Studies on the epidemiology of *Nematodirus spathiger* and *Nematodirus filicollis* in New Zealand

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Abstract

This thesis studies aspects of the epidemiology of *Nematodirus spathiger* and *Nematodirus filicollis*, both are common and potentially pathogenic parasites of lambs in New Zealand. Three studies were undertaken; the first a presence/absence survey to determine the distribution of the two species on farms, the second examined the prevalence of benzimidazole (BZ) resistance in the two species and the third experiment investigated the requirements for chilling for *N. filicollis* eggs to hatch.

The first two studies utilised faecal samples from routine faecal egg counts and/or routine faecal egg count reduction testing on farms. To identify the *Nematodirus* species involved, eggs from these samples were chilled and then incubated to facilitate hatching of *N. filicollis*, before larvae were recovered. The identity of the larvae was determined using PCR of the ITS-2 region of rDNA. The third experiment used a bulk collection of *N. filicollis* from naturally infected lambs. Extracted eggs were incubated at temperatures between 2.7°C - 9.9°C, for up to 224 days. The proportion of eggs hatching was assessed against chill units. Chill units (degree-day) were calculated by subtracting the culture temperature from a constant threshold of 11°C and multiplying by the number of days for which the sample was cultured.

In Study 1, *N. spathiger* was present on all farms tested, while *N. filicollis* was found on 76% of farms. Both species were distributed throughout New Zealand, with no regional differences. In Study 2, the BZ-resistance study, efficacies below 95% were recorded for *N. spathiger* and *N. filicollis* on 95% (20/21) and 40% (4/10) respectively of farms tested. In Study 3, the chilling experiment, the overall hatching of *N. filicollis* eggs was low, but increased with chill accumulation to plateau at about 11%, with 800-1000 chill units required for maximum hatching.

In conclusion the two species of *Nematodirus* were commonly found on most New Zealand farms. The prevalence of BZ-resistance in *N. filicollis* was lower than that in *N. spathiger*. *N. filicollis* required a considerable period of chilling to enable hatching to occur and this will influence their epidemiology.

Preface

In 2009-2010 two new anthelmintic families (monepantel and derquantel) were released to the market in New Zealand, which reduced the need for research into finding solutions to the anthelmintic resistance problem. It also created an opportunity to focus on minimising the impact of parasites in sheep through improved management strategies. In particular targeting specific nematodes species and understanding their epidemiology on pasture. This is poorly understood in some species. For this study the genus *Nematodirus* was targeted because it is pathogenic, it is often reported as resistant to drenches and it is found nation-wide. Further, the ecology of the genus is quite different to the other strongyle species and it has been poorly studied in New Zealand. Consequently this project was initiated with an aim of discovering the factors influencing the development and survival of the pre-parasitic stages of *Nematodirus spathiger* and *N. filicollis* and was undertaken as part of a Foundation for Research Science and Technology grant, under contract C10X0714.

The study commenced in November 2010, with a study on the effect of three temperature regimes using mixed *Nematodirus* species from different farms in tissue culture well plates. However this was unsuccessful, as many eggs did not hatch, therefore this experiment is not presented. The project commenced in late spring - early summer, when *Nematodirus* eggs are present in faeces, before a literature review could be undertaken; this resulted in not fully understanding the development and hatching requirements of *Nematodirus* and the failure of the first experiment.

At the same time faecal samples were collected and incubated from farms throughout the country for the distribution survey. Drench tests were also being sent to the laboratory for routine faecal egg count reduction testing (FECRT), which became part of the benzimidazole resistance study. As eggs had not hatched in the first experiment it was decided to halve the cultures from both these experiments. This allowed one half to be chilled and the other half processed.

Finally with the comprehension that *N. filicollis* eggs required significant chilling before they would hatch, an experiment was planned to determine what these requirements were. In late January 2012 an almost pure strain of *N. filicollis* was found on a local farm. After isolation from the field and a period of incubation to allow the eggs to

develop, the chilling experiment began in May 2012 with the eggs being placed at different temperatures.

The chapters relating to the experimental studies are presented as papers for journal publication. Chapter 3 has been published in the New Zealand Veterinary Journal (Oliver *et al*. 2014). My contribution for this published paper was 85%. Chapter 4 has been prepared for submission to the same journal; my contribution for this paper was 80%. Chapter 5 has been submitted to Veterinary Parasitology, my contribution for this paper was 75%. As a result there is some repetition of methods; also reformatting and modifications to these chapters by way of annotations and the re-numbering of sections and illustrations have been made, to reflect thesis style. Some raw data is also presented for Chapter 3, 4 and 5 in the appendices. The references for each chapter have been collated into the bibliography.

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Abbreviations

ABBREVIATION	DESCRIPTION
°C	Degree Celsius
µm	Micrometre
µL	Microlitre
ABZ	Albendazole
BZ	Benzimidazole
cm	Centimetre
CU	Chill units
DNA	Deoxyribonucleic acid
epg	Eggs per gram
FEC	Faecal egg count
FECR	Faecal egg count reduction
FECRT	Faecal egg count reduction test
g	Gram
HPLC	High performance liquid chromatography
ITS	Internal transcribed region
lb	Pound
kg	Kilogram
L2	Second stage larva
L3	Third stage larva
ML	Macrocyclic lactone
mL	Millilitre
mm	Millimetre
%	Percent
PCR	Polymerase chain reaction
pmol	Picomole
rDNA	Ribosomal Deoxyribonucleic acid
sg	Specific gravity
SOP	Standard operating procedure
SSU	Small subunit

Chapter 1
Introduction

Nematode parasites belonging to the genus *Nematodirus* cause disease in domesticated and wild ruminants throughout the world. Two species, *N. spathiger* and *N. filicollis*, are recognised as important parasites of lambs in New Zealand in that both species are common (Pomroy 1997; Vlassoff and McKenna 1994) and they are pathogenic in comparatively low numbers (Brunsdon 1967; Vlassoff and McKenna 1994; Anonymous 2010). The genus is believed to have naturalised in New Zealand when their host ruminants were brought to this country for agricultural purposes, with *N. filicollis* identified as an important parasite as early as 1895 (Vlassoff and McKenna 1994). Disease caused by *Nematodirus* along with the other gastro-intestinal nematodes remains a major animal health issue for sheep farmers in New Zealand (Lawrence *et al*. 2007).

Since the development of modern broad-spectrum anthelmintics in the 1970s and 1980s, there has been little research on the epidemiology of parasites in New Zealand. Recently, following the emergence of anthelmintic resistance as a serious problem for livestock farmers around the world (Waller 1997; Jackson and Coop 2000; Sutherland and Leathwick 2011) there has been a resurgence of interest in the biology of our important disease causing parasites. Studies have been conducted to quantify the dynamics of *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* on pasture (Leathwick *et al*. 2011; Waghorn *et al*. 2011; Reynecke *et al*. 2011) with the aim of building models (Leathwick 2013) capable of improving our ability to manage disease with reduced reliance on chemical interventions. Despite being important parasites of sheep, the majority of research carried out on *Nematodirus* spp. in New Zealand was completed prior to 1973. Early studies were conducted by Tetley (1935, 1941) but the majority of studies on *Nematodirus* in New Zealand were conducted in the 1950s and early 1960s by Brunsdon (1960, 1961, 1962, 1963, 1963b). At this time interest in *Nematodirus* was stimulated by the occurrence of acute outbreaks of disease, predominantly in Canterbury, Otago and Southland.

Based on these early studies it was concluded that *Nematodirus* spp. were present on pasture throughout the year (Tetley 1935; Brunsdon 1960) but that the two species differed in their seasonal patterns. *Nematodirus filicollis* had two seasonal peaks, a small spring rise and a larger autumn peak, while larval numbers of *N. spathiger* were highest from mid-summer to autumn, with few larvae present through winter and spring (Brunsdon 1963; Vlassoff 1973). Additionally *N. spathiger* and *N. filicollis* were both widely distributed throughout both the North and South Islands, with *N. filicollis* tending to dominate infections more often in the North Island than *N. spathiger* (Tetley 1935; Brunsdon 1961). In the South Island the two species tended to dominate infections equally (Brunsdon 1961).

