nearest to sequences from *Strongyluris calotis* (LC133186 to LC133190) with 100% cover and 96.9% pairwise identity (Table 5.3). In contrast, the "outlier" sequence from the fourth HT caecal nematode (#20-C(F1)) showed only 92% pairwise identity to the NIB/LSK 18S consensus and aligned nearest to a sequence from *Paraspidodera* sp. (AF083005) with 100% cover and 96.9% pairwise identity (Table 5.3).

Both tuatara nematode extracts produced a sequence with 18S primers that aligned 100% with each other. The tuatara 18S consensus sequence showed 96.3%, 95.9%, and 93.3% identity with the NIB/LSK 18S consensus, the "main" HT 18S consensus sequence, and the HT "outlier" 18S sequence (#20-C(F1)) respectively (alignment of all kiwi caecal and tuatara faecal nematode 18S sequences is presented in appendix J).

The top 100 discontiguous megaBLAST results of the 18S consensus sequences derived from the seven NIB/LSK sequences, the three "main" HT sequences, and the two tuatara sequences, as well as from the individual HT "outlier" sequence (#20-C(F1)), were combined and included predominantly a mixture of nematode sequences from the orders Ascaridida and Spirurida; these were filtered to include only ascarid sequences, leaving a total of 92 GenBank sequences. The oxyurid sequence identified as from *Aorurus agile* (MN190720) was used as outgroup and the final alignment was 334 characters in length. The tree was produced using the general time-reversible model with invariable sites and gamma distribution (GTR+G[0.466+I[0.543]). The resultant tree (Figure 5.4) showed all study sequences clustering among other members of the Heterakoidea superfamily, although the HT "outlier" sequence (#20-C(F1)) was notably distant from the remainder of the sequences. The tuatara sequences aligned closer to the identified *Heterakis* species included within the analysis than to the sequences from the kiwi caecal

nematodes (a list of all GenBank accessions included in the ascarid 18S tree is presented in appendix O).

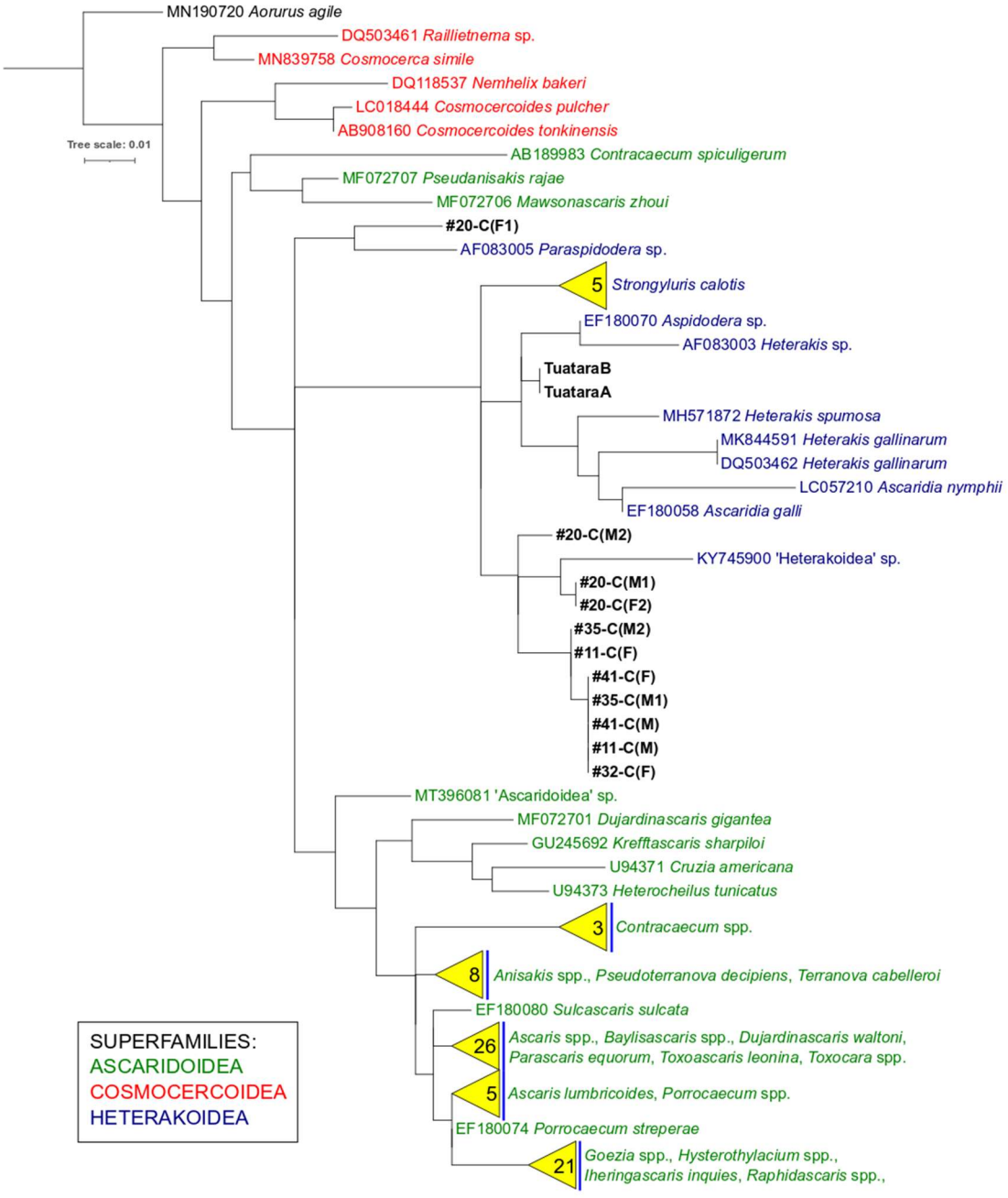

Figure 5.4. Maximum likelihood 18S phylogenetic tree of ascarid taxa including the sequences produced from this study and GenBank representatives as selected from discontiguous megaBLAST analysis results. Yellow triangles represent collapsed nodes containing multiple closely related sequences, the number of which is represented by the number in the triangle, and blue vertical lines are used to partition multiple different taxa present within the collapsed nodes (all GenBank accession numbers included within the uncollapsed tree are presented in appendix O). A sequence from *Aurorus agile* (order Oxyurida) was used as outgroup taxon. Taxonomic division into superfamilies is extrapolated from Anderson et al. (2009) and/or from NCBI submission data.

For multiple extracts from caecal nematodes, the JB3-JB4.5 primer set showed no or poor amplification with poor-quality sequences. In contrast, relatively good-quality sequences using the COIF-COIR primer set were obtained from most of the extractions from which 18S sequences were available, including sequences from two nematodes originating from each of the three NIB kiwi (#11 – BOP, #35 – N/L, and #41 – Taranaki), one from the LSK (#32-C(F)), and three of the four nematodes from the HT including the "outlier" female nematode which had showed disparate 18S results (#20-C(F1)). No two nematode COI sequences from the same host showed 100% identity with each other: the two sequences from #11 were 98.4% identical, from #41 were 97.3% identical, and two of the three sequences from the HT (#20-C(F2) and #20-C(M1)) were 99.1% identical. However, the two sequences from host #35 were only 90.6% identical to each other and, in comparison between those from other hosts, one of these (#35-C(M2)) was similarly different to all other sequences, ranging from 85.6-89.9% identity. The sequences from all other NIB and the LSK varied less, ranging from 97 to 100% identical with each other. Similarly, the "outlier" HT sequence (#20-C(F1)) showed only 87-87.6% identity with the other two HT sequences, and none of the HT sequences had greater than 89.6% identity with any of the NIB/LSK sequences. BLAST results were equally variable with no close matches; pairwise identities to the top alignments for each individual sequence ranged from 87.8 to 90.8% (Table 5.3).

Neither of the tuatara extracts amplified using the COIF-COIR primer set, and only one produced an amplicon with the JB3-JB4.5 primer set. The trimmed sequence was 434 bases in length and showed only 82.1-84.2% identity with any of the kiwi caecal nematode sequences (alignment of all kiwi caecal and tuatara faecal nematode COI sequences is presented in appendix K).

The top 100 discontiguous megaBLAST results from the individual COI sequences obtained in this study were combined and comprised predominantly a mixture of nematode sequences from the orders Ascaridida and Strongylida, with some rhabditids and free-living taxa also included;

these were filtered to include only ascarids, leaving a total of 283 GenBank sequences. As two different primer sets had been used to obtain the study sequences, and due to the degree of sequence disparity and the variable quality and length of the study sequences in this analysis, the final alignment was trimmed to cover only the shortest study sequence length, resulting in a final length of 357 characters. A strongylid sequence identified as from *Necator americans* (AJ417719) was retained from BLAST results as the outgroup. The tree was produced using the Hasegawa-Kishino-Yano model with invariable sites and gamma distribution (HKY85+G[0.415+I[0.455]).

The resultant tree (Figure 5.5) found all study sequences except the HT "outlier" sequence aligning nearest to other members of the Heterakoidea superfamily. Sequence #20-C(F1) aligned with sequences identified as *Subulura chinensis* although with a relative lack of comparative data as these were the only representatives of this superfamily included in the analysis (a list of all GenBank accessions included in the ascarid COI tree is presented in appendix P). The other kiwi caecal nematode sequences aligned among sequences from other avian heterakoids with clustering suggesting that at least two, possibly three different species may be present.

5.4.6. Visceral and neurological *larva migrans*

Samples including histologically confirmed larvae were selected from three kiwi, including both lung and brain from one (#36), and lung from the other two (#23 and #50); subsequent to the initial results, sections of liver from the latter two, which contained confirmed lesions but no obvious larvae, were also analysed. Examination of histological sections from immediately before and after the tissue scroll taken for DNA extraction confirmed presence of nematode larvae in one of the sandwich histology sections (taken before the tissue scroll) in three of the four organs for which larvae were confirmed in the original section, and presence of characteristic inflammatory lesions in both of the sandwich sections for all tissues.

PCR was performed using the primer sets 18SF-18SR, Nemo 18S F-Nemo 18S R, and Tcat1-NC2. (Table 4). For kiwi #36, only a very faint amplicon (not sequenced) was produced from the lung

using Nemo 18S F-Nemo 18S R, but robust amplicons were obtained from the brain section with all primer sets, and resultant sequences aligned 99.8-100% with those from *T. cati* present in GenBank (e.g. MT341314) (Table 5.4).

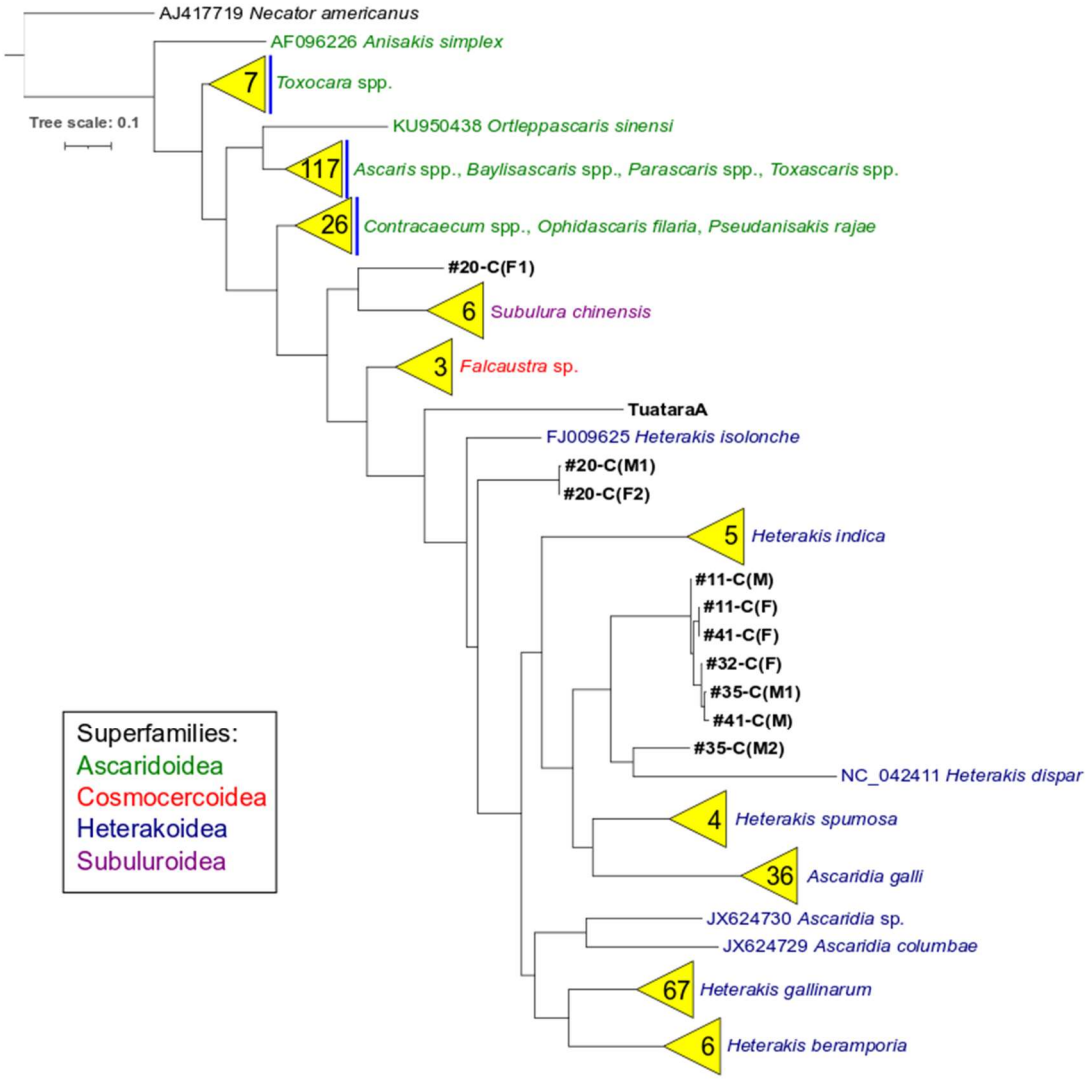

Figure 5.5. Maximum likelihood COI phylogenetic tree of ascarid taxa including the sequences produced from this study and GenBank representatives as selected from discontiguous megaBLAST analysis results. Yellow triangles represent collapsed nodes containing multiple closely related sequences, the number of which is represented by the number in the triangle, and blue vertical lines are used to partition multiple different taxa present within the collapsed nodes (all GenBank accession numbers included within the uncollapsed tree are presented in appendix P). A *Necator americanus* sequence (order Strongylida) was used as outgroup taxon. Taxonomic division into superfamilies is extrapolated from Anderson et al. (2009).

For kiwi #23 the initial 18S PCR (using 18SF-18SR) produced a sequence that was >99% identical to the 18S consensus sequence acquired from caecal nematodes found in NIB kiwi. However, this result was not repeatable and a duplicate PCR with the same primers produced a relatively poor-quality sequence that aligned among *Toxocara* spp. (e.g. *T. canis*, U94382), with no further amplification using alternate 18S (Nemo 18S F-Nemo 18S R) or the ITS-2 primers. Subsequent DNA extraction, PCR, and sequencing from the liver of the same case, in which larvae had not been identified histologically but multiple consistent inflammatory lesions plus areas of more acute inflammation were present, produced good-quality 18S and ITS-2 sequences aligning 100% to *T. cati* sequences in GenBank (e.g. MT341314) (Table 5.4).

In the case of #50, initial 18S PCR on the lung section (using 18SF-18SR primers) did produce a relatively poor-quality sequence that aligned with *T. cati* (EF180059). However, on duplicate PCR to confirm the result, a sequence was produced that was >99% identical to those obtained from the kiwi intestinal-type spirurids. Repeat PCR using the alternative 18S primer set (Nemo 18S F-Nemo 18S R) similarly produced both a sequence aligning with the intestinal-type spirurids and a sequence aligning with *T. cati* (MN585772) on duplicate PCR runs, and there was no amplification from this tissue with the ITS-2 primer set. Subsequent DNA extraction, PCR, and sequencing from the liver of the same case, in which few inflammatory lesions and no larvae were present, also produced robust 18S amplicons that aligned with the intestinal-type spirurids but no amplification from the ITS-2 primer set (Table 5.4).

Table 5.4. MegaBLAST results for tissue PCR, including results of duplicate PCR runs using the same primer sets in which disparate sequencing results were produced.

Kiwi # (tissue)	Forward primer	Amplicon	Sequence length (bp)	Aligned to GenBank #	Organism (target)	Cover	Pairwise identity	Bit-score	E-value
#23 (lung)	Nemo 18S F	+[a]	216	EF180070	*Aspidodera* sp. (18S)	100%	95.4%	342.750	3.92^{e-90}
#23 (lung)	Nemo 18S F	+	249	U94382	*Toxocara canis* (18S)	100%	99.8%	457.243	1.58^{e-124}
#23 (lung)	18SF	-							
#23 (lung)	Tcat1	-							
#23 (liver)	Nemo 18S F	+	253	MN585772	*Toxocara cati* (18S)	100%	99.8%	464.629	9.64^{e-127}
#23 (liver)	18SF	+	325	EF180059	*Toxocara cati* (18S)	100%	99.8%	597.588	1.21^{e-166}
#23 (liver)	Tcat1	+	291	MT341314	*Toxocara cati* (ITS-2)	100%	99.8%	534.802	8.55^{e-148}
#36 (lung)	Nemo 18S F	+*							
#36 (lung)	Tcat1	-							
#36 (brain)	Nemo 18S F	+	249	MN585772	*Toxocara cati* (18S)	100%	100%	460.936	1.22^{e-125}
#36 (brain)	18SF	+	313	EF180059	*Toxocara cati* (18S)	100%	100%	579.122	4.20^{e-161}
#36 (brain)	Tcat1	+	382	MT341314	*Toxocara cati* (ITS-2)	100%	99.8%	534.802	8.55^{e-148}
#50 (lung)	Nemo 18S F	+	101	EF180059	*Toxocara cati* (18S)	100%	98.0%	174.705	5.57^{e-40}
#50 (lung)	Nemo 18S F	+[b]	161	MG805661	*Diomedenema diomedeae* (18S)	100%	96.3%	261.498	7.94^{e-66}
#50 (lung)	18SF	+[b]	328	DQ094174	*Onchocerca cervicalis* (18S)	99.7%	98.2%	571.735	7.26^{e-159}
#50 (lung)	18SF	+	197	MN585772	*Toxocara cati* (18S)	100%	99.0%	357.524	1.25^{e-94}
#50 (lung)	Tcat1	-							
#50 (liver)	Nemo 18S F	+[b]	265	DQ094174	*Onchocerca cervicalis* (18S)	98.8%	97.7%	453.549	2.20^{e-123}
#50 (liver)	18SF	+[b]	326	DQ094175	*Onchocerca cervicalis* (18S)	100%	98.0%	568.042	9.53^{e-158}
#50 (liver)	Tcat1	-							

*not sequenced; [a]99.5% identical to "NIB/LSK caecal 18S consensus" sequence; [b]98.1-99.7% identical to "Intestinal 18S consensus" sequence

5.5. Discussion

Molecular evaluations were unable to definitively identify any of the gastrointestinal nematodes found within the survey population of kiwi. In the case of the gizzard and caecal nematodes, presumed to be host-adapted, native parasites of the kiwi, this is unsurprising as (to the author's knowledge) this is the first study to attempt the characterisation of these organisms by DNA sequencing. However, preliminary molecular evaluation does support the probability that there exists more than one heterakoid species infecting the caeca of kiwi, as has been previously indicated by Clark (1983a, b). The single sequence from a capillarid nematode originating from the small intestine of one NIB kiwi similarly did not allow a species-level identification, but did show close alignment with other *Capillaria* species of avian origin and was sufficiently distinct from the sequences obtained from capillarid nematodes associated with skin disease in juvenile rowi (French et al., 2020a; Chapter 3) to indicate a different organism, while the previously undescribed spirurid nematodes originating from proventriculus and small intestine had no close matches within the GenBank database; whether these may all reflect novel nematode species specific to kiwi or acquired infections therefore remains uncertain.

Gastrointestinal spirurids

The only formally identified spirurid nematode of kiwi is *Cyrnea apterycis* (Harris, 1975), with the presence of one or two further novel *Cyrnea* species subsequently suggested by Clark (1983a, b). In its original description, the gizzard nematode was identified as *Cyrnea (Cyrnea) apterycis* (family Spiruridae). At the time of publication, the genus *Cyrnea* contained three subgenera, *Cyrnea*, *Metacyrnea*, and *Procyrnea*; however, *Metacyrnea* and *Procyrnea* have since been raised to genus level and all three genera are now generally accepted to be placed within the family Habronematidae (Chabaud, 2009).

Within the prospective survey (Chapter 4), gizzard infections were the most prevalent nematodes encountered, with 44 out of 50 kiwi (88%) showing some evidence of infection although the burdens varied widely from one to well over 100 organisms found. The size and

superficial morphology of these nematodes resembled the published description of *Cyrnea apterycis* (Harris, 1975). However, in a small number of birds (five of 47, ~11%) a second, morphologically distinct spirurid nematode was identified in low numbers within the proventriculus and in 11 out of 47 (~23%), a third, larger spirurid nematode was identified grossly and/or histologically within the small intestine; neither proventricular nor intestinal spirurids have previously been described in kiwi.

Sequencing and BLAST analysis from a small selection of these proventricular-, gizzard-, and intestinal-type nematodes, together with creation of some basic phylogenetic trees to further evaluate the relationships between the study sequences and sequences derived from the BLAST analyses, support the origins of all three groups of agents within the order Spirurida but was unable to resolve their placement within this order even to family or superfamily level with any degree of confidence. This may be largely due to a lack of comparative data available within the GenBank database, combined with the limitation of only relatively short sequences obtained by use of the selected primer sets and quality of the extracted DNA.

Sequence comparison from the gizzard-type and proventricular-type nematodes does support that these reflect (at least) two distinct species, potentially relatively closely related. Based on the 18S sequences, the closest BLAST match for both was to a GenBank sequence identified as from *Cyrnea mansioni*; this nematode, primarily parasitising the gizzard of birds of prey, was originally designated *Cyrnea* (*Procyrnea*) *mansioni* (Quentin et al., 1983) and so contemporarily would be more accurately identified as *Procyrnea mansioni*. Similarly, the only other two *Cyrnea* species included within the 18S spirurid phylogenetic tree were originally formally identified as *Cyrnea* (subspecies *Procyrnea*) and are thus now classified as *Procyrnea* species (e.g. Borgsteede et al., 2003); no 18S (or COI) sequences belonging to *Cyrnea* (*Cyrnea*) spp. were included among the BLAST results, nor could be found on searching the database. Morphology of the female proventricular-type nematodes was not, however, suggestive of either *Cyrnea* or *Procyrnea*.

The presence of more than one species of *Cyrnea* parasitising the gizzard of kiwi, as previously suggested by Clark (1983a, b), could not be confirmed by molecular means. In other nematode groups, the 18S gene of nuclear ribosomal DNA may not allow resolution to species level particularly where only shorter fragments of the gene are sequenced (e.g. Guardone et al., 2013), although evaluation of longer or full 18S sequences (up to ~1800 bp) have been found to provide sufficient variation to allow assessment of interspecific relationships (e.g. Tamaru et al., 2015; Sakaguchi et al., 2020). In the relatively short 18S sequences (up to 326 bases) obtained from this study, there was a single base difference between the 18S sequence derived from the only *Cyrnea*-type nematode originating from a rowi and those from the majority of the remaining kiwi, and a different, single base substitution in the 18S sequences of two out of the total of ten *Cyrnea*-type nematodes derived from one LSK and four NIB kiwi. Such minor variations may appear to be of dubious significance, potentially within the bounds of individual variation or sequencing error – especially true in the case of the rowi sequence where only one sample was available for analysis. However, given that the 18S gene is a relatively highly conserved, "housekeeping" gene (i.e. required for cellular function) minor variations, especially if consistent between more than one organism, could potentially be indicators for species differentiation. As only short, partial 18S sequences were obtained, the true protein translation is impossible to know for certain making it more difficult to speculate upon whether or not this could indicate a small mutation existing between two different species.

It was, however, interesting that the only COI results obtained using the selected primer sets came from the same two NIB kiwi *Cyrnea*-type gizzard nematode samples that showed this single base difference. The mtDNA of some nematode groups has been shown to have a significantly higher mutation rate than that of many other animal taxa (Blouin et al., 1995; Anderson et al., 1998; Ahmed et al., 2019), a fact which makes it more difficult to design "universal" nematode COI primers. This could raise the possibility that the lack of amplification from the majority of gizzard nematode samples is due to sufficient divergence existing between

the COI sequences of the two nematodes which did amplify and those which did not to render the chosen primers inappropriate. This is pure speculation, however, and the minor difference in the 18S sequences and lack of COI amplification may simply be coincidental, compounded by poor-quality DNA – e.g. secondary to long-term storage prior to extraction and/or the method of extraction. If these features do indicate two different species, they would be present as mixed infections in the same host. Ideally, future investigations would include both detailed morphological examination of more samples from multiple different host species, coupled with more extensive molecular analysis including, if feasible, obtaining longer 18S sequences and trialling or designing alternative COI primer sets.

The COI sequences that were obtained from the two *Cyrnea*-type gizzard nematodes showed 96.5% identity with each other, within the realms of intraspecific sequence variation that has been previously described for nematode mitochondrial genes (up to 6%) – although these values appear largely based on study of the NADH dehydrogenase subunit 4 gene (Blouin et al., 1998; Blouin, 2002). The two *Cyrnea*-type gizzard nematode COI sequences in turn showed 91.2% and 92.6% identity with the only COI sequence obtained from a proventricular-type nematode; mtDNA sequence differences between closely related species has most commonly been found to be between 10 to 20% (Blouin et al., 1998; Blouin, 2002). However, once again, it must be taken into account that the sequences compared in this study are relatively short, and comparison of longer COI sequences could potentially allow for the identification of greater (or lesser) degrees of variation (e.g. Aly, 2014). It has also been suggested that, ideally, geographic- and taxon-specific thresholds for species differentiation using the COI gene should be established (Hebert et al., 2003). Comparison of the COI sequences between the gizzard-type and intestinal-type nematodes were similarly around the 88-91% identity mark, and there can be little doubt that these are different species (at the very least) based on differences in size and superficial morphology alone.

In the case of the intestinal-type spirurids, 18S sequences from the five individual nematodes analysed showed greater than 99% pairwise identity with each other, while the four COI sequences obtained ranged from only 91.6% up to 100% identical. BLAST analysis of both the 18S and COI sequences indicated a closest match to nematodes belonging to the superfamily Filarioidea, a group which typically produces microfilaria rather than ova, and of which multiple other members also featured prominently among the top 100 BLAST results. However, morphological examination and microdissection of several of the nematodes had confirmed that they contained larvated eggs, making a placement within this superfamily highly unlikely. Within the phylogenetic trees, comparing BLAST results from all study sequences as well as the study sequences themselves, the intestinal-type sequences did cluster separately from members of the filarioid group and, based on the COI tree especially, appeared more closely related to the other study sequences than to any of the GenBank sequences including those of the filarioids. These results highlight a potential pitfall in interpreting BLAST results in isolation, especially where there are no close matches.

Intestinal capillarids

The capillarid group have a complicated and contentious taxonomy, at different times consolidated into the single genus *Capillaria* or differentiated into up to 27 proposed genera (Gibbons, 2010). Following the classification system proposed by Moravec (1982), up to 10 genera have to date been identified in avian species (Yabsley, 2008; Tamaru et al., 2015), all infecting sites within the gastrointestinal tract (Yabsley, 2008; Anderson and Bain, 2009). Over 300 species of Capillaria *sensu lato* have been thus far described, and Clark (1983a, b) suggested the existence of one or two new species of *Capillaria* present in kiwi that had yet to be formally identified.

Capillarids were rarely found within the small intestine of the survey population (2 out of 47 kiwi, ~4%) and only one of the two specimens evaluated produced an 18S sequence, which on BLAST analysis aligned among other avian *Capillaria* species. Sequence comparison also

supported a different organism from that identified as causing cutaneous capillariasis in juvenile rowi (French et al., 2020a; Chapter 3), which aligned primarily among *Eucoleus* species within the capillarid group. Avian *Eucoleus* species have been predominantly identified in the upper gastrointestinal tract (e.g. oral cavity, oesophagus, crop, or one species in the gizzard) (Yabsely, 2008). In contrast, the capillarid from this survey aligned among avian *Capillaria* species that parasitise the intestine and/or caeca (e.g. *Capillaria madseni, C. anatis*, *C. spinulosa*, and *C. pudendotecta*) (Yabsley, 2008; Tamaru et al., 2015), although with no exact match based on available sequences. This lack of an exact match does raise potential for a novel *Capillaria* species, particularly as previous studies found that, at least from partial sequences as here, there is little variation in the 18S gene between closely related capillarid species (Guardone et al., 2013). However, even if so, whether this organism may reflect a novel capillarid specific to kiwi versus an acquired infection from another source remains unclear. Further assessment of these nematodes would therefore likely benefit most from expert morphological examination by a taxonomist familiar with the morphology of this complicated nematode group, as well as evaluation of longer 18S sequences.

Caecal heterakoids

The only formally identified heterakoid nematode of kiwi was originally designated *Heterakis gracilicauda*, within the family Heterakidae, by Harris (1975). It is possible, however, that many of the nematodes infecting the caeca in kiwi may not be *Heterakis* species at all. Work presented by Clark (1983a, b) suggested the existence of at least five novel heterakoid species in kiwi including two within the genus *Heterakis*, but also two requiring the creation of a new genus near *Spinicauda* or *Haterrianema* and one requiring a new genus near *Lauroia*. It was not made clear in the limited published information whether *Heterakis gracilicauda* was specifically identified along with these novel species, which does become an important question as the classification of *Heterakis gracilicauda* was revisited several years later by Inglis and Harris (1990) as morphological similarities between *H. gracilicauda* and the tuatara intestinal

nematode *Hatterianema hollandei* led to the creation of a proposed new genus, *Kiwinema*, for the kiwi caecal nematode, and a new family Kiwinematidae to encompass both. The morphological relationship between these two nematodes, both originating from ancient, native species in New Zealand that have been geographically isolated for millions of years, was used to discuss the possibility that these may reflect some of the most ancestral forms within the Heterakoidea superfamily.

Preliminary molecular evaluation was unable to provide support for a close relationship between the tuatara nematode and those originating from the caeca of several different kiwi hosts, with the caveat that only one (COI) or two (18S) sequences originating from tuatara nematode specimens were available for evaluation, and morphological examination was limited by specimen quality (sourced from faecal samples). Molecular results do, however, support the presence of more than one heterakoid species parasitising kiwi.