Interestingly, over the decades since these studies were undertaken a number of perceptions have come to be widely accepted. Firstly, it is commonly said that disease caused by *Nematodirus* is only really a problem in the South Island, and secondly that *N. filicollis* is seldom seen in the North Island. Given the widespread and intensive use of modern anthelmintics and changes in farming systems over the interceding decades, it was unclear whether or not these perceptions represented real changes in parasite dynamics.

Although there has been no New Zealand research on the dynamics and epidemiology of this genus in over 40 years, its importance has increased due to its ability to develop resistance to modern anthelmintics. An initial report identified BZ-resistance in *N. spathiger* (Middelberg and McKenna 1983), but more recent studies have only referred to the genera and not identified the resistant species (West *et al*. 1989; Macchi *et al*. 2001; Little *et al*. 2010; McKenna *et al*. 1995a; McKenna 1995b). There are also reports of the genus being resistant to one or more of the other anthelmintic classes. Two of these studies reported the prevalence of BZ-resistance on New Zealand farms as between 87-89% (Waghorn *et al*. 2006; McKenna 2013), so clearly BZ-resistance in this genus is widespread. In addition, resistance to levamisole was reported on 11-25% of farms tested and to ivermectin on 7-9% (Waghorn *et al*. 2006; McKenna 2013). Further, a combination BZ/LEV failed to achieve the required 95% efficacy on 7% of farms (Waghorn *et al*. 2006). Thus some populations of *Nematodirus* are now demonstrating resistance to multiple drug classes. This not only leaves few alternative

anthelmintics with which to maintain control, but also shows that the parasite is very capable of becoming resistant to all these drugs.

The genus *Nematodirus* has a unique lifecycle amongst the Strongylid nematodes found in small ruminants, in that development to the third larval stage occurs within the egg. The species differ, however, in that *N. filicollis* requires a period of chilling before hatching (Thomas and Stevens 1960; van Dijk and Morgan 2009), while *N. spathiger* hatches almost immediately after development has been completed (Kates 1950; Kates and Turner 1955). As the epidemiology of *N. filicollis* is poorly understood in New Zealand isolates, it is important to determine the level of chilling that is required for hatching to occur.

Given that the genus is often reported as being resistant to anthelmintics, has the potential to be highly pathogenic and regularly occurs in infections in young lambs, then a greater understanding of the population dynamics will be required to facilitate control. The expectation is that eventually the findings of this study will assist in developing strategies to manage the individual parasites better, thereby reducing the reliance on anthelmintics. The overall aim of this thesis was to study the dynamics of the two commonly found *Nematodirus* species in New Zealand lambs: *N. spathiger* and *N. filicollis*. The main focus was on three areas, their distribution, their BZ-resistance status and the chilling requirements for hatching of *N. filicollis*.

Chapter 2
Literature Review

2.0.1 Introduction

This review reports on some aspects of *N. spathiger* and *N. filicollis*, including their taxonomy, distribution, biology, and anthelmintic resistance. It then focuses on the molecular techniques and identification of anthelmintic resistance, both are methods used in the experimental sections of the thesis.

2.1 Taxonomy

The naming and grouping of organisms are useful biological tools and though taxonomists may debate the placement of certain species, the practice is essential for unambiguous communication. In this section, the classifications for *N. spathiger* and *N. filicollis* are presented, with brief comments on the taxonomic ranks that are contentious. Also the distinguishing features of the genus are highlighted, along with the two species descriptions at different life stages, with an emphasis on their differences.

The taxonomic positions for *Nematodirus filicollis* and *N. spathiger* are taken from Anderson (2000), which are based on the widely used CIH keys to the nematode parasites of vertebrates and are reported below (Column A). This classification is largely a result of morphological structures and in light of molecular studies a tentative classification of the higher ranks is also presented (Column B) and is based on phylogenetic relationships proposed by De Ley and Blaxter (2002). This alternative classification though tentative, is an important inclusion as it acknowledges modern methods in use which have the capacity to clarify taxonomic relationships.

	A	B
Kingdom:	Animalia	Animalia
Phylum:	Nematoda (round worms)	Nematoda
Class:	Secernentea	Chromadorea
Subclass:	Secernentea	Chromadoria
Order:	Strongylida (the bursate nematodes)	Rhabditida
Superfamily:	Trichostrongyloidea Cram 1927	Strongyloidea
Family:	Molineidae	
Subfamily:	Nematodirinae	
Genus:	*Nematodirus* Ransom 1907	
Species:	*filicollis*	
	spathiger	

Nematode classifications are often controversial, some of these issues around the classifications are mentioned below. Firstly there is debate on the phylum; the name Nematoda is contentious, with some taxonomists using Nemata (De Ley and Blaxter 2002). The latter name was given when nematodes were proposed for phylum rank, while Nematoda has the longer usage, initially used at family level in 1837. As the International Code for Zoological Nomenclature provides no guidance either name appears legitimate (De Ley and Blaxter 2002), therefore in this literature review Nematoda was selected as the older term. In some publications such as Kassai *et al*. (1988), the phylum Nemathelminthes was used, however nematodes have been linked on molecular data with several other phyla of moulting animals therefore this name is not currently used (De Ley and Blaxter 2002).

At the class and subclass level, there has been a long history of different classifications proposed. Chitwood (1933) divided nematodes into those with phasmids: variably known as Phasmidia, Secernentea or Rhabditea and those without: the Adenophorea, which was split into Enoplida and Chromadorida (Inglis 1983; Adamson 1986, Meldal *et al*. 2007). The class Secernentea (secreters) was widely used for a long time, as shown in the classification (Column A) from Anderson (2000). However Inglis (1983) and Adamson (1986) ceased using Secernentea and Adenophorea, with Inglis (1983) proposing Rhabditea, Enoplea and Chromadorea. Following this De Ley and Blaxter (2002) have encompassed the bulk of nematodes (including Secernentea) into the class

Chromodorea based on phylogenetic studies using SSU-rDNA. The change to the higher ranks is a considerable shift in thinking.

Older classifications placed *Nematodirus* under the family Trichostrongylidae; however Durette-Desset and Chabaud (1977, 1981) established the family Molineidae (under the superfamily Trichostrongyloidea) by elevating Molineinae. The subfamilies of Nematodirinae, Molineinae, Anoplostrongylinae and Ollulaninae were created. Within Nematodirinae six genera were recognised: *Nematodirus*, *Nematodirella*, *Murielus*, *Lamanema*, *Nematodiroides* and *Rauschia* (Durette-Desset and Chabaud 1977, 1981; Hoberg *et al*. 2005b). Further revision by Durette-Desset and Chabaud (1993) elevated the superfamily Trichostrongyloidea to suborder, with a name change to Trichostongylina within the order Strongylida and three superfamilies were established Molineoidea, Trichostrongyloidea and Heligmosomoidea to recognise monophyletic lineages (Hoberg *et al*. 2005b). Though these modifications were not universally accepted i.e. Lichtenfels *et al*. (1997) reverted to the CIH keys in their assessment of taxonomy on the gastrointestinal nematodes. *Nematodirus* is considered to be contained in the Nematodirinae, within Molineidae and Molineoidea (Durette-Desset *et al*. 1994).

Nematodirus filicollis is the type species of the genus proposed by Ransom in 1907, after originally being described as an *Ascaris* in 1802 by Rudolphi, using adult females (May 1920; Thomas 1959a; Beckland and Walker 1967). *N. spathiger* Raillet 1896 was also repositioned into *Nematodirus* from the genus *Strongylus* by Raillet and Henry in 1909 (May 1920; Thomas 1959a). Given the length of time the genus and species have existed, both appear stable and widely accepted in the scientific community.

2.1.1 Species within the genus *Nematodirus*

In comparison with the other members of the Nematodirinae, *Nematodirus* is a species rich genus, with 40-50 species identified since its inception (Rossi 1983; Hoberg 2005). By 1937, 17 species had been recognised (Tucker 1942), these numbers have increased steadily, with four new species described by Rossi (1983). Although not surprisingly synonymy has also occurred (Samson 1968; Hoberg *et al*. 1988) that has subsequently reduced numbers. This was the case in the latter study where clarification to species identity using molecular technology resulted in *Nematodirus tortuosus* becoming a synonym for *N. neotoma* (Hoberg *et al*. 1988). Overall the range in the number of species is rather extended and presumably reflects the fluidity of taxonomy.

2.1.2 Defining characteristics - morphological

Previously the identification of nematode species relied mainly on morphological characteristics. These unique features in *Nematodirus* spp. were structures of the adult male, in particular the spicule tip and bursa, as the females possessed limited features of taxonomic value (Samson 1968; Rickard and Lichtenfels 1989). Of the bursa both the rays and bosses were key characteristics (Rickard and Lichtenfels 1989). Several researchers have contributed information on the morphology of *Nematodirus* spp., with the majority of research published more than 50 years ago (for example: Boulenger 1915; Dikmans and Andrews 1933; Thomas 1957). However more recent studies have added details on the synlophe, which was found to be a characteristic that separated species, both in females and males (Lichtenfels and Pilitt 1983; Rossi 1983; Hoberg *et al*. 1988; Rickard and Lichtenfels 1989).

The following paragraphs describe the features of the genus and some of the morphological differences between *N. spathiger* and *N. filicollis*.

2.1.2.1 Generic characteristics of the adult

May (1920) and Boulenger (1915) described adult *Nematodirus* as filiform with the cuticle of the head being inflated, while on the body there are between 14 and 24 synlophe. The mouth is oval with six associated small papillae; the buccal cavity is very short. In the male: the bursa has two large lateral lobes and two small dorsal lobes; the ventral rays are slender, parallel and close together. Dorsal rays (one in each dorsal lobe) are short and bifid at the tip. The spicules are: slender, tubular, filiform, greater than 0.5 mm long and united by a membrane either throughout the length or distally. The female is larger than the male with the vulva a transverse slit that opens in the middle third of the body. The posterior is truncate with a short slender spine. The eggs are large, usually over 150 μm long.

2.1.2.2 Characteristics of the adult N. spathiger and N. filicollis

Historically, the evidence for accurate identification of the two species is inauspicious, with both Thomas (1957) and Beckland and Walker (1967) noting cases where *N. spathiger* and *N. filicollis* were mistaken for one another. The early mistakes in identity that were then written into descriptions have propelled further errors in identification.

Males: Interestingly May (1920) described both *N. spathiger* and *N. filicollis* as having the same length, with the adult male 10-15 mm long, while the female was larger measuring 15-23 mm in length. Lichtenfels and Pilitt (1983) reported that the range was wider, for female *N. spathiger*, 12.8-19.0 mm compared to 14.3-17.9 mm for *N. filicollis*. One of the primary differences between the adults of the two species is the male spicules. In *N. spathiger* they are 0.7-1.10 mm long and end in a spoon-shaped enlargement whereas the spicules of *N. filicollis* are 0.68-0.95 mm long and end in a narrow, pointed piece (May 1920). The differences in the bursa are: *N. filicollis* has a large bursa with thin rays and the dorsal lobules of the dorsal lobe are small, often indistinct, the lateral lobes are round and large; in *N. spathiger* the bursa is relatively small and the dorsal lobules are medium sized with distinct lateral lobes elongate and small (May 1920; Beckland and Walker 1967). *N. spathiger* has bursal bosses distributed between the postero-lateral and latero-ventral rays, they are few in number with an average of 15 per bursal lobe and small – the largest averaging 12 μ (May 1920; Stringfellow 1968). The numerous bosses of *N. filicollis* are between the ventro-ventral and postero-lateral ray; averaging 56 per bursal lobe, they are large. The largest averaging 20 μ and form an overall fan-shaped appearance (Stringfellow 1968). Though the nature of the bosses is a useful characteristic this study only examined a small number of specimens (10-12), with the range of the number and size of bosses overlapping considerably in the six species studied, they therefore should be used cautiously. However, used alongside the other characteristics mentioned they would assist in differentiating the species.

General characteristics of *N. spathiger* and *N. filicollis*: The characteristics of the synlophe are: the cervical region has a total of 18 ridges in both species; *N. filicollis* has 10 ridges that reach the head capsule, the ventral ridge is described as finlike and a fine lateral ridge is present; comparatively in *N. spathiger* only six ridges reach the head capsule, the ventral ridges are not finlike nor a lateral ridge present; the former species has 14 mid-body ridges and the latter 18 (Lichtenfels and Pilitt 1983; Rickard and Lichtenfels 1989). A small number of perioral denticals are noted for *N. filicollis*, while they are numerous in *N. spathiger* (Lichtenfels and Pilitt 1983).

2.1.2.3 Characteristics of N. spathiger and N. filicollis eggs

Several studies have reported the egg size of *N. filicollis* and *N. spathiger* (Table 2.1). Some of these (Tetley 1935; Thomas 1957; Lichtenfels and Pilitt 1983) report the egg size of the two species clearly differing, while others report overlapping measurements (May 1920; Shorb 1939). Egg length appears to overlap between the two species more frequently than the width. The range Boulenger (1915) reported for *N. filicollis* is larger than that of the other workers and Thomas (1957) suggested that he used a mixed culture of the two species. Tetley's (1935, 1941) New Zealand studies reported measurements representing the majority of eggs that were observed (91%) and under these circumstances the two species could be distinguished. However, the extreme ranges did overlap. Interestingly, eggs found in each of the different uteri of one female fell centrally in the range of both *N. filicollis* and *N. spathiger* (Tetley 1941). While unusual, this illustrates that identifying the two species based on egg size alone is not always precise. Some of these studies sourced eggs directly from dissected female worms while others measured eggs from faeces; however egg measurements were similar from either method (Tetley 1941). In general *N. filicollis* do have smaller eggs compared to *N. spathiger*; however the slight size overlap makes it difficult to identify the two species free of error. If *N. abnormalis* or *N. helvetianus* are part of a mixed infection it becomes more complex, with more ranges overlapping (Table 2.1) and it seems only the very experienced researcher could identify the species with accuracy based on egg size alone.

Other notable features of eggs of the two species are the transparent, colourless, smooth shell is relatively thick (Boulenger 1915; May 1920; Thomas 1959a; Onar 1975). The shell of *N. filicollis* was approximately 3-4.1 µm (Boulenger 1915; Shorb 1939) and for *N. spathiger*, 5.0 µm (Shorb 1939). Conversely of 300 *N. spathiger* eggs measured the shell thickness ranged between 1.4-2.7 µm (2.6 µm) (Viljoen 1972). Eggs of New Zealand *N. filicollis* had evenly oval shells of 3 µm at the sides and slightly thicker poles and *N. spathiger* not so evenly oval, 3-4 µm at the sides and 5-8 µm at the poles (Tetley 1935). The majority of studies report *N. spathiger* eggs with polar thickening, some mention it as slight or more or less (May 1920; Kates and Shorb 1943) others report it as a distinguishing feature (Thomas 1957), yet this paper cites Kates and Shorb (1943) so this is not clear. In *N. filicollis* some have reported a slight thickening at the poles (Tetley 1935) this has also been refuted (Boulenger 1915; Kates and Shorb 1943).

Kates and Turner (1955) conclude the eggs of *N. spathiger*, *N. filicollis*, *N. battus* and others of the genus look similar and Onar (1975) stated that reliable identification between *N. abnormalis*, *N. helvetianus* and *N. spathiger* eggs was impossible.

Table 2.1. Summary of the literature on egg dimensions (length x width) of *Nematodirus* spp. found in New Zealand sheep in micrometres. Mean reported in brackets when available.