Evaluation of four individual caecal nematodes from the only HT included within the study population found one nematode with a significantly different sequence, both 18S and COI, from the other three, raising the likelihood of two different species present in this single host. While the 18S phylogenetic tree showed this outlier sequence clustering among other members of the Heterakoidea superfamily, albeit some distance from the other study sequences, in the COI tree it appeared to show more affinity to several sequences identified as from *Subulura chinensis*; it is entirely possible this is an artefact of the lack of comparative data, particularly as these sequences were the only ones from the subulurid family that were included in the analysis. It is worth noting though that Clark (1983a, b) does suggest the existence of a novel species of *Primasubulura* in kiwi but its anatomic origin was not specified, and on superficial morphological examination the individual nematode in question strongly resembled the other heterakoid nematodes harvested from the same host and site. However, obviously, further investigation

examining both the detailed morphology and sequence data from multiple caecal nematodes in HT would be suggested to investigate this possibility further.

In contrast, the 18S sequences from caecal nematodes originating from three NIB kiwi and a single LSK nematode were virtually identical to each other, and ~98.4% identical to the three more similar nematodes from the HT. There was, unsurprisingly, more variation among the COI sequences. Although the majority of COI sequences originating from the NIB kiwi and the single COI sequence from a LSK showed 97-100% identity with each other, again within the realm of previously reported intra-specific sequence variation (Blouin, 2002), one sequence from one of the NIB kiwi showed only 85.6-90.6% identity with the others suggesting potentially a second species present as a mixed infection in a single host. There was a similar degree of difference (pairwise identities ranging from 86.0-89.6%) between the COI sequences from all nematodes originating from NIB and LSK versus the nematodes from the HT, again supporting a different species of heterakoid nematode infecting these kiwi of North and South Island origin respectively.

Overall, these preliminary molecular results from caecal nematodes suggest the presence of up to four distinct species: one infecting NIB kiwi and a LSK from several sites around the North Island; a potential separate species present as part of a mixed infection from a single NIB kiwi originating from Northland; and two separate species infecting a single HT originating from a reserve within the South Island – although one of these may potentially not be a member of the Heterakoidea, based on the COI analysis. The suggestion that the caecal nematodes of tokoeka (aka South Island brown kiwi) and those of NIB kiwi are different species is easy to justify, given their relative geographic and genetic separation; phylogenetic studies estimate that the two species diverged around 1.5 million years ago (Weir et al., 2016). The fact that LSK carry the same caecal nematode as NIB kiwi may be similarly easy to explain, as the LSK experienced a severe genetic bottle neck in the early 1900s, with the population dwindling to as few as five to

seven individual birds that were placed on an island sanctuary along with other kiwi species (Jolly and Colbourne, 1991; Ramstad et al., 2010; Ramstad et al., 2013). It is entirely possible that even if LSK carried their own, host-specific parasites historically, the parasites themselves may have become extinct where the kiwi survived. However, only a single nematode from the LSK was evaluated in this study, and that from a bird recently translocated from an off-shore island sanctuary to a mainland sanctuary in the North Island, so more recent acquisition of a parasite of NIB kiwi origin may also be a potential explanation.

While phylogenetic studies also suggest the existence of four genetically and geographically distinct clades within the NIB kiwi (Weir et al., 2016), there have been considerably more translocations and intermixing of these populations, making the evaluation of any potential geographically-specific, host-adapted nematode species among this group more complicated. Further investigation of this group of nematodes would benefit from expert, comparative morphological evaluation within and between differing geographical locations. Cophylogenetic studies comparing molecular data from both parasite and host to further explore the diversity of the caecal nematodes of kiwi and their co-evolutionary history may also be worth consideration (e.g. Mockett et al., 2017).

Nematode larva migrans

It has been previously established through molecular evaluation that a primary cause for visceral and neural LM in NIB kiwi is the aberrant migration of *Toxocara cati* larvae (French et al., 2020b; Chapter 2). However, it was noted that among the 17 cases of LM diagnosed among the survey population of kiwi were included nine out of the 11 kiwi in which the previously undescribed small intestinal spirurid nematodes were also identified on gross and/or histological examination, and for this reason three such cases were chosen for DNA extraction and PCR to try and confirm the origin of the larvae involved in the lesions of LM. While PCR, sequencing, and BLAST analysis did ultimately produce results that supported *T. cati* as the causative agent

in all three cases tested, confounding sequences were also produced in two of the cases, raising some potentially significant sample handling considerations for future testing.

In one case (kiwi #36), the results were straightforward and entirely supportive of the previous study (French et al., 2020b; Chapter 2). Larvae had been identified within lesions present in both lung and brain, and within the lung larval cross-sections were present for which both the size and morphology (presence of bilateral alae) were consistent with *T. cati.* Molecular evaluation of the brain section produced both 18S and ITS-2 sequences that aligned with sequences from *T. cati* available in GenBank.

However, the initial PCR result from the lung of kiwi #23 produced an 18S sequence that aligned almost exactly with the 18S sequence obtained from other NIB caecal heterakoids in this study. This finding was not repeatable, however, and duplicate PCR of the lung section, and subsequently further analysis of the liver section from the same bird (in which multiple and extensive inflammatory lesions but no obvious larval organisms were identified), confirmed an 18S and both 18S and ITS-2 sequences respectively aligning among *Toxocara* spp. or to *T. cati* specifically. The initial result is therefore considered likely to reflect contamination of the formalin-fixed lung specimen. Caecal nematodes were found in moderate numbers at gross necropsy, and the caecal samples had been collected into the same formalin container as the visceral specimens. It was even specifically noted in this case that caecal nematodes were identified histologically present outside the caecal lumen within the mesenteric fat (Chapter 4), raising potential that free caecal nematodes may well have become associated with other fixed tissue samples as well.

In the final case, kiwi #50, the section of lung (which did contain a larval form histologically) was initially tested, and duplicate PCR runs using two different 18S primer sets each produced one sequence that aligned with *T. cati* and another that was >99% identical to the 18S sequence produced from the small intestinal-type spirurids. Subsequent DNA extraction and testing of

liver from the same bird (in which a few localised inflammatory lesions but no larval organisms were present) both produced only the spirurid sequence, and a *T. cati*-specific ITS-2 sequence could not be produced from either tissue. In this case, contamination of the fixed organ sections was also strongly suspected. The production of *T. cati* sequences from the lung supports this as the causative agent for visceral LM in this bird, as (disregarding a slight possibility for DNA contamination between cases) there is no other explanation for this sequence material to be present. The host kiwi had multiple small intestinal spirurids identified grossly, as well as the highest number of small intestinal nodules (many of which were histologically confirmed to contain nematode larvae), multiple of which had been collected into the same formalin container as the visceral specimens, once again raising the potential for contamination between tissues. While true visceral migration of the intestinal spirurid can't be entirely discounted, it is considered unlikely. If future investigations are undertaken, however, these findings do highlight the need to avoid the potentially confounding result of sample contamination caused by the collection of gastrointestinal samples into the same container as visceral samples.

5.6. Conclusion

No species-level identifications of the nematodes originating from the gastrointestinal tract of kiwi were obtained based on the limited molecular analyses performed. However, results were able to provide support for the presence of more than one species of heterakoid nematode parasitising the caeca of kiwi, and for the identification of *Toxocara cati* as cause for lesions of visceral and neural *larva migrans*.

Chapter 6: General discussion

The impetus for this research arose from the increased recognition of cases of nematode *larva migrans* (LM) affecting kiwi (*Apteryx* spp.) presented for necropsy to the Wildbase Pathology Service (Palmerston North, New Zealand). This does not necessarily reflect an increased incidence of the disease, which would be difficult to assess due to the sporadic and often serendipitous nature of its diagnosis by histological examination, but may instead be a result of increased disease surveillance associated with rising concern for the conservation status of this iconic native bird. Through investigation into potential causes of LM and other cases of aberrant nematode migration in kiwi, it also became clear that, despite the national prominence of the host, there was a surprising dearth of information regarding the expected gastrointestinal nematode fauna in these species. These studies aimed to expand the current knowledge base, addressing not only the identification and potential source of nematodes causing aberrant migratory lesions in kiwi but also investigating the diversity, prevalence, and pathogenicity of their gastrointestinal nematodes – as well as any potential relationship between these two presentations of nematodiasis – through integrated and accessible diagnostic techniques including histopathology and molecular evaluations.

From the outset, a precise definition for the disease entity of LM was difficult to pin down and the term may be considered to some extent an historical one[1], superseded in many cases by the growing body of knowledge identifying potential causes and refining diagnostic techniques that allow more specific aetiological descriptors to be applied to the common syndromes. Contemporarily, the term "*larva migrans*" remains most associated with the disease presentations it was first introduced to describe – cutaneous lesions caused by aberrant

[1] Even the use of the term "larva" to describe immature nematodes is considered now by some to be inaccurate in and of itself, the argument being that the larval form of an insect is one that undergoes a marked metamorphosis before maturing to the adult form while, in contrast, nematodes undergo a series of moults but retain a similar basic form as they mature (Wharton, 1986); the term "juvenile" has been touted as a more appropriate alternative but this does not appear to have been widely adopted in literature as yet.

migration of hookworm larvae and the visceral forms of infection by *Toxocara* larvae and related ascarid species. With the establishment of *Toxocara cati* as a primary cause for the disease in kiwi (French et al., 2020b; Chapter 2), the diagnosis of LM would therefore be appropriate but could equally be termed as visceral and neurological toxocariasis (or, per some authors, toxocarosis). In contrast, the histological identification of adult nematodes migrating within the skin of juvenile rowi (French et al., 2020a; Chapter 3) suggests the initial diagnosis of this particular syndrome as a form of cutaneous LM is strictly speaking incorrect, and with histological and molecular support that the lesions are caused by a species of *Capillaria sensu lato*, the term cutaneous capillariasis is more accurate.

The identification of *T. cati* DNA within archival (French et al., 2020b; Chapter 2) and prospectively collected (Chapter 5) formalin-fixed, paraffin-embedded (FFPE) tissue blocks refutes results of a preliminary study into the causative agent of LM in kiwi (van Zyl, 2014). In that investigation, despite the histomorphological suggestion of a *Toxocara* species as the most likely cause, PCR using specific primer sets developed to identify and distinguish between *T. canis* and *T. cati* was negative in all cases and it was concluded that neither of these agents were likely to be involved. There were limitations to this study, which did not attempt to assess the quality of the DNA being extracted from the FFPE tissue sections, some of which had been archived for up to 14 years. Additionally, of the 29 FFPE tissue blocks containing multiple "affected" organs from 18 kiwi used in the analysis, it was described that only 13 of the examined slides contained granulomas suspicious for LM, and only three showed cross-sections of larvae. The results of the current study (French et al., 2020b; Chapter 2) found that PCR was more likely to be successful where there was histological confirmation of larvae directly adjacent to the sections taken for molecular analysis, although it was also successful in a small number of cases where larvae were not histologically evident, some of which could perhaps be explained by a marked, florid inflammatory response that may have obscured the presence of larval fragments. However, this is an obvious disadvantage to the application of PCR to confirm that the cause of

such histological lesions (e.g. the classic “eosinophilic granuloma”) is definitively due to LM, in such cases where larval organisms are not already obvious in histological section.

A further, potential confounding factor to the use of PCR in this setting was highlighted in the prospective study (Chapter 5), which (in part) aimed to confirm *T. cati* as the cause for LM in light of the concurrent findings of unidentified spirurid nematodes invading the small intestinal wall in just over half of the diagnosed cases. Of three cases tested, while all did produce sequences aligning with *T. cati* thereby supporting the results of the retrospective study, two also produced sequences compatible with two different types of gastrointestinal nematodes identified from the survey kiwi. It was concluded that this was almost certainly due to contamination – not only were increased numbers of gastrointestinal samples than would perhaps be normally collected at routine necropsy placed into the same formalin sample container as the visceral sections, but examination of the gastrointestinal tract was often performed at the same time (and potentially using the same instruments) as for the collection of sections of viscera. This potential for tissue contamination needs to be considered in the collection of samples where molecular analysis may be indicated in future diagnostics, but is often overlooked at the stage of routine diagnostic post-mortem examination.

In working with FFPE samples, the section taken for DNA extraction and molecular analysis is most commonly a 10 µm thickness microtome tissue “scroll” taken from the face of the paraffin-tissue block. An alternative approach that could be trialled in the future is the technique of tissue coring to collect the sample for DNA extraction, which has been developed for a more precise, localised collection of affected diseased tissue (primarily neoplastic) from FFPE blocks for molecular analysis (e.g. Patel et al., 2016). The desired lesion is localised in the block and a needle or punch biopsy instrument is used to sample the exact site, thereby theoretically harvesting a sample that may be more likely to contain increased amounts of the DNA of interest. In the context of LM there is no guarantee, and no easy way to assess, whether an

identified larval migratory tract would persist, remain linear, and/or be contained within a core sample such as this. Nonetheless, it is a method that may be worth future evaluation to try and increase the probability of including amplifiable amounts of nematode DNA within the extraction, as well as an approach that could minimise the possibility of amplifying contamination from the capsular or cut surface of the organ section.

Given confirmation that at least a majority of cases of LM in kiwi are caused by *T. cati*, use of immunohistochemistry could also be considered as a potential confirmatory test. This technique has been applied successfully in human cases of LM due to *T. canis* (Kaplan et al., 2001; Musso et al., 2007), where it was found to identify the presence of antigenic material in the macrophages of inflammatory granulomas even where larval fragments were not present. However, to the author's knowledge, such a method is not currently commercially available for *T. cati* and would have to be specifically developed.

In the absence of tissue biopsy, none of these techniques would be applicable to the antemortem diagnosis of LM in kiwi. Such a test would be desirable for kiwi presenting alive with suggestive neurological signs, as treatment of LM requires long-term anthelmintic medication with currently no method to assess whether this is appropriate. If future incidence of such presentations increases, it may be worth looking into the development of an ELISA assay capable of detecting circulating *T. cati* excretory-secretory antigens in kiwi serum (e.g. per Keenan, 1992).

While the application of molecular tools to archival pathology specimens has strongly implicated *T. cati* as a primary cause for aberrant internal larval migratory lesions in kiwi, what could not be elucidated was the role of the kiwi as a potential definitive host for the same agent, as has been previously described in a single North Island brown (NIB) kiwi (Clark and McKenzie, 1982). The prospective survey of gastrointestinal nematodes (Chapter 4) did not identify any kiwi carrying adult *Toxocara*. Although human infection with *Toxocara* spp. also most commonly

presents as aberrant larval migration, there are sporadic reports in which adult *Toxocara* have been identified from the gastrointestinal tract of humans, predominantly children, which (in contrast to the syndromes of LM) have been attributed more commonly to *T. cati* than *T. canis* (e.g. Rodan and Buckley, 1969; Wiseman and Lovel, 1969; von Reyn et al., 1978). The adult nematodes were typically passed only in low numbers, either in vomitus or faeces, and potential explanations for their presence included the ingestion of the nematodes either already in their adult form or as late-stage infective larvae. The possibility that the nematodes developed in the gastrointestinal tract following ingestion of infective eggs, successful completion of the internal migratory route through liver and lungs, and ultimate maturation to adult form in the intestine was considered unlikely as there was no clinical indications of systemic migratory disease (Wiseman and Lovel, 1969; von Reyn et al., 1978).