	N. spathiger µm	*N. filicollis* µm	*N. abnormalis* µm	*N. helvetianus* µm
Boulenger 1915		130-210(160) x 65-95 (80)		
May 1920	150-220 x 80-110	130-200 x 70-90	160-230 x 85-115	
Tetley 1935	180-210 x 90-105	140-165 x 70-85		
Shorb 1939*	181-230 (200) x 91-107 (98)	149-194 (171) x 74-107 (88)	178-223 (212) x 91-107 (96)	184-233 (212) x 84-110 (97)
Tetley 1941[†]	179-210 (195) x 88-107 (97)	133-176 (154) x 69-86 (77)		
Herlich 1954			185-245 (208) x 92-113 (106)	
Thomas 1957	183-214 x 87-99	134-168 x 71-87		
Soulsby 1968	175-260 x 106-110	130-200 x 70-90		160-230 x 85-121
Viljoen 1972	173-238 (218) x 97-119 (103)			
Onar 1975			190-246 (209) x 80-118 (95)	
Lichtenfels & Pilitt 1983	172-217 x 95-114	146-167 x 68-88	172-200 x 101-116	186-224 x 86-111

[†] Distribution of 91.2% of eggs.

* Kates & Shorb (1943) reported the same values.

2.1.2.4 Characteristics of the infective larvae

The notable characteristics of *Nematodirus* spp. third stage larvae (L3) are: the long sheath tail, which contributes significantly to a longer larva overall, especially in comparison with other trichostrongyloid genera; the intestine is composed of only eight cells and the tail has noticeable terminal appendages (van Wyk *et al*. 2004). Thomas (1957) provided the following descriptions and measurements for the two species. The overall length of the L3 stage of *Nematodirus spathiger* is slightly longer than *N. filicollis* (Table 2.2) being 976-1130 µm and 752-1018 µm respectively. In this description almost all measurements are longer for *N. spathiger*, the exception being the length between the anus and tip of the sheath which measured between 294-406 µm in *N. filicollis* and 310-390 µm in *N. spathiger*. Both species have simple buccal cavities that open into the oesophagus which measures 192-224 µm for *N. spathiger* and 170-210 µm for *N. filicollis*. A slender rectum opens 64-74 µm from the posterior of the body of *N. spathiger* and between 32-48 µm in *N. filicollis*. The appendage of the tail of *N. spathiger* is notched with a 15 µm long finger-like projection between the lobes. *N. filicollis* lacks this protrusion, instead the tail is deeply notched with a dorsal and a larger ventral lobe, the latter being subdivided, resulting in a tricuspid tail (Thomas 1957; van Wyk *et al*. 2004). Measurements from different authors are summarised in Table 2.2 and are in accord with each other except for those by Boulenger (1915) who Thomas (1957) concluded described *N. spathiger* and not *N. filicollis*, which given the presence of the terminal process is a feasible explanation.

2.1.2.5 New Zealand data

Apart from the egg measurements of Tetley (1935, 1941) it appears there is no published work on New Zealand isolates describing the size of structures for *N. spathiger* or *N. filicollis* adult worms or L3. It must be presumed then that when New Zealand isolates are identified they are within the published ranges.

Table 2.2. Summary of the literature on the measurements of the infective larvae (L3) of *N. spathiger* and *N. filicollis* in micrometres.

	N. spathiger				*N. filicollis*		
	Monnig 1931	Thomas 1957	Viljoen 1972	Dikmans & Andrews 1933	Boulenger 1915	Maupas & Seurat 1913[†]	Thomas 1957
Total length	1119-1177 (1158)	976-1130 (1018)	969-1282 (1075)	922-1118 (1009)	850-1150 (1000)	768	752-1018 (912)
Length of larva		723-810 (751)			530-700	560	496-672 (600)
Oesophagus length	227-235 (235)	192-224 (204)	103-351 (188)	160-225 (195)	180-220	150	170-210 (193)
Anterior end – genital primordium	509	394-480 (428)	356-540 (433)	365-500 (417)	459 [†]	310	304-416 (369)
Larval tail	43-59 (59)	64-74 (69)		52-65 (60)	50	36	32-48 (39)
Anus – tip of sheath	325-340 (340)	310-390 (332)	270-416 (331)	315-350 (328)	342[†]	244	294-406 (370)
Process		15		10-15	13		Absent

[†] Measurements taken from the drawing.

2.1.2.6 Molecular characteristics

Although morphological characteristics can and have been used to distinguish the different species, the majority of characters are microscopic and identification is time consuming. Recent development of molecular technologies now allows species to be identified using specific genetic markers – see Section 2.7.

2.2 Biology of *Nematodirus* with an emphasis on *N. spathiger* and *N. filicollis*

The biology of the free-living stage of *Nematodirus*, like other helminths is a complex interaction between the nematode, the environment and the host. The climate influences the development of eggs and survival of larvae on pasture (Vlassoff 1982).

2.2.1 Definition of parasite and host

A parasite is a smaller organism that lives in or on and at the expense of a larger organism – the host (Bowman 1999). Though there are variations of the definition (Zelmer 1998), the basic concept is covered by this statement and is suitable for the purpose of this literature review. Therefore *N. filicollis* and *N. spathiger* are parasites, being smaller than the host, living (for a portion of the lifecycle) within and at the expense of their larger ruminant hosts. As only part of the lifecycle is technically parasitic the term free-living or non-parasitic is applied to the phases that occur outside the host. This terminology is used throughout this thesis.

2.2.2 Lifecycle – free living stages

In biology an important aspect is to understand the succession of the species, in particular the life stages passed through, culminating in the organism reproducing the next generation. In simple terms how the species survives. Nematodes follow a basic path: starting from an egg, followed by four larval stages and ending as an adult in the fifth stage. However each species has its own set of intricacies for attaining each life stage, these will be discussed in relation to *N. spathiger* and *N. filicollis*.

The lifecycle of *Nematodirus* spp. has been investigated in several studies (Boulenger 1915; Herlich 1954; Kates and Turner 1955; Thomas 1959a). *Nematodirus* spp. have a direct, but atypical lifecycle that is divided into the parasitic and free-living states. It is atypical because the third stage larva develops inside the egg (Boulenger 1915; Kates and Turner 1955), unlike most other trichostrongylid species that hatch as first stage larvae. Prior to this, when *Nematodirus* eggs are passed onto pasture they contain two-eight large undifferentiated granular cells (Kates and Turner 1955; Viljoen 1972). These cells commence division at this stage and start to differentiate after 2-4 days (Boulenger 1915; Thomas 1959a) with the eventual development of a very active first stage larva (L1). This is followed by the larva moulting and becoming a second stage larva (L2). Another moult occurs before the L3 hatches from the egg, with the sheath of the first moult being shed but the second retained until after transmission into the host (Kates and Turner 1955; Thomas 1959a). The L3 stage is reached between day 6-14 in *N. spathiger* (Kates and Turner 1955) and day 24-27 in *N. filicollis* (Thomas 1959a), though this is largely dependent on temperature. This is discussed in further detail in section 2.2.3.

Post hatching the L3 is infective to animals and like other trichostrongylids this stage is non-feeding. The method of transmission is by ingestion (Rickard *et al*. 1989). Within the host's small intestine the larva develops to an adult, by first undergoing two moults (Kates and Turner 1955), it is non-migratory. The pre-patent period for *N. spathiger* and *N. filicollis* is 14 days in sheep for both species (Kates and Turner 1955; Thomas 1959a). Though some *N. spathiger* and *N. filicollis* worms develop to sexual maturity in 14 days, greater numbers of eggs are produced during the third week after infection (Kates and Turner 1955; Thomas 1959a).

2.2.3 Free living stages - development and hatching - laboratory experiments

The rate of development to L3 and hatching of the egg is largely dependent on the climate it is expelled into. Several laboratory studies have provided information on the temperature profiles and development rates for the key stages in the lifecycle.