It has been described that following ingestion of infective larvae within a paratenic host, in which they have already undergone a migratory step, *Toxocara* spp. may in some cases go on to complete their life cycle within the intestine of the final host without undergoing further migration (Strube et al., 2013). This may be a possible origin for the *T. cati* found in the small intestine of the kiwi, but in the described case over 100 nematodes were present within the intestinal lumen, including both adult and larval forms (Clark and McKenzie, 1982), making this explanation perhaps less likely unless there was repeated exposure. The presence or absence of any evidence suggesting that visceral larval migration had also occurred was not addressed in the report. While the possibility of misidentification of the nematodes was discussed by the authors, this was based on detailed morphological examination by multiple experienced parasitologists (Clark and McKenzie, 1982). The deposition of voucher specimens of the nematodes at two different repositories could allow for future molecular confirmation of the identification although, assuming it was confirmed, this would provide no further explanation as to their presence in the kiwi. It may simply be that under certain, uncommon, environmental and physiological conditions the kiwi may indeed act as a definitive host for this nematode, but

unless other confirmed cases of adult *Toxocara* within the intestinal tract are described, further speculation is difficult. It may be worth noting that the island origin of the affected kiwi is now free from mammalian predators including the feline definitive host of *T. cati*.

Even downplaying any potential role as definitive host, the identification of *T. cati* as a cause for parasitic disease in kiwi should be considered in strategies regarding the control of feral cats (*Felis catus*), which are also a cause for the direct mortality of kiwi chicks and juveniles through predation (McLennan et al., 1996). There would be value in advocating strict worming protocols in domestic cats, especially those living adjacent to mainland reserves holding kiwi, where cats have been recorded patrolling boundary fences. In a broader context, there may also be implications for aberrant infection of other ground-dwelling native avian species, not to mention the zoonotic potential to humans.

In fact, that the application of PCR and BLAST analysis was able to identify the causative agent of lesions of visceral and neural LM in kiwi as *T. cati* with some degree of certainty is due in no small part to the significance of human toxocariasis as "one of the most widespread public health and economically important zoonoses" (Macpherson, 2013; p. 999). The molecular identification and differentiation of *Toxocara* and other related ascarid species have therefore received a fair amount of research attention with multiple published primer sets and abundant database sequence information available. In contrast, when similar techniques were applied to the cutaneous nematode migratory lesions in rowi, a species-level identification of the causative agent was not possible (French et al., 2020a; Chapter 3), which may be attributable to the fact that, in general, databases of nematode genomics are still relatively sparsely populated (Poon et al., 2017). It is clear that, at least in the field of nematology, the use of molecular tools is not yet a diagnostic panacea and the integration of genetic analyses with other techniques including morphological and/or histomorphological evaluation remains of immense value.

Limitations to the application of PCR in nematode identification includes the choice of appropriate primers, particularly as there is, as yet, no one specific target region of DNA that has been established to allow reliable species-level identification, and for targets which do show sufficient variability for species differentiation, the development of "universal" primers that will amplify across a wide range of taxa becomes problematic. Therefore, when approaching the identification of an unknown nematode, some initial knowledge that may at least narrow down the broad taxonomic group to which the parasite belongs is advantageous. As an example of this, preliminary investigation into the rowi cutaneous skin lesions (van Zyl, 2014; Gartrell et al., 2015) performed PCR using a "universal" primer developed from the ITS-2 region of rDNA of the free-living nematode *Caenorhabditis elegans* (Gasser et al., 1993), which had also been applied successfully to multiple parasitic members of the order Strongylida (e.g. Hoste et al., 1993; Gasser et al., 1994; Stevenson et al., 1995). In the case of the rowi skin samples, amplification was successful and subsequent sequencing and BLAST analysis suggested a nematode that aligned among members of the strongylid genus *Trichostrongylus* (van Zyl, 2014; Gartrell et al., 2015). This result was quite rightly greeted with some scepticism by the authors, as members of this genus have not previously been associated with aberrant migratory lesions in any host, and indeed this result was unable to be replicated as part of the current research (French et al., 2020a; Chapter 3). More detailed histomorphological examination of the lesions in question was, however, able to discern that the nematodes belonged to not only a different order than *Trichostrongylus* spp., but a different class of nematode (Adenophorea versus Secernentea). This initial identification allowed the selection of primer sets that had been previously successfully applied to nematodes from the order Enoplida, and eventual development of primers designed to identify members of the *Eucoleus* genus more specifically.

The successful amplification of capillarid DNA in this case was, however, still unable to identify the nematode in question to species level. Initial PCR targeted the 18S gene of rDNA, a region which is, in general, highly conserved and therefore considered suitable for higher-level

taxonomic classification, but which may not contain sufficient variability to allow species-level differentiation (Guardone et al., 2013). True to this, there was little to no variation in the relatively short sequences obtainable from the FFPE samples comparative to published sequences available in the GenBank database originating from multiple different species (of both avian and mammalian origin) of the capillarid genus *Eucoleus*. In contrast, mitochondrial DNA (mtDNA) is generally agreed to exhibit increased interspecific variation, but when COI primers were developed and applied, no exact match to the obtained sequences were found in the database – which indeed contained only relatively few *Eucoleus* COI sequences (none of which originated from avian parasites). Furthermore, two similar but distinct COI sequences were obtained from the rowi samples, bringing to light a dilemma that has arisen in the use of mtDNA for metabarcoding purposes – the potential over-estimation of species diversity due to the concurrent amplification of mitochondrial pseudogenes or "numts", defined as non-functional copies of mtDNA that have become integrated into the nuclear genome (Derycke et al., 2010; Deagle et al., 2014). In the case of rowi, the very small biopsy samples available for analysis coupled with the limited geographical location of the disease argued against the likelihood of there being two different species of nematode at play, but under other circumstances this would have been far more difficult to interpret.

Results leave unresolved the question of whether the capillarid nematode causing skin disease in juvenile rowi may reflect a novel, undescribed *Eucoleus* species, or whether it represents aberrant infection by an established nematode originating from another source, i.e. described but not yet represented in the genomic database. While Clark (1983a, b) previously indicated the existence of one or two novel capillarid species infecting kiwi, the unusual presentation – a crusting dermatitis over the ventral abdominal skin and vent margin – has only ever been diagnosed among the juvenile rowi present on this particular island, and as *Eucoleus* species in birds typically infect the upper gastrointestinal tract (Yabsley, 2008), the very atypical site of migration within the epidermis in these cases is more suggestive of an aberrant infection from

another source. Speculatively, this unique presentation may require a combination of factors. The island fauna could provide exposure to a nematode infection not likely to be encountered by other kiwi populations, while the sharing of burrows by the juvenile rowi may allow increased environmental contamination and magnification of infections in individuals. There may also be genetic, physiological, or immunological factors perhaps relating to the somewhat unusual biological features of kiwi in general, the young age of the rowi specifically, and/or the fact that they were reared from eggs in a controlled, conservation setting prior to their translocation to the island.

The prospective survey of gastrointestinal nematodes in kiwi (Chapters 4 and 5) confirmed that a different capillarid species does parasitise the small intestine of kiwi, although these were identified in low numbers from only two out of 47 (~4%) of the survey birds, both of which were NIB kiwi. While the single 18S sequence obtained from one such organism aligned among *Capillaria* species of avian intestinal origin, genetically distinct from the *Eucoleus* group, an exact match was not found and once again it could not be established whether this may reflect a novel, kiwi-specific nematode as suggested by Clark (1983a, b), or an acquired infection. Given the previous study indicating that partial sequences from the 18S gene show little genetic variability between closely related capillarid species (Guardone et al., 2013), the lack of an exact genetic match in this case could well support a novel organism, but gathering of further specimens for both more extensive molecular analysis and expert morphological examination will likely be required to establish this for certain.

Similarly, while significant progress was made in determining the diversity of gastrointestinal nematodes present in kiwi, identification to the species level could not be obtained when molecular tools were applied to specimens collected during the prospective survey (Chapter 5). In the case of the gizzard spirurids and caecal heterakoids, which comprised the most commonly identified infections, it is highly likely that these are predominantly host-specific kiwi nematodes

– as described by Harris (1975) – and as, to the author's knowledge, this was the first attempt at molecular characterisation of these agents the lack of exact comparative matches in the database is unsurprising. However, in the case of the spirurid nematodes in general, for which morphological examination suggested the presence of at least three differing species parasitising proventriculus, gizzard, and small intestine (Chapter 4), BLAST analysis and creation of phylogenetic trees to further compare sequence data were unable to even align the kiwi nematode sequences within a particular nematode superfamily with any degree of certainty (Chapter 5).

While the limited precision in these molecular analyses is considered in a large part to result from the lack of comparative data available within the database, it is also worth noting that phylogenetic relationships as implied by tree-building depend on a number of factors including the model used, taxons included, sequence length, method of alignment, and the choice of outgroup used to root the tree (Stevens and Schofield, 2003; Luo et al., 2010). It is possible that a different approach to the creation of the phylogenetic trees may have been able to imply better defined groupings, but the primary goal for the purposes of this study was to examine more closely the relationship between the sequences obtained from the kiwi nematodes and the results of their BLAST alignments. This was particularly of interest for the intestinal-type spirurids where closest BLAST alignments of both the 18S and COI sequences were to filarioid nematodes, but where morphological evaluation (primarily the production of ova) made membership within this group highly unlikely – ultimately supported by distinctly separate clustering in both the 18S and COI trees.

While the traditional, morphology-based taxonomy of the nematode phylum – largely per Anderson et al. (2009) – has been referenced throughout these studies, it is also important to acknowledge that molecular-based systematics are becoming more and more widely adopted[2]

[2] While this (so far) has not dramatically affected the classification of many animal-parasitic nematodes at the higher level, the terminology does differ with, for example, the division of the phylum into five Clades (defined as a group of organisms related by

(e.g. Blaxter and Koutsovoulos, 2015). While such systems have so far concentrated mainly on the higher-level classifications as defined by full-length 18S sequences, it seems inevitable that as more molecular data becomes available there will be some reorganisation within the lower-level systematics also. Among the spirurids, there is already suggestion that, based on available molecular data, the traditional classification of some taxa belonging to superfamilies Habronematoidea, Physalopteroidea, and Thelazioidea require re-evaluation (Pereira et al., 2018).

There were some obvious limitations beyond the lack of comparative genetic data in the approach to these preliminary studies of kiwi gastrointestinal nematodes (Chapters 4 and 5). Superficial morphology, largely restricted to evaluation of size, tail conformation, and external cuticular ornamentation such as cervical alae, was performed at the time of nematode collection, with microdissections to evaluate egg morphology also performed in a small number of cases. However, for the most part, the large numbers of nematodes found were collected directly into 70% ethanol and stored for up to three years prior to any further evaluations. Ethanol fixation, chosen in this case with a view to potential future molecular analysis, is not ideal for morphological examination as may cause some shrinkage and morphological distortion (Naem et al., 2010). Conversely, formalin-fixation, which is preferable for morphological preservation, is not recommended for the application of molecular tools due to the formation of cross-linkages between DNA and protein (Williams et al., 1999). Collection of specimens into both ethanol and formalin could have been performed but, ideally, molecular evaluation would be coupled with detailed morphological examination and description of the same specimen. An alternative fixative that preserves both morphology and genetic integrity has been proposed in

descent from a common ancestor) containing infraorders: relevant to the nematodes of kiwi, the orders Ascaridida and Spirurida appear analogous to infraorders Ascaridomorpha and Spiruromorpha, both placed within Clade III (or the Spirurina), while the capillarids reside within the infraorder Trichinellida (or Trichocephalida) placed within Clade I (e.g. Blaxter et al., 1998; Bowman, 2014; Blaxter et al., 2015).

the form of DESS (Yoder et al., 2006), which was considered but never properly trialled in this study.

In the absence of access to expert morphological examination at the time of collection, detailed photographs of the specific nematodes used for the molecular analyses could have allowed for consultation and re-evaluation of the features of interest after sequencing results had been analysed – this would have especially been useful in the case of the "outlier" caecal nematode from the Haast tokoeka, for which sequencing, BLAST, and phylogenetic analyses suggested a distinctly different species (possibly even a different superfamily, based on the COI sequence) than the other caecal heterakoids even harvested from the same bird (Chapter 5). While some photographs were taken of the specimens used in the molecular analyses, these were at low-magnification and uncleared, insufficient for any meaningful comparative examination after the fact. The increasing use of molecular analyses, especially where small nematodes are likely to be entirely consumed by the process of DNA extraction, has resulted in the acceptance of digital imaging techniques as an alternative to the physical preservation of voucher specimens, and there are many descriptions on methods for taking high-resolution photographs (e.g. Eisenback, 2012) or even video capture (De Ley and Bert, 2002) which could have been employed here.

But even with the best of equipment and techniques, the quality of the specimen itself will affect the ability to evaluate and describe morphological detail, and to some extent this was limited by the nature of the available samples. Preservation of nematodes in a "relaxed" state, for ease of morphological evaluation of key identifying features, is generally recommended through killing the collected nematodes via their placement into a heated fixative (e.g. Grewal et al., 1990). However, live nematodes were almost never encountered in this survey; as is so often the case for wildlife specimens, the kiwi submitted for necropsy had been dead and the bodies sometimes stored chilled or frozen prior to evaluation, resulting in a highly variable degree of preservation of their parasites. This was most obvious in the case of the caecal nematodes which

were often noted to be degenerate in appearance at the time of collection, with degradation likely accelerated as a result of the fermentative environment within the caeca.

Relatively short sequence reads and variable sequence quality were also limiting factors to the molecular aspects of this study (Chapter 5). Application of next-generation sequencing, which is becoming increasingly accessible and affordable, may be worth considering in future studies although any advantages to its use would still be constrained by the quality of the extracted DNA. As with morphology, DNA quality will have been adversely affected by the poor state of preservation of many of the nematodes at harvest, particularly (once again) the caecal nematodes, but the prolonged period of alcohol fixation prior to analysis was also not ideal. It is possible that trialling alternative extraction protocols and/or the addition of a DNA purification step following enzymatic lysis of the smallest nematodes may have been able to improve sequence integrity, but this was not investigated further.

The results obtained were able to support the presence of different species of heterakoid nematodes parasitising the caeca of NIB kiwi versus Haast tokoeka, a finding not entirely surprising given the genetic and geographic divide between these two species (Weir et al., 2016). Results also raised the possibility of two different heterakoid species occurring as a mixed infection in the caeca of one NIB kiwi, and a genetically distinct nematode – possibly heterakoid or possibly from a different superfamily based on 18S and COI sequencing respectively – occurring as part of a mixed infection in caeca of the Haast tokoeka; however, the gathering of further sequences from additional hosts to support these isolated findings would certainly be advisable. Clark (1983a, b) previously suggested the existence of up to six different species of heterakoid nematodes belonging to at least three different genera in kiwi, but unfortunately no comparative details of these organisms, either morphological or regarding their host of origin, is available in the published material. Specific collection for expert morphological and more extensive molecular evaluations, potentially even co-phylogenetic studies between the caecal

nematodes and their kiwi host species, would be a fascinating study and legacy to the work of Dr. Clark. Despite the apparent low pathogenicity of these caecal parasites, such further investigation may have value in understanding the evolution of these well-adapted host-parasite relationships, particularly in contrast to the pathogenic effects of other nematode parasites identified in this thesis.