It is difficult to draw comparisons between the two species as many studies only relate to one or the other, methodology differs between the studies and the isolates are from different areas of origin. Development to the L3 stage within the egg in *N. spathiger* is generally more rapid, taking 6-14 and 18-22 days at 24°C and 21°C respectively (Kates and Turner 1955; Thomas 1959a). For *N. filicollis* this process took 24-28 days between 19-27°C (Boulenger 1915; Thomas 1959a; van Dijk and Morgan 2009) but was slower at cooler temperatures being 35-42 days at 13-15°C (Boulenger 1915) and 47-50 days at 12-15°C (van Dijk and Morgan 2009). Conversely at warmer temperatures the development rate was more rapid for both species; at 28°C *N. filicollis* took 20 days (Thomas 1959a) and *N. spathiger* took only 5 days at 33°C (Viljoen 1972). Interestingly, Viljoen (1972) indicated that for *N. spathiger* development would only occur between 21-36°C, taking 12 days at the lower temperature and 6 days at the higher. In contrast, van Dijk and Morgan (2009) found the thresholds for *N. filicollis* were lower being from 12-25°C, taking 50 days at the lower temperature and 25 days at the higher temperature, for 50% of eggs to complete development.

Another factor besides temperature considered for larval development has been moisture. Eggs of *N. filicollis* require some level of moisture in their early stages of development, with desiccation killing them (Boulenger 1915). In contrast, excess moisture did not cause any issues. In *N. spathiger*, partially embryonated eggs survived desiccation for 3 months at room temperature but not for 4 months and slightly longer at

a cooler temperature (Turner 1953). As Boulenger (1915) concluded temperature conditions are considered of greatest importance to development, however the amount of literature on temperature effects on development far outweighs those on the effect of moisture or desiccation in *N. spathiger* or *N. filicollis* so a balanced view on this is difficult to assess.

Hatching of L3 from the egg can be differentiated from development to the L3 stage. For some species there are indications that some form of preconditioning can influence the rate at which L3 hatch from the egg. There appears to be almost no pause between the appearance of the L3 within the egg and the hatching for *N. spathiger* as long as conditions are suitable. The time from egg deposition to hatching for *N. spathiger* larvae has been reported as between 10 and 22 days at temperatures between 21-28°C (Viljoen 1972; Kates and Turner 1955; Thomas 1959a) but only 8 days when at 33°C (Viljoen 1972). For *N. spathiger*, 33°C was considered the optimal temperature required for hatching (Viljoen 1972). In this case "optimal" was defined as the shortest time taken before a stage occurred and not necessarily when the highest numbers of L3 were present. Unfortunately no actual data was provided for mortality at this "optimal" time and the only data given on the success of eggs developing and hatching was for Day 21, when at 33°C 93% of eggs had hatched and a similar number at 27°C and 30°C. It therefore appears that between 27°C and 33°C was the "optimal" temperature for hatching success. The thresholds for hatching in *N. spathiger* were between 21°C and 36°C; by 39°C it had ceased (Viljoen 1972). At the lower temperature of 21°C it took 19 days to hatch and at 36°C, hatching was completed in 10 days. At this higher temperature on Day 21 approximately 45% of larvae hatched, so within 3°C hatching success approximately halved. Overall it seems *N. spathiger* has a broad temperature range in which it can hatch.

It is more difficult to get *N. filicollis* L3 to hatch after they have completed development. Boulenger (1915) managed to get L3 to develop but under his experimental conditions could not get them to hatch from the egg. Even after seven months 90% of the larvae remained active but unhatched. Unfortunately Boulenger (1915) reported little information on the methods used but did acknowledge the limited nature of the experiments and fairly assessed the results as "general effects" of different temperatures on cultures. Of note, though Boulenger mistook *N. spathiger* for *N. filicollis* in an earlier section of his work, as mentioned in section 2.1.2.4 of this review,

it appears he was working with *N. filicollis* for the development and hatching experiments due to the similar nature of the results compared to Thomas (1959a). Thomas (1959a) had a similar lack of success in getting L3 to hatch from eggs, even with incubator temperatures up to 36°C. Additionally van Dijk and Morgan (2009) found very few eggs hatched (3.5%) that contained L3 and had been incubated at 20°C for 33 days. It is obvious from these studies that hatching of *N. filicollis* does not follow immediately once L3 are present within the egg and are indicative that other factors or processes are required before hatching will occur. The details around hatching of *N. filicollis* will be discussed in Section 2.2.4

In summary, the two species are somewhat different in both development and hatching rates. The biggest difference is the inability of *N. filicollis* to hatch without suitable preconditioning. Consequently *N. spathiger* has the overall shorter timeframe before it can be ingested by a host. It will develop and hatch over a wide range of conditions which allows the species to have a wider distribution and also a greater ability to infect animals throughout the different seasons of the year.

2.2.4 Diapause - chilling

Several laboratory studies have described difficulties in hatching the eggs of *N. filicollis* (Boulenger 1915; Thomas 1959a; Christie 1962; van Dijk and Morgan 2009). This is because *N. filicollis* and also the related *N. battus* enter a state of diapause while in the egg (Thomas and Stevens 1960; Christie 1962). Diapause is a hypometabolic state similar to dormancy, quiescence and hibernation (Padilla and Ladage 2012) in which the biological processes, like development are arrested or slowed. The characteristics that distinguish diapause from the other forms of arrested development are, it is irreversible once commenced, even in environments that would usually sustain growth and it is often induced by environmental cues, which normally relate to the seasons (Sommerville and Davey 2002). While in diapause intrinsic changes must occur before development can continue. It should be noted though, that even when this process is completed reactivation will only occur if conditions are favourable.

There are two main reasons suggested for organisms to have the adaptation of diapause. Firstly the development of the parasite becomes synchronised with the seasonal abundance of the host (Perry 1989). For example, *N. filicollis* by hatching in the spring has a greater opportunity of being ingested by a lamb, as that is when lambs are more

numerous. Thus the strategy enhances reproductive success. Secondly it allows the organism to withstand unfavourable conditions, as *N. filicollis* remains in the egg over the winter it is protected from the severe climatic conditions by the egg membranes (Thomas and Stevens 1960). Essentially diapause is a part of the phenological strategy and is considered an escape in time, like migration is an escape in space; it provides flexibility to the lifecycle (Sommerville and Davey 2002).

Diapause can be obligate i.e. it is inherent and considered genetically fixed; or facultative and governed by seasonal signals (Sommerville and Davey 2002). However, it is not entirely clear whether diapause in *N. filicollis* is inherent or facultative. It is similarly unclear for the related species *N. battus*. Various experiments have indicated that eggs of *N. filicollis* require a period of cold chilling before L3 will hatch in reasonable numbers. It appears that exposure to colder temperatures speeds up diapause (van Dijk and Morgan 2008, 2009) so eggs can hatch as soon as temperatures rise in the spring, suggesting obligate diapause. Alternatively diapause could be induced by chilling in autumn-winter which would make it facultative (Sommerville and Davey 2002). Whether the species are obligate or facultative, chilling is involved as an important requirement in the diapause process and ability to hatch. It is clear that disease with *N. filicollis* is usually seen in young lambs in spring implying that cool winter conditions are important for conditioning the larvae to emerge in some way.

There are a few examples where some level of chilling had a positive influence on hatching *N. filicollis*. In a comparison of eggs containing L3 stored for prolonged periods at 6°C, 8°C and 11°C there were more larvae hatching in those stored at the lower temperature (52% of eggs hatching at 6°C but only 3% of eggs hatching at 11°C; van Dijk and Morgan 2009). A temperature of 11°C was determined to be the upper threshold for chilling (van Dijk and Morgan 2009). This same trend was observed when hatch rates were compared between eggs chilled at 3°C for 6-8 months which resulted in 35% of developed eggs hatching compared to less than 3% hatching when incubated at 21°C for this same period (Thomas and Stevens, 1960). This latter study is somewhat confused as the text indicated the species involved was *N. filicollis* whereas the related figure implied the species involved was *N. battus*. Overall it appears chilling is vital to allow hatching to subsequently occur for *N. filicollis*. It is not clear as to how to achieve the necessary chilling and this may vary between different provenances of *N. filicollis*.

Once development and chilling has been completed for *N. filicollis* the hatching temperatures and rates can be then be assessed. In one study hatching occurred most successfully at 13ºC with 72% of larvae hatching (van Dijk and Morgan 2009); with 60% of eggs hatching within 3 days. Hatching occurred between 4ºC and 17ºC, taking 19 days and 4 days respectively at these two extreme values though only 8% hatched at 4°C and 18% at 17ºC. In this same study alternating temperatures between 7-13ºC and 14-20ºC resulted in similar hatching levels to the constant 11ºC and 13ºC implying a measure of heat accumulation over a range of temperatures rather than a critical temperature *per se* is required. In a similar experiment with eggs containing L3 incubation at 15ºC resulted in a higher hatch rate (75%) compared to 5ºC and 10ºC (Thomas and Stevens 1960). Indeed a negligible number of eggs hatched at 5ºC indicating the lower threshold for hatching was 10ºC in this experiment which is not that dissimilar to the later study by van Dijk and Morgan (2009). In comparing the reported temperature ranges that *N. filicollis* and *N. spathiger* hatch at, it is evident that *N. spathiger* has a considerably higher range (section 2.2.3), admittedly this is based on a limited number of (two) reports.

Interestingly, though *N. spathiger* eggs do not enter diapause and chilling is not required for it to hatch, a positive effect has been recorded in this species too. When eggs were exposed to 0ºC for 7 days followed by an incubation of 36ºC for 14 days the hatching rate (82%) was over double that for eggs just incubated at the higher temperature (34%) (Viljoen 1972).

2.2.5 Chill accumulation

Temperature has a major influence on the development of the stages of the lifecycle and especially the rate at which these stages occur, as presented above. The concept of temperature (both heat and chill) accumulation that affects different physiological processes has been widely studied in the fields of plant biology (Alburquerque *et al*. 2008; Rose and Cameron 2009; Kuden *et al*. 2013) and insect phenology (Nealis *et al*. 1984; Lobinske *et al*. 2002; Lewis *et al*. 2003; Hartley and Lester 2003), and occasionally nematology (Vrain *et al*. 1978). A form of heat unit accumulation was first reported for fruit trees by Reaumur, nearly 300 years ago (Wang 1960; Idso *et al*. 1978; McMaster and Wilhelm 1997). Many subsequent studies have built on this same concept. Heat units are accumulated above a certain threshold usually by summing the

daily mean temperature minus the threshold temperature (Wang 1960). These are also known as degree-days. The threshold temperature is that below which physiological activity is assumed to be inhibited (Idso *et al*. 1978). A prediction of the date that development/maturity is complete can be made when sufficient growing degree-days have been accumulated i.e. that a crop can be harvested. For example if the threshold temperature was 10°C and the mean temperature for the day is 15°C, the heat unit or "degree-day" is 5 degree-days and a plant that requires 80 degree-days for maturity should reach maturity by the time that value has accumulated.

A similar and related concept to heat accumulation is chill accumulation, which essentially is its inverse. As acknowledged above for *N. filicollis,* while in diapause a certain amount of exposure to colder temperatures is required by an organism before development can continue which has been termed its chilling requirement (Schwartz 1997; Cesaraccio *et al*. 2004). In order to break dormancy individual species have a unique set of time–temperature combinations that form their chilling requirement (Cesaraccio *et al*. 2004). Chill accumulation has been calculated in different ways. Some measure the time, often in hours, below a threshold, essentially the same way that growing degree-days are calculated. Other more complex methods, weight the hours spent at different levels of temperature below the threshold, this is known as cumulative chill units (Schwartz 1997; Cesaraccio *et al*. 2004). Chill hours and chill units are calculated and used in models to predict the release of dormancy, i.e. for bud burst in fruit and nut trees (Alburquerque *et al*. 2008; Kuden *et al*. 2013).

2.2.6 Seasonality

The presence or absence of larvae on pasture and sequentially adults within the host, over a year, is important knowledge; both for understanding the biology of *Nematodirus* and for developing controls to minimise damage to the host. In this section these fluctuations in numbers will be discussed, with particular interest in the peaks and troughs. The peaks suggest environmental conditions are most favourable for L3s and also show when animals are at the highest risk of disease. The attention to the troughs are for the reverse purpose, i.e. knowledge on the conditions that are unfavourable for larvae and when animals are at the lowest risk. The following review is based principally on New Zealand studies.

Early observations indicated that most *Nematodirus* infections in lambs occur in spring and early summer which lead to the earliest epidemiology studies. These were undertaken in the Manawatu, New Zealand (Tetley 1935). Interestingly, these indicated that *Nematodirus* L3 were available to lambs in similar numbers throughout the year (Tetley 1935) but there was limited information on different species within this genus. These studies can also be criticised as having small numbers of lambs and using FEC to monitor the prevalence and rate of contamination. Despite these limitations they did provide an indication of the overall trend which was that *Nematodirus* are present on pasture year round. This provided a starting point on the seasonal availability to lambs in New Zealand.

Further studies (Tetley 1959) in the Manawatu discovered the genus did have well defined seasonal fluctuations in abundance. Worm counts of tracer lambs on pasture for two week intervals from summer to autumn (early December 1950 - May 1951), showed that the adult *Nematodirus* population increased sharply to a peak in early autumn (late March) then declined quickly to a similar level observed in mid-summer.

A slightly different pattern was observed, in a three year study (Brunsdon 1960) undertaken in the Wellington region, New Zealand, using FEC in lambs and pasture larval counts. For the latter, peaks occurred in spring (October) and then a larger peak in autumn (March). The FEC results showed the same initial spring peak, albeit later (mid-November), but dropped to a low level over summer and by late autumn levels were diminished further. By early winter, when lambs were 9-10 months old *Nematodirus* FECs were very low. Thus two larval peaks were recognised, however differentiation to species was not made in either study.

In a later study Brunsdon (1963) did study the seasonal availability of the two species (*N. filicollis* and *N. spathiger*) separately and over the course of two years. On pasture which carried infected lambs during spring-early summer, *N. filicollis* occurred in numbers throughout the following years, with a small peak in larvae in November. After this spring peak, numbers were low until mid-January when they increased quickly to a large peak in late summer and early autumn (January and March). By comparison the annual pattern of *N. spathiger* larvae on pasture was somewhat different. Few *N. spathiger* larvae were present from winter-spring (May-December). From December the number of larvae then increased, peaking in both January and March; this summer-

autumn peak was similar to *N. filicollis*. Numbers dropped rapidly and were low from autumn until winter (April-June) after which they were almost absent. Although the late summer-autumn peaks of the two species coincide with one another, *N. spathiger* numbers rise before *N. filicollis* (Brunsdon 1963). An explanation for this is *N. spathiger* develops and hatches much faster than *N. filicollis* as described by Gibson (1958).

Nematodirus are one of the first nematodes lambs obtain in large numbers in New Zealand, with the peak infection at or just prior to weaning. This spring infection depends on the availability of larvae on pasture (Brunsdon 1960). The source of infection for the spring lambs comes from larvae that hatched in autumn and overwintered and a portion of eggs that are passed the previous spring/summer and remain unhatched, overwintering as eggs on pasture (Brunsdon 1963). Tetley (1959) thought the spring infection was predominantly caused by overwintering larvae, as larvae are abundant in late autumn. The late summer-autumn peaks resulted from eggs deposited in the spring and early summer of the current season (Brunsdon 1963; Tetley 1959). Though there is some suggestion that a few unhatched eggs persisted from the previous year.

A further study on the seasonal availability of *Nematodirus* spp. larvae in coastal Otago, New Zealand (McDonald 1966), was similar to Brunsdon's (1963) results for *N. filicollis*, with larvae found throughout the following year and similar larval peaks in spring and autumn even though the species were not identified. Interestingly, larvae were recovered 70 weeks post deposition. Vlassoff's (1973) study of seasonal incidence of the two species generally agrees with Brunsdon (1963) who also described the seasonal pattern of *N. filicollis* as having two seasonal peaks in spring and autumn and *N. spathiger* just one in autumn. Similar patterns have been observed for *N. filicollis* in other geographical areas such as Northern Ireland (Baxter 1957, 1958). However, the spring peak in these reports was larger than the autumn peak - the opposite of that observed in New Zealand studies (Baxter 1957, 1958). In the south of England Gibson (1963) found larvae were present on pasture in fluctuating but relatively high numbers throughout the year. Similarly in Oregon, using tracer lambs, year round transmission occurred with *N. filicollis*, although highest burdens were observed in late fall-winter and a minor peak in summer (Rickard *et al*. 1989). Conversely in Northern England only a single peak in spring was found for *N. filicollis* (Thomas and Stevens 1960;

Thomas 1959b, 1960). This suggests only one generation per year is produced, especially with the lifecycle requiring a dormant phase (Thomas 1959b). Nevertheless, overall it can be concluded that *N. filicollis* larvae are generally available throughout the year and thus more than one generation per year is capable of being produced.