The formal description of novel nematode species (e.g. Šlapeta, 2013) was beyond the scope and expertise of this study. Moving forward, for formal identification and description of the gastrointestinal nematodes of kiwi to be advanced beyond the results of this thesis, greater effort should be made to harvest nematodes from the freshest of necropsy specimens, with detailed morphological examination or high-quality digital imaging performed either at the time of collection or following a considered choice of the preservative used in order to maintain both the morphology and genetic integrity of the organisms. Nematode systematics have been previously described as being at a cross-roads, where a worldwide decline in the number of experienced morphological taxonomists has been coupled with a rise in the development, accessibility, and application of molecular tools (Ferris, 1994; Abebe et al., 2011), to the extent of suggesting the future may even lie in the redefinition of the concept of "species" as a measure of biodiversity, moving more toward a "molecular operating taxonomic unit" (MOTU) based on an as yet undetermined molecular marker or markers (Blaxter, 2004; Abebe et al., 2011). However, at least at the current time, integrative approaches to the identification of nematodes utilising both morphological and molecular data where possible, still appear to be most desirable (e.g. De Sousa et al., 2019).

The pursuit of specific or formal nematode identification is certainly of at least academic interest; however, another, perhaps more practical perspective to the relevance of these studies is that of the wildlife clinician or pathologist whose main interest lies more in the significance of such infections to the host – whether or not the parasite itself can be given a name. To this latter

end, the prospective survey (Chapter 4) did bring some noteworthy findings to light. Of most concern, despite the presence of several different types of spirurid nematodes, spirurid eggs were never identified in faecal flotations using the routine diagnostic protocol of a 33% zinc sulphate flotation solution. While these infections on the whole appeared to be relatively well-tolerated by the host, during the course of the study two very similar cases were presented of juvenile kiwi being raised in captive facilities diagnosed with erosive/ulcerative ventriculitis in association with heavy gizzard spirurid burdens. Similar findings have been described in young, captive-reared rhea (*Rhea americana*), a fellow palaeognath, infected with *Procyrnea* (syn. *Sicarius*) *uncinipenis* (e.g. Ederli and de Oliveira, 2014). In the case of the juvenile kiwi, the exact nature of the relationship between clinical illness/mortality and the presence of the parasites could not be definitively established, but these cases do raise potential that in some circumstances such infections may be able either to incite or to exploit and exacerbate clinically significant gizzard lesions. Importantly, this potential association between nematode infection and clinical illness may historically have been missed, even where repeat faecal flotations were performed in hospitalised patients, due to the inherent difficulty in floating spirurid eggs. To further assess for the presence, prevalence, and potential significance of gizzard nematodiasis, more work is clearly indicated to establish a reliable method for the antemortem diagnosis of spirurid infection.

Of the other nematode species encountered in the survey, no histological suggestion of disease was found in association with caecal heterakoid infections, and heterakoid eggs were readily identified by routine faecal flotation although with a relatively low sensitivity – if antemortem diagnosis of infection is considered clinically important, for which there is (as yet) no indication, repeat faecal flotations would therefore be recommended. Capillarid eggs were also quite commonly found but with no established association to presence of intestinal capillarids, based on the small number of such nematodes identified – it thus remains unclear as to whether in some cases the capillarid eggs found in routine faecal flotations may reflect ingestion of the ova

from other species that have passed through the gastrointestinal tract or whether capillarid infections were significantly underestimated by this survey due to the very fine, hair-like nature of the nematodes and the fact that adult capillarids commonly burrow within the intestinal mucosa. In either case, the apparent low burden of infection suggested both by this survey and also previously by Clark (1983b), and the lack of any histopathological evidence of infection in any of the cases presented here, suggests that such infections are likely to be of negligible pathological significance to the kiwi host.

More sporadically encountered on routine testing, the presence of either oxyurid (pinworm) or strongylid eggs are considered compatible with their ingestion in soil or the faeces of other species, and such findings can therefore be discounted by clinicians. The occasional presence of non-heterakoid, non-*Toxocara*, ascarid-type eggs is similarly likely to be secondary to soil ingestion, although potential infection with acquired ascarids such as *Porrocaecum* sp., as previously described by Clark (1983b) – although only in immature form – while not confirmed in this survey, could also not be entirely discounted. Of the existence of the first described but never since encountered (potentially mythical) kiwi nematode, *Ascaris apterycis* (Chatin, 1884, 1885), there remains no indication.

Conclusion

The research presented in this thesis covers the use and validation of methodology using archival formalin-fixed, paraffin-embedded tissues sections that allowed the molecular identification of the feline nematode *Toxocara cati* as a primary cause of visceral and neural *larva migrans* in the North Island brown kiwi. Similar techniques were then applied to an unusual syndrome of cutaneous nematodiasis in juvenile rowi, leading to confirmation of the causative agent as a member of the *Capillaria sensu lato*, although a precise identification of the nematode species involved and its original source do remain elusive.

In conjunction with these investigations into aberrant nematode infections, a prospective collection of parasitological and histological specimens from kiwi presented for necropsy was initiated, and the resultant 18-month survey evaluated the presence and prevalence of gastrointestinal nematodes along with results from routine faecal flotations and the histological examination of infected tissues. While five distinct nematode types were identified, two of which have not been previously described from kiwi, no specific identifications could be reached through use of molecular tools, highlighting deficiencies in comparative sequence material within nematode databases (particularly regarding parasites of wildlife origin) and emphasising a contemporary need for skilled taxonomic parasitologists.

While there is still a lot of work that would be required in order to unravel the true diversity of these parasites and their host-relationships, the results presented herein do offer some clinical insights that may benefit future kiwi management, and which expand the current, somewhat sparse body of knowledge regarding the cause and significance of nematodiasis in our national bird.

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Appendices

Appendix A

Identification numbers for samples tested in Chapter 2.

Chapter case identifier	SoVS database information		Tissue	Laboratory (PCR) sample identifier
	Case identifier	Block identifier		
Kiwi #1	54238	-1	Lung	4
Kiwi #2	52933	-1	Liver	5
		-3	Lung	6
		-6	Brain	7
		-7	Brain	8
Kiwi #3	52838	-4	Skeletal muscle	9
		-5	Skeletal muscle	10
		-6	Lung	11
		-7	Liver	12
		-12	Brain	13
Kiwi #4	52209	-1	Liver	14
		-5	Brain	15
		-7	Brain	16
Kiwi #5	46460	-1	Lung	17
		-2	Liver	18
		-6	Brain	19
Kiwi #6	54420	-2	Brain	21
Kiwi #7	53074	-1	Lung	22
Kiwi #8	50269	-3	Lung	23
		-6	Brain	24
Kiwi #9	48790	-2	Lung	25
Kiwi #10	47257	-5	Lung	26
		-6	Brain	27
Kiwi #11	46820	-D	Liver	28
Kiwi #12	46540	-1	Lung	29
Kiwi #13	42795	-A	Heart	30
		-D	Liver	31
Kiwi #14	38174	-A	Brain	32
		-B	Liver	33
		-C	Lung	34
Kiwi #15	37334		Liver	35
Kiwi #16	37189		Brain	36
			Lung	37
Kiwi #17	35683	-A	Liver	38
		-B	Lung	39
		-C	Brain	40

Appendix B

Identification numbers for samples tested in Chapter 3.

Chapter case identifier	SoVS database information		Tissue	Laboratory (PCR) sample identifier
	Case identifier	Block identifier		
Rowi #1	50062	-1	Skin	42
Rowi #2	50062	-2	Skin	43
Rowi #3	50062	-3	Skin	44
Rowi #4	50062	(P13015609)-1[a]	Skin	45
Rowi #5	-	P13015819-1[b]	Skin	47
Rowi #6	50113	-5	Skin	48
Rowi #7	52481		Skin	49
Rowi #8	56782		Skin	50
Kahu #1	44839		Oral	H1
Kahu #2	40753		Crop	H2
Kahu #3	37340	-C	Oral	H3
Kahu #4	34688	-A	Oral	H4
Kahu #5	34402		Oral	H5
Kahu #6	34265	-A	Oral	H6
Kahu #7	34049	-D	Oral	H7
Kahu #8	33538	-A	Oral	H8
Kahu #9	33315	-C	Crop	H9
Kahu #10	33314		Oral	H10
Kahu #11	33142	-C	Crop	H11

[a]submitted through New Zealand Veterinary Pathology Laboratory, reported through SoVS database; [b]submitted and reported through New Zealand Veterinary Pathology Laboratory.

Appendix C

Identification numbers for cases described in Chapter 4.

Chapter case identifier	SoVS database case identifier
#1	54246
#2	54356
#3	54472
#4	54674
#5	54675
#6	54698
#7	54782
#8	54907
#9	54950
#10	54977
#11	54978
#12	54982
#13	55069
#14	55074
#15	55229
#16	55284
#17	55321
#18	55415
#19	55417
#20	55418
#21	55480
#22	55551
#23	55552
#24	55553
#25	55554

Chapter case identifier	SoVS database case identifier
#26	55573
#27	55666
#28	55677
#29	55688
#30	55720
#31	55732
#32	55748
#33	55789
#34	55790
#35	55957
#36	56040
#37	56041
#38	56056
#39	56067
#40	56130
#41	56282
#42	56340
#43	56411
#44	56419
#45	56420
#46	56423
#47	56474
#48	56475
#49	56486
#50	56567

Appendix D

Identification numbers for nematodes tested in chapter 5.

Chapter specimen identifier	Laboratory (PCR) specimen identifier
#15-PV	PV1
#36-PV	PV2
#47-PV	PV3
#11-G(M)	G3
#11-G(F)	G4
#15-G(M)	G5
#15-G(F)	G6
#32-G(M)	G7
#32-G(F)	G8
#35-G(M)	G9
#35-G(F)	G10
#41-G(M)	G11
#41-G(F)	G12
#38-G(F)	G13
#9-SI	Kiwi1
#15-SI	S1
#22-SI	S2
#36-SI	S3
#48-SI	S4
#21-Cp	Cp1
#29-Cp	Cp2
#11-C(F)	C2
#11-C(M)	C3
#15-C(F1)	C4
#15-C(F2)	C6
#15-C(M1)	C28
#15-C(M2)	C29
#20-C(F1)	C7
#20-C(F2)	C8
#20-C(M1)	C9
#20-C(M2)	C10
#32-C(M)	C18
#32-C(F)	C20
#35-C(M1)	C40
#35-C(M2)	C41
#41-C(F)	C12
#41-C(M)	C30

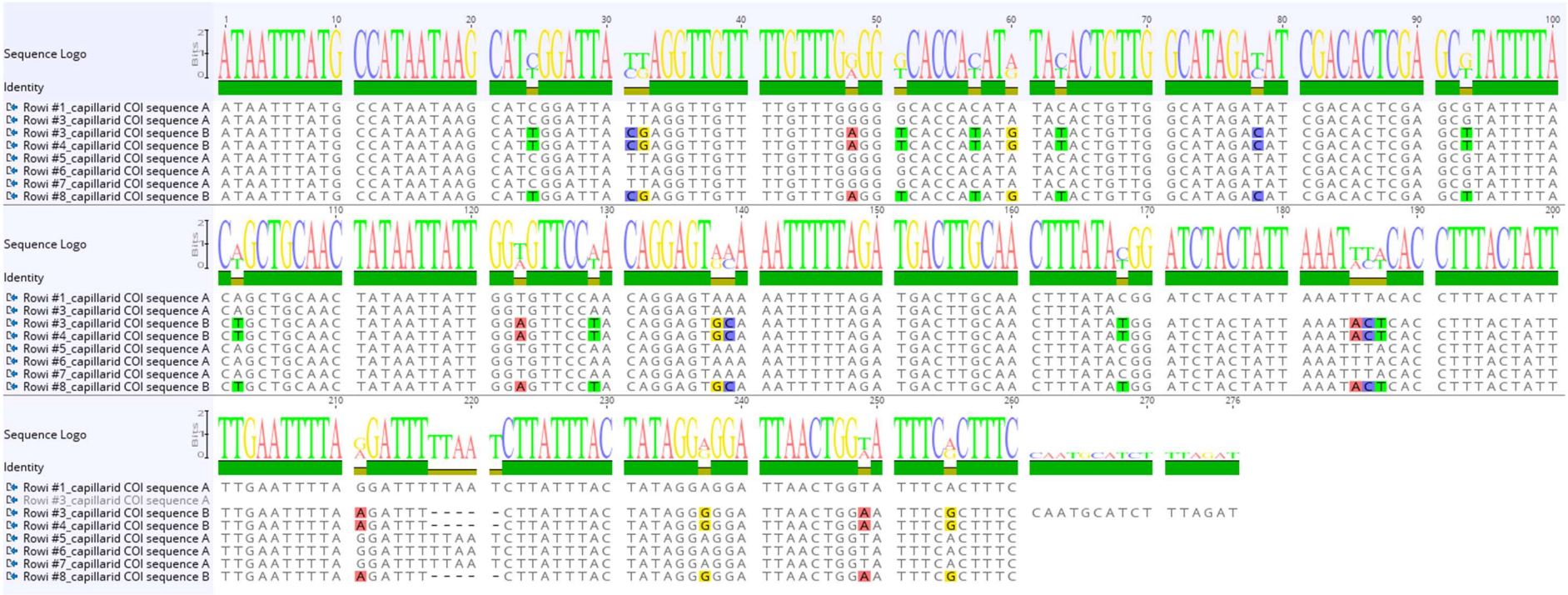
Appendix E

Identification numbers for tissue samples tested in Chapter 5.

Chapter case identifier	SoVS database information		Tissue	Laboratory (PCR) sample identifier
	Case identifier	Block identifier		
#23	55552	-4	Lung	51
		-6	Liver	55
#36	56040	-5	Lung	52
		-9	Brain	53
#50	56567	-E	Lung	54
		-F	Liver	56

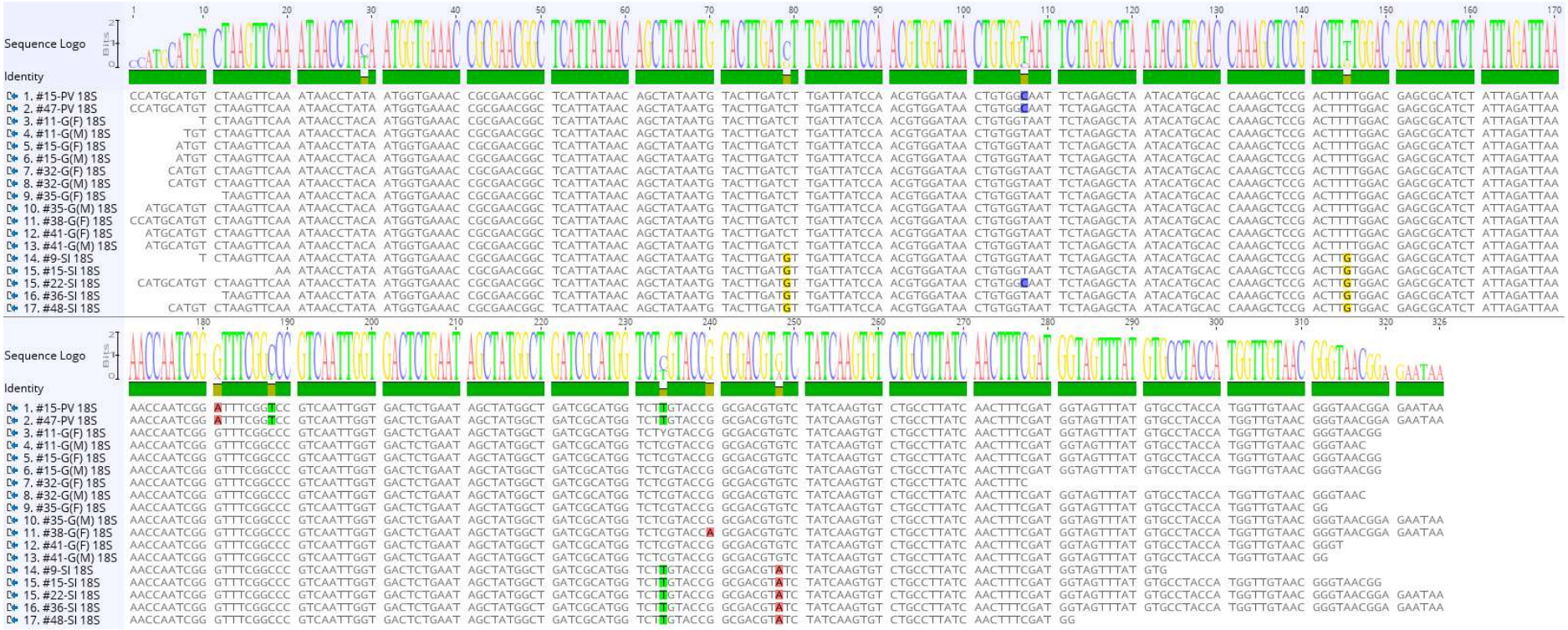
Appendix F

Capillarid COI sequences obtained from rowi skin samples in Chapter 3, presented aligned using Geneious v. 10.2.3 with disagreements to the consensus highlighted. The dashes in the three "B" sequences (at base positions 217-221) reflect the five base gap resulting from the alignment, as discussed in Chapter 3.