In conclusion *N. filicollis* and *N. spathiger* appear as L3 at almost any time of the year. Larval peaks occur in spring, summer and autumn for *N. filicollis* and the latter two seasons for *N. spathiger*, on pastures grazed by lambs born in spring (Brunsdon 1963). Vlassoff (1973) surmised that *Nematodirus* is a parasite of lambs, being transmitted from one season to the next by eggs and larvae that overwinter each year. Fewer *Nematodirus* eggs are seen in lambs as they get older and ewes do not contaminate pastures with *Nematodirus* as eggs are rarely found in mature animals unlike most of the other trichostrongylid genera (Vlassoff 1973). Re-infection does not occur in the autumn as acquired resistance and possibly age resistance protect the lamb from serious re-infection.

2.2.7 Survival on pasture of larvae

Resistance to the environment by *Nematodirus* spp. is well-known (Marquardt *et al.* 1959). Kates (1950) assessed adult worm counts from sheep and found *Nematodirus* larval survival was higher in cool months but adversely affected by summer conditions. In contrast, a slightly different result was found on test plots in Maryland, USA, involving only *N. spathiger* (Turner 1953), more larvae were recovered during summer-fall than in winter-spring although those recovered in summer-fall were considered to be relatively inactive and some were exsheathed compared to those recovered in winter-spring. Interestingly, these larvae were also found in different locations with those in the winter-spring period predominantly recovered from grass (70%) while in summer-fall most larvae were recovered from the humus and sub-soil surface (90%). The difference in the results of Kates (1950) and Turner (1953) may be a reflection of the methods used or alternatively the differentiation of larvae. However, Marquardt *et al.* (1959) also studied *N. spathiger* but in Montana, USA, and the results were dissimilar to Turner (1953), in that the L3 were observed to survive longest in the cooler seasons with 22% surviving from November compared to only 1% surviving from June until July when summer conditions were present. This has greater similarity to the initial study (Kates 1950) discussed, so perhaps the differences between these studies relate to differences

in the isolates. Interestingly, if an artificial cover was applied to the pasture to protect the larvae from sunlight, survival over both periods increased being 46% and 10% respectively (Marquardt *et al*. 1959), indicating that both hot and dry conditions as well as sunlight are detrimental for larval survival. This observation is in agreement with both Kates (1950) and Turner (1953). By comparison with other trichostrongylid larvae it has been concluded that *Nematodirus* larvae are more resistant to environmental conditions than those of *Haemonchus*, *Cooperia*, *Trichostrongylus* and *Ostertagia* and can survive the annual weather variations sufficiently well (Kates 1950; Turner 1953).

Results from laboratory experiments show similar trends. Poole (1956) described that some *N. filicollis* larvae survived when exposed to different temperatures with the range from -65 to 52°C although the time at the extreme temperatures was generally short. If the larvae were desiccated to a relatively anhydrous state the higher range increased to 74°C. Some larvae (22%) also survived 22 weeks continuously at -6.5°C. Larvae also survived when exposed to cycles of different temperatures with some larvae surviving 12 sequences of freezing at -6.5°C for 24 hours followed by thawing for 1 hour at 30°C. Overall, these results indicated that *N. filicollis* L3 can survive under reasonably extreme temperatures. Van Dijk and Morgan (2009) also studied the survival of *N. filicollis* L3 and found that the daily mortality rate increased from 9°C to 30°C. Very few equivalent laboratory-based studies exist for *N. spathiger*. In one (Turner 1953), it was reported that after two months of desiccation at room temperature, 82% of larvae survived but none were alive past 5 months. Additionally larvae survived a temperature of -2°C, 99% and 25% were alive after 3 and 10 months respectively. Also in this study the effect of freeze-thawing was examined, though the cycles were extremely sporadic. However, after 7 months 72% of larvae had survived and this decreased to 37% after 10 months (Turner 1953). These results are broadly similar to those found for *N. filicollis*.

Overall, on evidence from studies on pasture and in the laboratory the results indicate that a proportion of the population of *N. filicollis* and *N. spathiger* larvae can tolerate a wide range of environmental conditions.

2.3 Host species

Usually *Nematodirus* spp. are generally parasites of even-toed ungulates (Artiodactyla), usually in pecoran ruminants although there are a small number of species in rodents (Hoberg *et al*. 1988; Hoberg 2005). *N. spathiger* have been recorded from sheep, cattle, goats, deer, some gazelles, camels and rodents and *N. filicollis* has been recorded from sheep, goats, cattle and deer (May 1920; Lichtenfels and Pilitt 1983). Both species have been found in domesticated ruminants and their wild counterparts (van Dijk and Morgan 2009). Several species of *Nematodirus* have been recorded from the host of interest, the domesticated sheep, including: *N. spathiger*, *N. filicollis*, *N. abnormalis, N. helvetianus* and *N. battus* (Brunsdon 1967; McKenna 2009). The latter is not found in New Zealand sheep and *N. helvetianus* is more commonly found in cattle (Brunsdon 1967; Pomroy 1997; McKenna 2009).

2.3.1 Host age and immunity

Nematodirus spp. are found in young lambs, being one of the first parasites that infect the lamb in spring (Brunsdon 1960; Vlassoff and McKenna 1994). Infections of *Nematodirus* occur mostly in lambs less than nine months old (Tetley 1935; Brunsdon 1963b) and in particular between two and five months old (Brunsdon 1967). Initially lambs have little capacity to resist infection (Vlassoff *et al*. 2001). However, resistance eventuates and results in: increased prepatent period, stunted adult worms, fewer numbers establishing in the host and inhibited larval development and fewer eggs produced (Brunsdon 1962). Resistance develops rapidly, with egg output declining suddenly and in certain cases within two months after the initial infection (Brunsdon 1963). Additionally it is a strong response and in general lasts throughout the animal's life. Adult ewes rarely have *Nematodirus* burdens, even during the post-parturient rise *Nematodirus* are absent, though most other trichostrongyloid genera are found (Brunsdon and Vlassoff 1971; Vlassoff 1973). It seems both age and acquired resistance contributes to the response to *Nematodirus*. Age resistance *per se* was reported in previously worm-free two-tooth sheep (18 months), with a lower FEC, fewer worms and a longer prepatent period, than in lambs (6 months) (Brunsdon 1962). It also appeared that age-related resistance was stronger against *N. filicollis* than *N. spathiger*. However Tetley (1935) concluded that age-related resistance did not occur, as a 10 month old lamb that was naïve to nematodes gained an infection of *Nematodirus*.

Though Tetley's studies are conducted with few animals they instead suggest acquired immunity was most important as he had already shown infections occur more predominately in lambs less than 9 months. Brunsdon (1963b) also discovered that the stimulation of resistance to *N. filicollis* and *N. spathiger* was due to the pre-patent development of larvae and not the results of an established patent infection.

2.3.2 Pathology

Only a few studies have given details of the pathology related to *N. spathiger* and *N. filicollis*. The clinical signs of disease in lambs with *Nematodirus* infections are: loss of appetite, sometimes failure to gain weight or weight loss, profuse diarrhoea and dehydration (Thomas 1959a; Kates and Turner 1953, 1955; Brunsdon 1961). The outbreak of disease commonly occurs suddenly (Brunsdon 1961). Lambs that were given an experimental dose of *N. spathiger* presented with tissue damage to the intestinal wall. In histological sections of the jejunum, the mucosa was eroded (Kates and Turner 1955). Damage to the mucosa occurred within five days of infection, with the loss of epithelium from the villi and necrosis of the lamina propria. Damage was so severe in some parts few villi remained. Slightly longer infections resulted in similar damage though some of the anterior ends of the immature worms penetrated the muscularis mucosa (Kates and Turner 1955). The consensus is that tissue damage is caused mostly by the developing larvae and the clinical signs are a result of this damage. The disease is more difficult to diagnose due to these immature worms, as eggs are not being produced the FEC does not reflect the actual parasitic burden (Brunsdon 1961), however it could be argued that FEC never reflects the true worm burden for *Nematodirus* as they are poor egg-producers as evidenced by Chalmers (1985) study.