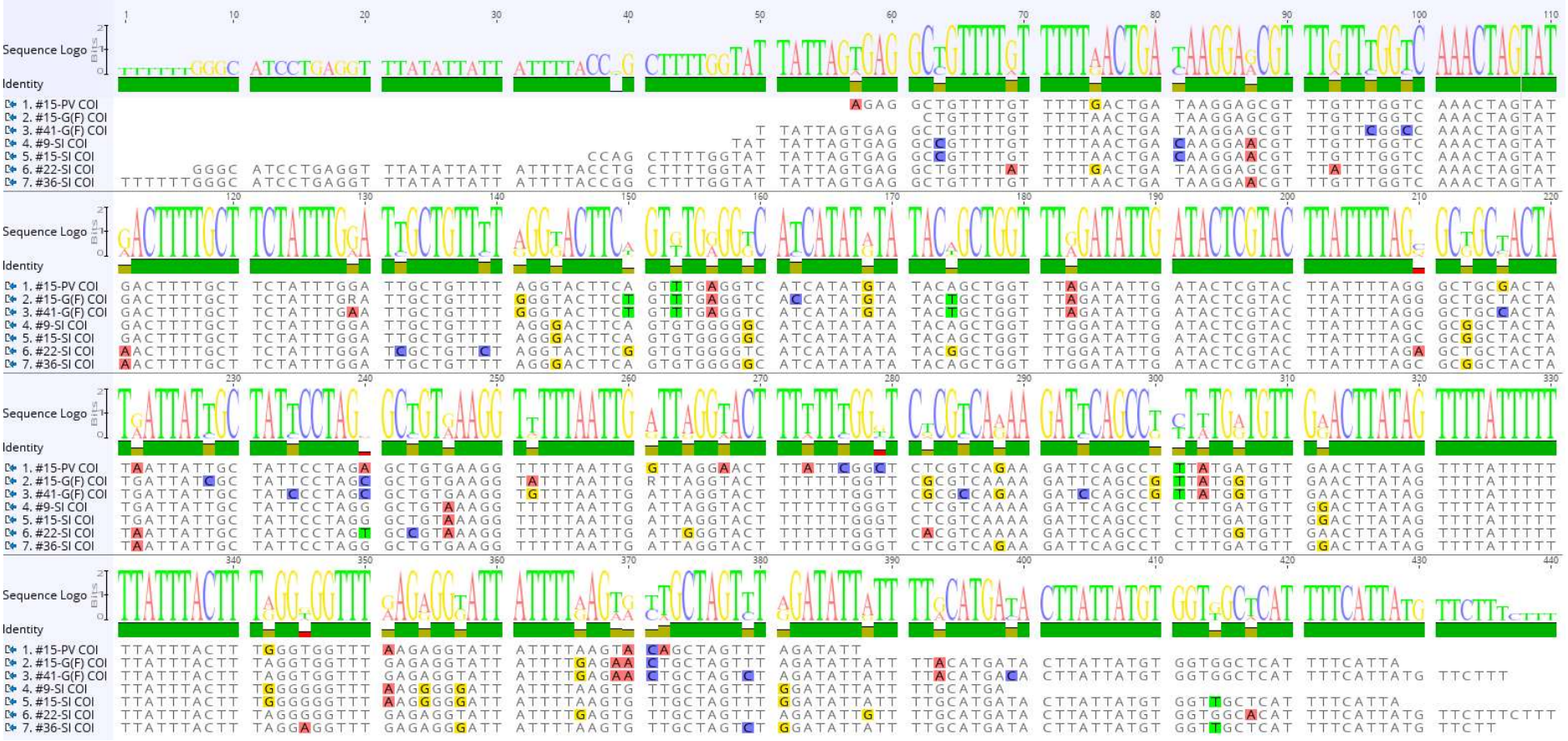
Appendix G

18S sequences obtained from spirurid nematodes originating from the proventriculus, gizzard, and small intestine of kiwi, as described in Chapter 5, presented aligned using Geneious v. 10.2.3 with disagreements to the consensus highlighted.



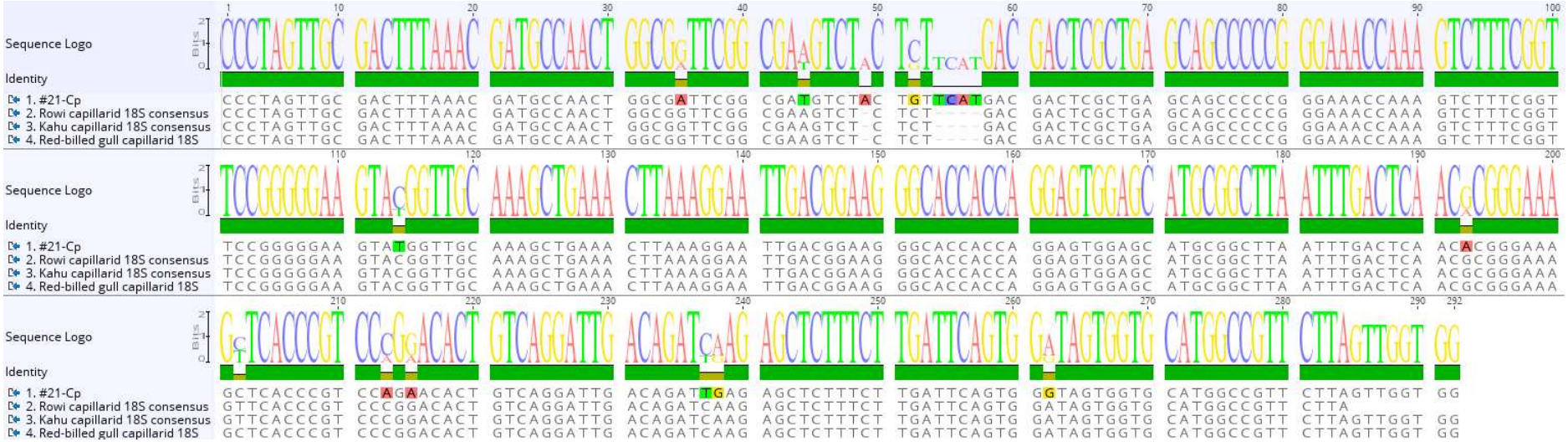
Appendix H

COI sequences obtained from spirurid nematodes originating from the proventiculus, gizzard, and small intestine of kiwi, as described in Chapter 5, presented aligned using Geneious v. 10.2.3 with disagreements to the consensus highlighted.



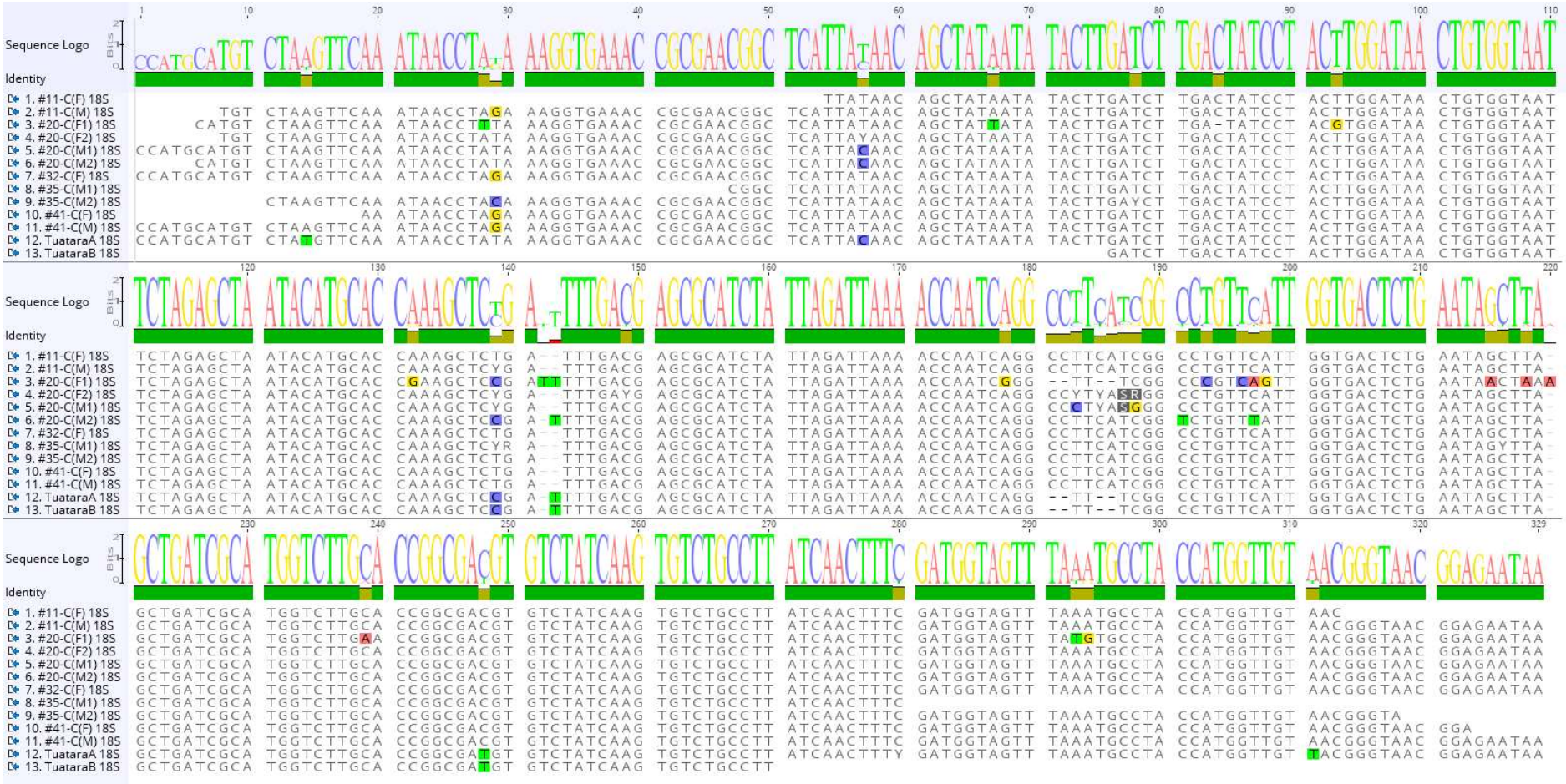
Appendix I

18S sequence obtained from capillarid nematode originating from the small intestine of a kiwi as described in Chapter 5, aligned with 18S consensus sequences obtained from capillarids originating from rowi skin lesions and kahu oesophagus and crop and an 18S sequence from a capillarid originating from proventriculus of a red-billed gull, as described in Chapter 3. Presented aligned using Geneious v. 10.2.3.



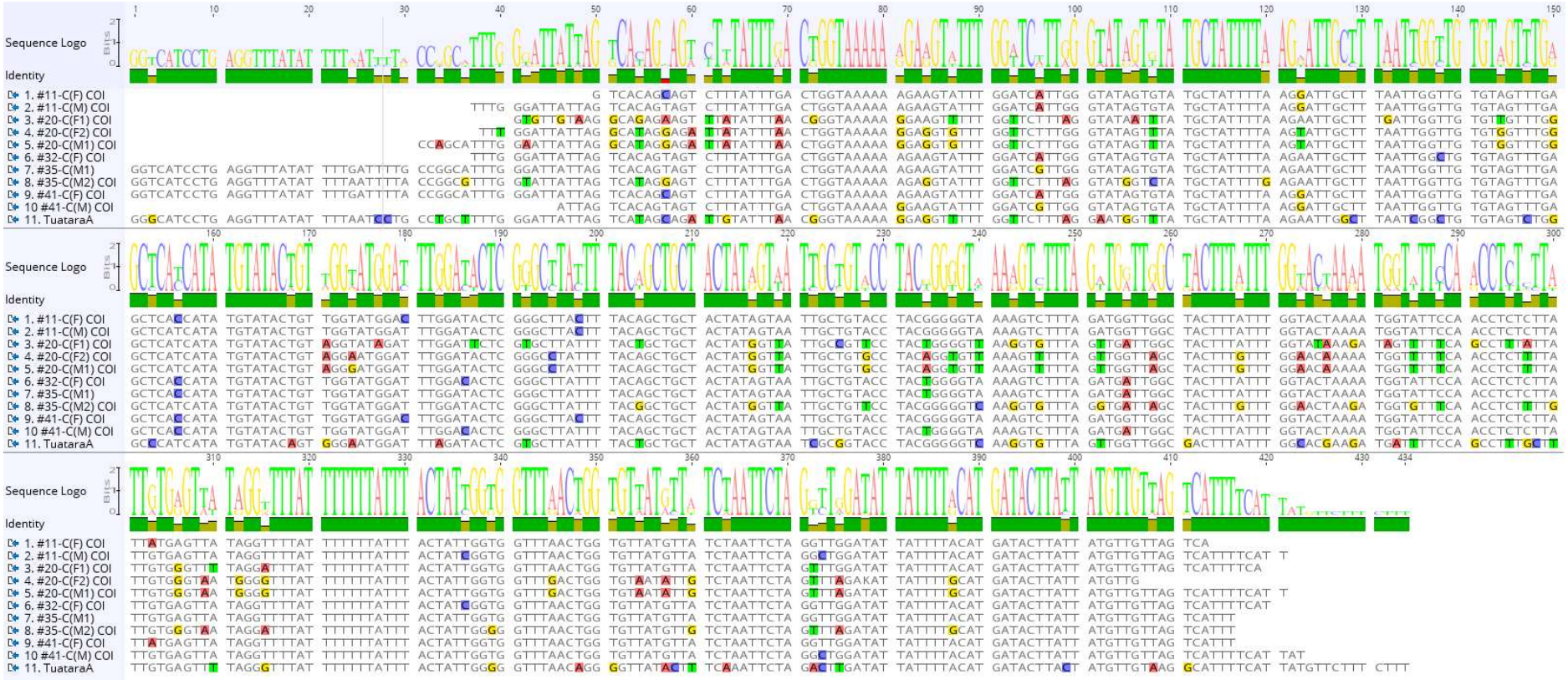
Appendix J

18S sequences obtained from ascarid nematodes originating from the caeca of kiwi and from the faeces of tuatara as described in Chapter 5, presented aligned using Geneious v. 10.2.3 with disagreements to the consensus highlighted.



Appendix K

COI sequences obtained from ascarid nematodes originating from the caeca of kiwi and from the faeces of tuatara as described in Chapter 5, presented aligned using Geneious v. 10.2.3 with disagreements to the consensus highlighted.



Appendix L

List of all GenBank accession numbers (#) included in the spirurid 18S phylogenetic tree analysis presented in Chapter 5 as Figure 5.1.

Nematode name	GenBank #
Acanthocheilonema viteae	DQ094171
Acuariidae sp.	MK301119
Ascarophis arctica	DQ094172
Breinlia mundayi	JF934735
Brugia malayi	AF036588
Capillospirura sp.	MG594289
Cyrnea leptoptera	EU004815
Cyrnea mansioni	AY702701
Cyrnea seurati	EU004816
Cystidicola farionis	MG594291
Diomedenema diomedeae	MG805661
Dipetalonema sp.	DQ531723
Dirofilaria repens	AB973229
Dirofilaria repens	MK192092
Dirofilaria repens	MH981970
Dirofilaria repens	MH981971
Echinuria borealis	EF180064
Filarioidea sp.	MK868471
Filarioidea sp.	MN129783
Gongylonema nepalensis	LC388743
Gongylonema nepalensis	LC388747
Gongylonema nepalensis	AB646107
Gongylonema nepalensis	AB646109
Gongylonema nepalensis	LC278392
Gongylonema pulchrum	AB646059
Gongylonema pulchrum	AB646062
Gongylonema pulchrum	AB646055
Gongylonema pulchrum	AB646106
Gongylonema pulchrum	AB646064
Gongylonema pulchrum	AB646069
Gongylonema pulchrum	LC026017
Gongylonema pulchrum	LC026018
Gongylonema pulchrum	LC026019
Gongylonema pulchrum	LC026020
Gongylonema pulchrum	LC026021
Gongylonema pulchrum	LC026024
Gongylonema pulchrum	LC026025
Gongylonema pulchrum	LR215834
Gongylonema pulchrum	LC388753

Nematode name	GenBank #
Loa loa	XR_002251421
Loa loa	DQ094173
Mansonella ozzardi	MN432519
Mansonella perstans	MN432520
Onchocerca cervicalis	DQ094174
Oxyspirura petrowi	KF110799
Oxyspirura petrowi	KF110800
Oxyspirura petrowi	LC316613
Oxyspirura sp.	LC508119
Physaloptera alata	AY702703
Physaloptera apivori	EU004817
Physaloptera mirandai	KT894816
Physaloptera retusa	KT894814
Physaloptera sp.	MG808040
Physaloptera sp.	HM067978
Physaloptera sp.	EF180065
Physaloptera thalacomys	JF934734
Physaloptera turgida	DQ503459
Proleptus sp.	JF934733
Rhabdochona guerreroensis	JF934732
Rhabdochona kisutchi	MG594298
Rhabdochona milleri	MG594294
Salmonema sp.	MG594295
Salmonema sp.	MG594290
Salmonema sp.	MG594299
Serratospiculum tendo	AY702704
Setaria digitata	DQ094175
Spinitectus gracilis	MG594297
Spirocerca lupi	AY751497
Spirocerca sp.	AY751498
Spirurida sp.	MG594293
Streptopharagus sp.	HM067977
Synhimantus cf. *laticeps*	KP861914
Synhimantus hamatus	EU004819
Synhimantus laticeps	EU004818
Turgida torresi	EF180069
Wuchereria bancrofti	AF227234
Wuchereria bancrofti	AY843436

Appendix M

List of all GenBank accession numbers (#) included in the spirurid COI phylogenetic tree analysis presented in Chapter 5 as Figure 5.2.

Nematode name	Genbank #
Acanthocheilonema odendhali	KF038145
Acanthocheilonema odendhali	KF038146
Acanthocheilonema odendhali	KF038147
Acanthocheilonema odendhali	KF038148
Acanthocheilonema odendhali	KF038149
Acanthocheilonema odendhali	KF038151
Acanthocheilonema odendhali	KF038153
Acanthocheilonema reconditum	KY347823
Acanthocheilonema spirocauda	KF038155
Acanthocheilonema viteae	AP017679
Acanthocheilonema viteae	HQ186249
Brugia malayi	AF538716
Brugia pahangi	AP017680
Brugia timori	AP017686
Chandlerella quiscali	HM773029
Dirofilaria immitis	AF181893
Dirofilaria immitis	AJ537512
Dirofilaria repens	KR071802
Dirofilaria repens	KX265047
Dirofilaria repens	KX265048
Dirofilaria repens	KX265049
Dirofilaria repens	MK210632
Dirofilaria repens	MW206367
Dirofilaria repens	MW206368
Dirofilaria repens	MW206371
Dirofilaria repens	MW206374
Dirofilaria repens	MW206376
Dirofilaria sp. 'hongkongensis'	KX265050
Dirofilaria sp. 'Thailand'	KX912163
Dirofilaria sp. 'Thailand'	KY971621
Filarioidea sp.	JX870433
Gongylonema neoplasticum	LC331001
Gongylonema neoplasticum	LC331005
Gongylonema neoplasticum	LC331015
Gongylonema neoplasticum	LC331016
Gongylonema neoplasticum	LC331019
Gongylonema neoplasticum	LC331022
Gongylonema neoplasticum	LC331025
Gongylonema neoplasticum	LC331027
Gongylonema neoplasticum	LC331039
Gongylonema neoplasticum	LC331043
Gongylonema neoplasticum	LC334451
Gongylonema neoplasticum	LC334453
Gongylonema neoplasticum	LC334454

Nematode name	Genbank #
Gongylonema pulchrum	AP017685
Habronema muscae	KJ819944
Litomosoides sigmodontis	AP017689
Loa loa	HQ186250
Mansonella perstans	MT361687
Mastophorus muris	MG386206
Mastophorus muris	MG821081
Mastophorus muris	MK867474
Mastophorus muris	MK867478
Mastophorus muris	MK867480
Onchocerca flexuosa	AP017692
Onchocerca flexuosa	HQ214004
Onchocerca ochengi	AP017693
Onchocerca ochengi	AP017694
Onchocerca ochengi	KX181289
Onchocerca ochengi	KX181290
Onchocerca ochengi	NC_031891
Onchocerca volvulus	AF015193
Onchocerca volvulus	AP017695
Onchocerca volvulus	KT599912
Oxyspirura mansoni	LC538187
Oxyspirura mansoni	LC538188
Oxyspirura mansoni	LC538189
Oxyspirura mansoni	LC538190
Oxyspirura mansoni	LC538191
Parabronema smithii	MH445383
Setaria digitata	GU138699
Setaria digitata	KY284626
Setaria labiatopapillosa	NC_044071
Setaria tundra	DQ097309
Setaria tundra	EF661848
Spirocerca lupi	HQ674751
Spirocerca lupi	HQ674752
Spirocerca lupi	HQ674753
Spirocerca lupi	HQ674754
Spirocerca lupi	HQ674755
Spirocerca lupi	HQ674756
Spirocerca lupi	HQ674757
Spirocerca lupi	HQ674758
Spirocerca lupi	HQ674759
Spirocerca lupi	HQ674760
Spirocerca lupi	HQ674761
Spirocerca lupi	KC305876
Spirocerca lupi	KY495493

Appendix M continued.

Nematode name	Genbank #
Spirocerca lupi	KY495494
Spirocerca lupi	KY495495
Spirocerca lupi	KY495496
Spirocerca lupi	KY495497
Spirocerca lupi	KY495498
Spirocerca lupi	KY495499
Spirocerca lupi	KY495500
Spirocerca lupi	KY495501
Spirocerca lupi	KY495502
Spirocerca lupi	KY495503
Spirocerca lupi	KY495504
Spirocerca lupi	KY495505
Spirocerca lupi	KY634868
Spirocerca lupi	KY634869
Spirocerca lupi	KY634870
Spirocerca lupi	MH633995
Spirocerca lupi	MH633996
Spirocerca lupi	MH633997
Spirocerca lupi	MH633998
Spirocerca lupi	MH633999
Spirocerca lupi	MH634000
Spirocerca lupi	MH634001
Spirocerca lupi	MH634002
Spirocerca lupi	MH634003
Spirocerca lupi	MH634004
Spirocerca lupi	MH634005
Spirocerca lupi	MH634006

Nematode name	Genbank #
Spirocerca lupi	MH634007
Spirocerca lupi	MH634008
Spirocerca lupi	MH634009
Spirocerca lupi	MH634010
Spirocerca lupi	MH634011
Spirocerca lupi	MH634012
Spirocerca lupi	MH634013
Spirocerca sp.	KJ605487
Spirocerca sp.	KJ605489
Spirocerca vulpis	MH633991
Spirocerca vulpis	MH633992
Spirocerca vulpis	MH633993
Spirocerca vulpis	MH633994
Spirocerca vulpis	MH634014
Spirocerca vulpis	MH634015
Spirocerca vulpis	MH634016
Thelazia callipaeda	AP017700
Thelazia callipaeda	JX069968
Thelazia callipaeda	KY908318
Thelazia callipaeda	KY908319
Thelazia callipaeda	KY908320
Wuchereria bancrofti	AP017705
Wuchereria bancrofti	HQ184469
Wuchereria bancrofti	JF775522
Wuchereria bancrofti	JN367461
Wuchereria bancrofti	JQ316200

Appendix N

List of all GenBank accession numbers (#) included in the capillarid 18S phylogenetic tree analyses presented in Chapter 3 as Figure 3.2 and in Chapter 5 as Figure 5.3.

Nematode name	GenBank #
Aonchotheca (Aonchotheca) sp.	LC052366
Aonchotheca (Aonchotheca) sp.	LC052367
Aonchotheca (Aonchotheca) sp.	LC052368
Aonchotheca (Aonchotheca) sp.	LC052373
Aonchotheca (Aonchotheca) sp.	LC052374
Aonchotheca annulosa	KY488185
Aonchotheca erinace	KY488186
Aonchotheca musimon	LC052379
Aonchotheca paranalis	MF621021
Aonchotheca putorii	LC052349
Aonchotheca putorii	LC052350
Aonchotheca putorii	LC052352
Aonchotheca putorii	LC052356
Aonchotheca putorii	LC052361
Aonchotheca putorii	LC052363
Aonchotheca putorii	LC052364
Aonchotheca putorii	LC052365
Aonchotheca riukiuensis	LC052377
Baruscapillaria obsignata	LC052336
Baruscapillaria obsignata	LC052337
Baruscapillaria obsignata	LC425004
Baruscapillaria spiculata	MT068208
Baruscapillaria spiculata	MT068209
Calodium hepaticum	KY488184
Calodium hepaticum	LC425008
Calodium hepaticum	MF287972
Calodium hepaticum	MG686613
Calodium splenaecum	KC753538
Capillaria anatis	LC052334
Capillaria anatis	LC052335
Capillaria anatis	LC425001
Capillaria bursata	LC425006
Capillaria hepatica	JX456633
Capillaria hepatica	JX456634
Capillaria hepatica	KT875351
Capillaria hepatica	LC389878
Capillaria madseni	LC052344
Capillaria madseni	LC052346
Capillaria madseni	LC052347
Capillaria madseni	LC052348
Capillaria navoneae	KP063585
Capillaria plica	JX456614[a]
Capillaria plica	JX456615[a]
Capillaria plica	JX456616[a]
Capillaria plica	JX456618[a]

Nematode name	GenBank #
Capillaria plica	JX456619[a]
Capillaria plica	JX456623[a]
Capillaria plica	KF836607
Capillaria plica	KX962319[a]
Capillaria plica	KX962320[a]
Capillaria plica	KX962324[a]
Capillaria plica	KX962326[a]
Capillaria plica	KX962327[a]
Capillaria plica	KX962328[a]
Capillaria plica	KX962336
Capillaria plica	KX962352[a]
Capillaria pudendotecta	LC052338
Capillaria pudendotecta	LC052339
Capillaria putorii	JX456624
Capillaria putorii	JX456625
Capillaria putorii	JX456626[a]
Capillaria sp.	MG859285
Capillaria spinulosa	LC424999
Capillaria suis	LC052375
Capillaria tenuissima	EU004822
Capillaria xenopi	KJ415283
Eucoleus aerophilus	JX411622[b]
Eucoleus aerophilus	JX411628[b]
Eucoleus aerophilus	JX411629[b]
Eucoleus aerophilus	JX411630[b]
Eucoleus aerophilus	KJ188166
Eucoleus aerophilus	KX962317[b]
Eucoleus aerophilus	KX962318[b]
Eucoleus aerophilus	KX962323[b]
Eucoleus aerophilus	KX962329[b]
Eucoleus aerophilus	KX962340[b]
Eucoleus aerophilus	KX962344[b]
Eucoleus aerophilus	KX962356[b]
Eucoleus aerophilus	MF599385
Eucoleus boehmi	JX456628
Eucoleus contortus	LC424996
Eucoleus dispar	EU004821
Eucoleus garfiai	LC484432
Eucoleus perforans	LC424997
Eucoleus perforans	LC424998
Eucoleus sp.	KY488350
Eucoleus sp.	LC052380[a]
Eucoleus sp.	LC052381[a]
Eucoleus sp.	LC052382[a]
Eucoleus sp.	LC052383[a]

[a]included in figure 5.3 but not 3.2; [b]included in figure 3.2 but not 5.3.

Appendix N continued.

Nematode name	GenBank #
Eucoleus sp.	LC052384
Eucoleus sp.	LC052385
Pearsonema plica	MF621034
Pearsonema sp.	LC052386
Pearsonema sp.	LC052387
Pearsonema sp.	LC052388
Pearsonema sp.	LC052389
Pearsonema sp.	LC052390
Pseudocapillaria tomentosa	KU987805
Trichuris arvicolae	HF586908[b]
Trichuris discolor	HF586910[b]
Trichuris leporis	HF586913[b]
Trichuris muris	HF586907[b]
Trichuris ovis	HF586911[b]
Trichuris serrata	KM986321[b]
Trichuris serrata	KM986322[b]
Trichuris sp.	MG356472[b]
Trichuris vulpis	GQ352557[b]
Trichuris vulpis	GQ352558[b]
Trichuris vulpis	HF586909[b]
Trichuris vulpis	GQ352556[b]

[b]included in figure 3.2 but not 5.3.

Appendix O

List of all GenBank accession numbers (#) included in the ascarid 18S phylogenetic tree analysis presented in Chapter 5 as Figure 5.4.

Nematode name	GenBank #
Anisakis nascettii	JX486103
Anisakis pegreffi	EF180082
Anisakis pegreffii	MF072697
Anisakis simplex	MF072711
Anisakis sp.	U81575
Anisakis sp.	U94365
Ascaridia galli	EF180058
Ascaridia nymphii	LC057210
Ascaridoidea sp.	MT396081
Ascaris lumbricoides	KM079631
Ascaris lumbricoides	U94366
Ascaris sp.	JN256985
Ascaris suum	AF036587
Ascaris suum	MN558962
Ascaris suum	U94367
Aspidodera sp.	EF180070
Baylisascaris ailuri	JN256991
Baylisascaris procyonis	U94368
Baylisascaris schroederi	JN256992
Baylisascaris transfuga	JN256988
Baylisascaris transfuga	U94369
Contracaecum eudyptulae	EF180072
Contracaecum microcephalum	AY702702
Contracaecum multipapillatum	U94370
Contracaecum spiculigerum	AB189983
Cosmocerca simile	MN839758
Cosmocercoides pulcher	LC018444
Cosmocercoides tonkinensis	AB908160
Cruzia americana	U94371
Dujardinascaris gigantea	MF072701
Dujardinascaris waltoni	EF180081
Goezia pelagia	U94372
Goezia spinulosa	KY198732
Heterakis gallinarum	DQ503462
Heterakis gallinarum	MK844591
Heterakis sp.	AF083003
Heterakis spumosa	MH571872
Heterakoidea sp.	KY745900
Heterocheilus tunicatus	U94373
Hysterothylacium aduncum	MF072693
Hysterothylacium deardorffoverstreetorum	JF718550
Hysterothylacium fabri	MF072709
Hysterothylacium fortalezae	U94374
Hysterothylacium liparis	MF072708
Hysterothylacium longilabrum	MF072696
Hysterothylacium pelagicum	U94375

Nematode name	GenBank #
Hysterothylacium reliquens	U94376
Hysterothylacium sinense	MF072694
Hysterothylacium sp.	HM545896
Hysterothylacium sp.	MF072698
Hysterothylacium tetrapteri	MF072705
Hysterothylacium thalassini	MF072702
Hysterothylacium zhoushanense	MF072703
Iheringascaris inquies	U94377
Krefftascaris sharpiloi	GU245692
Mawsonascaris zhoui	MF072706
Nemhelix bakeri	DQ118537
Parascaris equorum	U94378
Paraspidodera sp.	AF083005
Porrocaecum angusticolle	EU004820
Porrocaecum depressum	U94379
Porrocaecum reticulatum	MF072700
Porrocaecum sp.	MT141136
Porrocaecum streperae	EF180074
Pseudanisakis rajae	MF072707
Pseudoterranova decipiens	U94380
Raillietnema sp.	DQ503461
Raphidascaris acus	DQ503460
Raphidascaris longispicula	MF072704
Raphidascaris lophii	MF072692
Raphidascaroides nipponensi	MF072710
Strongyluris calotis	LC133186
Strongyluris calotis	LC133187
Strongyluris calotis	LC133188
Strongyluris calotis	LC133189
Strongyluris calotis	LC133190
Sulcascaris sulcata	EF180080
Terranova caballeroi	U94381
Toxascaris leonina	JN256978
Toxascaris leonina	JN256979
Toxascaris leonina	JN256982
Toxascaris leonina	JN256984
Toxascaris leonina	U94383
Toxascaris leonina	JN256980
Toxocara canis	AF036608
Toxocara canis	JN256976
Toxocara canis	JN256977
Toxocara canis	U94382
Toxocara cati	JN256973
Toxocara cati	JN256975
Toxocara cati	EF180059
Toxocara vitulorum	EF180078

Appendix P

List of all GenBank accession numbers (#) included in the ascarid COI phylogenetic tree analysis presented in Chapter 5 as Figure 5.5.

Nematode name	GenBank #
Anisakis simplex	AF096226
Ascaridia columbae	JX624729
Ascaridia galli	JX624728
Ascaridia galli	KT613888
Ascaridia galli	KT613889
Ascaridia galli	KT613890
Ascaridia galli	KT613891
Ascaridia galli	KT613892
Ascaridia galli	KT613893
Ascaridia galli	KT613894
Ascaridia galli	KT613895
Ascaridia galli	KT613896
Ascaridia galli	KT613897
Ascaridia galli	KT613898
Ascaridia galli	KT613899
Ascaridia galli	KT613900
Ascaridia galli	KT613901
Ascaridia galli	KT613902
Ascaridia galli	KX266841
Ascaridia galli	KX266842
Ascaridia galli	KX266843
Ascaridia galli	KX266844
Ascaridia galli	KX266845
Ascaridia galli	KX266846
Ascaridia galli	KX266847
Ascaridia galli	KX266848
Ascaridia galli	KX266849
Ascaridia galli	KX266850
Ascaridia galli	KX266851
Ascaridia galli	KX266852
Ascaridia galli	KX266853
Ascaridia galli	KX266854
Ascaridia galli	KX266855
Ascaridia galli	KX266856
Ascaridia galli	KX266857
Ascaridia galli	KX266858
Ascaridia galli	KX266859
Ascaridia galli	KX266860
Ascaridia sp.	JX624730
Ascaris lumbricoides	KY368757
Ascaris lumbricoides	KY368758
Ascaris lumbricoides	KY368759
Ascaris lumbricoides	KY368760
Ascaris lumbricoides	KY368761
Ascaris lumbricoides	KY368763

Nematode name	GenBank #
Ascaris lumbricoides	KY368764
Ascaris lumbricoides	LN600400
Ascaris lumbricoides	MH674442
Ascaris lumbricoides	MH800223
Ascaris lumbricoides	MH800236
Ascaris lumbricoides	MH800241
Ascaris lumbricoides	MH800242
Ascaris lumbricoides	MH800244
Ascaris lumbricoides	MH800258
Ascaris lumbricoides	MH800265
Ascaris lumbricoides	MH800276
Ascaris sp.	JN575632
Ascaris sp.	MH059555
Ascaris suum	AB591803
Ascaris suum	KF719131
Ascaris suum	KY045804
Baylisascaris ailuri	HQ671080
Baylisascaris ailuri	MH795153
Baylisascaris columnaris	KC543472
Baylisascaris columnaris	KC543474
Baylisascaris columnaris	KY580736
Baylisascaris columnaris	KY580738
Baylisascaris columnaris	KY580739
Baylisascaris columnaris	LN600401
Baylisascaris columnaris	MH795147
Baylisascaris columnaris	MH795148
Baylisascaris devosi	KM216978
Baylisascaris devosi	KM216979
Baylisascaris devosi	KM216980
Baylisascaris devosi	KM216981
Baylisascaris devosi	KM216982
Baylisascaris devosi	KM216983
Baylisascaris devosi	KM216984
Baylisascaris devosi	MH795151
Baylisascaris procyonis	JF951366
Baylisascaris procyonis	KC543476
Baylisascaris procyonis	KP843600
Baylisascaris procyonis	KP843601
Baylisascaris procyonis	MH795149
Baylisascaris procyonis	MH795150
Baylisascaris schroederi	HQ671081
Baylisascaris schroederi	KJ587806
Baylisascaris schroederi	KJ587807
Baylisascaris schroederi	KJ587808
Baylisascaris schroederi	KJ587810

Appendix P continued.

Nematode name	GenBank #
Baylisascaris schroederi	KJ587811
Baylisascaris schroederi	KJ587812
Baylisascaris schroederi	KJ587813
Baylisascaris schroederi	KJ587814
Baylisascaris schroederi	KJ587815
Baylisascaris schroederi	KJ587817
Baylisascaris schroederi	KJ587819
Baylisascaris schroederi	KJ587821
Baylisascaris schroederi	KJ587827
Baylisascaris schroederi	KJ587828
Baylisascaris schroederi	KJ587834
Baylisascaris schroederi	KJ587836
Baylisascaris schroederi	KJ587837
Baylisascaris schroederi	KJ587838
Baylisascaris schroederi	KJ587842
Baylisascaris schroederi	KJ587849
Baylisascaris schroederi	KJ587853
Baylisascaris schroederi	KJ587858
Baylisascaris schroederi	KJ587861
Baylisascaris schroederi	KJ587862
Baylisascaris schroederi	MH795152
Baylisascaris tasmaniensis	MH795156
Baylisascaris transfuga	EU628683
Baylisascaris transfuga	EU740387
Baylisascaris transfuga	HM594948
Baylisascaris transfuga	HQ671079
Baylisascaris transfuga	KC543477
Baylisascaris transfuga	KY973960
Baylisascaris transfuga	MF419818
Baylisascaris transfuga	MH795154
Baylisascaris transfuga	MH795155
Baylisascaris transfuga	MK558921
Baylisascaris transfuga	MT881703
Contracaecum ogmorhini	AJ616895
Contracaecum ogmorhini	KU558727
Contracaecum ogmorhini	MT941430
Contracaecum ogmorhini	NC_031649
Contracaecum ogmorhini	AJ616896
Contracaecum ogmorhini	AJ616897
Contracaecum osculatum	AJ405303
Contracaecum osculatum	JN786330
Contracaecum osculatum	JN786332
Contracaecum osculatum	JN786333
Contracaecum osculatum	JN786334
Contracaecum rudolphii	FJ416644
Contracaecum rudolphii	FJ416645
Contracaecum rudolphii	FJ416646
Contracaecum rudolphii	FJ416649

Nematode name	GenBank #
Contracaecum rudolphii	FJ416650
Contracaecum rudolphii	FJ416651
Contracaecum rudolphii	FJ866816
Contracaecum rudolphii	FJ905109
Contracaecum septentrionale	FJ416652
Contracaecum septentrionale	FJ416653
Contracaecum septentrionale	FJ416653
Contracaecum septentrionale	FJ866817
Falcaustra sp.	MN729570
Falcaustra sp.	MN729571
Falcaustra sp.	MN729572
Heterakis beramporia	KU529972
Heterakis beramporia	LC592867
Heterakis beramporia	LC592900
Heterakis beramporia	LC592901
Heterakis beramporia	LC592902
Heterakis beramporia	LC592868
Heterakis dispar	NC_042411
Heterakis gallinarum	KP308308
Heterakis gallinarum	KP308310
Heterakis gallinarum	KP308311
Heterakis gallinarum	KP308312
Heterakis gallinarum	KP308313
Heterakis gallinarum	KP308314
Heterakis gallinarum	KP308315
Heterakis gallinarum	KP308316
Heterakis gallinarum	KP308317
Heterakis gallinarum	KP308318
Heterakis gallinarum	KP308319
Heterakis gallinarum	KP308320
Heterakis gallinarum	KP308321
Heterakis gallinarum	KP308322
Heterakis gallinarum	KP308323
Heterakis gallinarum	KP308324
Heterakis gallinarum	KP308325
Heterakis gallinarum	KP308326
Heterakis gallinarum	KP308327
Heterakis gallinarum	KP308328
Heterakis gallinarum	KP308329
Heterakis gallinarum	KP308330
Heterakis gallinarum	KP308331
Heterakis gallinarum	KP308332
Heterakis gallinarum	KP308333
Heterakis gallinarum	KP308334
Heterakis gallinarum	KP308335
Heterakis gallinarum	KP308336
Heterakis gallinarum	KP308337
Heterakis gallinarum	KP308338

Appendix P continued.

Nematode name	GenBank #
Heterakis gallinarum	KP308339
Heterakis gallinarum	KP308340
Heterakis gallinarum	KP308341
Heterakis gallinarum	KP308342
Heterakis gallinarum	KP308343
Heterakis gallinarum	KP308344
Heterakis gallinarum	KP308345
Heterakis gallinarum	KP308346
Heterakis gallinarum	KP308347
Heterakis gallinarum	KP308348
Heterakis gallinarum	KP308349
Heterakis gallinarum	KP308350
Heterakis gallinarum	KP308351
Heterakis gallinarum	KP308352
Heterakis gallinarum	KP308353
Heterakis gallinarum	KP308354
Heterakis gallinarum	KP308355
Heterakis gallinarum	KP308356
Heterakis gallinarum	KP308357
Heterakis gallinarum	KP308358
Heterakis gallinarum	KP308359
Heterakis gallinarum	KP308360
Heterakis gallinarum	KP308361
Heterakis gallinarum	KP308362
Heterakis gallinarum	KP308363
Heterakis gallinarum	LC592855
Heterakis gallinarum	LC592856
Heterakis gallinarum	LC592857
Heterakis gallinarum	MF066712
Heterakis gallinarum	MF066713
Heterakis gallinarum	MF066714
Heterakis gallinarum	MF066715
Heterakis gallinarum	MF066716
Heterakis gallinarum	MF066717
Heterakis gallinarum	MF066718
Heterakis gallinarum	MF066719
Heterakis gallinarum	MF066720
Heterakis indica	LC592870
Heterakis indica	LC592871
Heterakis indica	LC592873
Heterakis indica	LC592875
Heterakis indica	LC592903
Heterakis isolonche	FJ009625
Heterakis spumosa	KF765409
Heterakis spumosa	KF765410
Heterakis spumosa	MH571870
Heterakis spumosa	MH571871
Ophidascaris filaria	MH285588
Ophidascaris filaria	MH285589
Ophidascaris filaria	MH285591
Ortleppascaris sinensi	KU950438
Parascaris equorum	MH795158
Parascaris equorum	MK209651
Parascaris equorum	MK209654
Parascaris equorum	MK209657
Parascaris equorum	MK209658
Parascaris equorum	MK209661
Parascaris equorum	MK209662
Parascaris univalens	MK209667
Parascaris univalens	MK209668
Parascaris univalens	MK209669
Parascaris univalens	MK209670
Parascaris univalens	MK209671
Pseudanisakis rajae	MF113228
Subulura chinensis	MK770150
Subulura chinensis	MK770151
Subulura chinensis	MK770152
Subulura chinensis	MK770153
Subulura chinensis	MK770154
Subulura chinensis	MK770155
Toxascaris leonina	AJ920063
Toxascaris leonina	AJ920064
Toxascaris leonina	JF780946
Toxascaris leonina	JF780947
Toxascaris leonina	JF780950
Toxascaris leonina	JF780951
Toxascaris leonina	KC293926
Toxascaris leonina	KC293927
Toxascaris leonina	KC293929
Toxascaris leonina	KC293930
Toxascaris leonina	KC293934
Toxascaris leonina	KC293935
Toxascaris leonina	KX963448
Toxascaris leonina	MH795159
Toxascaris leonina	MK516267
Toxascaris leonina	MT895786
Toxascaris leonina	MT942619
Toxascaris leonina	MT942620
Toxascaris leonina	MT942621
Toxascaris sp.	KT223040
Toxocara canis	AJ920052
Toxocara canis	AJ920053
Toxocara canis	AJ920056
Toxocara malaysiensis	AJ920058
Toxocara malaysiensis	AJ920059
Toxocara malaysiensis	AJ920060
Toxocara malaysiensis	AJ920061

Appendix Q

Statements of Contribution for Chapters 2 and 3.

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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

Name of candidate:	Adrienne French
Name/title of Primary Supervisor:	Professor Brett Gartrell
Name of Research Output and full reference:	
Nematode larva migrans caused by Toxocara cati in the North Island brown kiwi (Apteryx mantelli). International Journal for Parasitology: Parasites and Wildlife, 11 (2020), 221-228.	
In which Chapter is the Manuscript /Published work:	Chapter 2
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Project concept and design, histological analysis, processing and molecular analysis samples, manuscript writing and corresponding author	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Adrienne French Digitally signed by Adrienne French Date: 2021.02.22 10:55:58 +13'00'
Date:	22 Feb 2021
Primary Supervisor's Signature:	Gartrell, Brett Digitally signed by Gartrell, Brett Date: 2021.02.25 10:15:12 +13'00'
Date:	25th February, 2021

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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

Name of candidate:	Adrienne French
Name/title of Primary Supervisor:	Professor Brett Gartrell
Name of Research Output and full reference:	
Ventral dermatitis in rowi (Apteryx rowi) caused by cutaneous capillariasis. International Journal for Parasitology: Parasites and Wildlife, 13 (2020), 160-170.	
In which Chapter is the Manuscript /Published work:	Chapter 3
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Project concept and design, histological analysis, processing and molecular analysis samples, manuscript writing and corresponding author	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Adrienne French Digitally signed by Adrienne French Date: 2021.02.22 11:20:15 +13'00'
Date:	22 Feb 2021
Primary Supervisor's Signature:	Gartrell, Brett Digitally signed by Gartrell, Brett Date: 2021.02.25 10:14:12 +13'00'
Date:	25th February, 2021

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)