2.4 Pathogenicity

Nematodirus is described as a genus with pathogenic potential (Kates and Turner 1953); however there appears to be a lack of experimental studies conducted on the individual effects of *N. filicollis* and *N. spathiger* in New Zealand lambs. References to their pathogenicity are related to reports of clinical disease and mortality when acute outbreaks occur. Of such reports, infections of up to 45,000 worms per host have been found with mortality rates of 6-10% (Brunsdon 1961; Anonymous 2010). International studies on pathogenicity of *N. spathiger* in experimental infections have shown effects on growth. After three weeks, lambs were on average 7lb heavier in a group of

uninfected lambs compared to lambs administered a large dose (300,000-900,000) of *N. spathiger* larvae (Kates and Turner 1953). Similarly a 26.5lb weight difference occurred after 10 weeks with a mixed *Haemonchus contortus* and *N. spathiger* infection compared to the uninfected control (Kates and Turner 1960). Evidently this is an additive effect of the dual species, as the study showed the weight gain was not as pronounced in a pure *H. contortus* infection (Kates and Turner 1960). It would be thought a similar effect would be found to these studies using New Zealand isolates of *N. spathiger*, given the similarity in clinical signs and mortality.

Before 1950 *Nematodirus* was considered non-pathogenic in New Zealand, however since this time, acute outbreaks occurred, as mentioned above (Brunsdon 1961). The more current thinking is that these two species are classed as of major importance in New Zealand sheep (Pomroy 1997). Brunsdon (1961) indicated *Nematodirus* generally only occur in lambs in moderate numbers. It seems this would be true today, possibly as a result of interventions with anthelmintics; and as a UK-based study suggested, *Nematodirus* normally contributes to the mixed nematode burden that a lamb experiences (van Dijk and Morgan 2009).

2.5 Distribution

Knowledge on where *Nematodirus* species occur geographically within New Zealand is important so farmers can manage the parasite population that they have on their farm. Also it is important as a reference point so future studies can compare changes to distributions, especially with the changing climate and changing farming practices.

2.5.1 World-wide distribution

Nematodirus spp. infect ruminants throughout the world, though they are generally associated with the high latitudes of the Holarctic and are absent from Africa and Southern Asia (Hoberg 2005). However, some species have spread globally with the movement of domestic livestock and are now cosmopolitan in their distribution. The distribution of *N. filicollis* and *N. spathiger* is worldwide (Thomas 1959a). Both species have colonised extreme environments i.e. they have been reported from the northern latitudes of Iceland (Richter 2002; Skirnisson 2011) to the semi-arid environments in eastern Ethiopia (Sissay *et al*. 2007).

2.5.2 *Nematodirus* distribution in New Zealand

Like the other common helminths of ruminants found in New Zealand, *Nematodirus* were introduced when their hosts were brought to New Zealand for domestic livestock purposes. Now, *Nematodirus* is found regionally throughout the country, as dispersion occurred with the movement of stock (Vlassoff *et al*. 2001). Brunsdon (1961) found three *Nematodirus* species to be widely spread throughout New Zealand, with *N. spathiger* and *N. filicollis* the most important as they caused outbreaks of disease. The third species, *N. abnormalis* was of minor significance though widely distributed. *N. filicollis* tended to dominate (i.e. it was greater than 60%) infections from the North Island (52% compared to 26% for *N. spathiger*), while in the South Island the prevalence of both species was similar (44% and 48% for *N. filicollis* and *N. spathiger*, respectively). Neither species dominating accounted for the remaining infections. This indicates that both species are present in both islands in significant numbers.

Regional differences have been found at both species and genus level. Between 1976 and 1979 the larvae available on herbage was recorded from five New Zealand sites: Dargaville, Ruakura, Wallaceville, Winchmore and Woodlands. Interestingly, the distribution of the two species indicated that *N. spathiger* was more common in the north and *N. filicollis* more common in the south (Vlassoff 1982), however no numerical evidence was provided with this statement. In terms of the variation in national distribution of disease, *Nematodirus* generally has a greater impact in the South Island, with outbreaks of disease occurring mostly in Canterbury, Otago, and Southland in the 1950s (Brunsdon 1967; Vlassoff *et al*. 2001). However preventative drenching reduced the incidence of such outbreaks (Brunsdon 1967). Another study reported *Nematodirus* were at lower abundance levels in the North Island regions compared to those in the South Island. This study used percent compositions of the relative abundance of all gastrointestinal nematode genera found in different areas of New Zealand (Vlassoff and McKenna 1994). The cause of these differences could relate to climatic factors, with the north-south temperature and west-east moisture gradient thought to influence species distribution (Pomroy 1997; Vlassoff *et al*. 2001). However the overall climate variations in different areas of New Zealand are small in comparison to other countries like Australia and conditions are favourable for development at least for part of the year in all districts (Vlassoff 1982; Vlassoff *et al*. 2001). The current distribution has not been assessed in recent times, though reports of *Nematodirus* being

an issue arise periodically, especially in the South Island (Anonymous 2010). A perception exists that *N. filicollis* is found only in the South Island.

2.6 *Nematodirus* resistance to anthelmintics

The control of nematodes, such as *Nematodirus* spp. in lambs, has been mainly by the administration of anthelmintics (Lawrence *et al*. 2007), though control through grazing management has also been used. This can be particularly effective for *Nematodirus* with the knowledge that the disease is largely transmitted from one year's lambs to the following year's lambs. These anthelmintics contain different active ingredients that eliminate the nematode from the host. There are five classes of broad spectrum anthelmintics with activity against *Nematodirus* that are available for use in New Zealand: benzimidazoles, imidazothiazoles, macrocyclic lactones, amino-acetonitrile derivatives and spiroindoles. One of these classes, the Benzimidazoles (BZ) is discussed in further detail. The compounds of the BZ act by binding to the nematode tubulin, disrupting the tubulin-microtubule balance, thus causing depolymerisation of the microtubules (Pritchard 1990). At recommended dose rates the efficacy of albendazole against adult *N. spathiger* and *N. filicollis* in sheep has been reported as between 94-100% (Theodorides *et al*. 1976; Schalkwyk *et al*. 1979; Diez Banos *et al*. 1979; Louw and Reinecke 1991).

As a result of the overuse (Leathwick *et al*. 2001) and/or misuse of anthelmintics, resistance in gastrointestinal parasites has developed and is well documented in all the major classes of anthelmintics. Resistance is heritable and occurs when there is a greater frequency of individuals in a population able to tolerate a dose of compound than in a normal population (Pritchard *et al*. 1980; Sutherland and Leathwick 2011). Generally resistance is detected through the use of FECRTs, which compare pre and post treatment egg counts (Coles *et al*. 1992). Resistance is diagnosed if the percent reduction is less than 95% (Coles *et al*. 1992). The mechanism for BZ-resistance in a population of worms is associated with a mutation in the β-tubulin genes that prevents the drug binding to the cytoskeletal protein, tubulin (Pritchard 1990; Obendorf *et al*. 1991; Wolstenholme *et al*. 2004).

Resistance in *Nematodirus* was discovered against the BZ class of anthelmintics and has been known for over 30 years (Middelberg and McKenna 1983; Vlassoff and Kettle 1985; Chalmers 1985). The first *Nematodirus* species implicated with resistance to

oxfendazole was *N. spathiger* (Middelberg and McKenna 1983). BZ-resistance in this genus has also been confirmed in other international studies. In Tasmania, Australia several isolates of *N. spathiger* were found to be BZ-resistant (Obendorf *et al.* 1986, 1991), the latter study using *in-vitro* egg development tests to determine resistance with thiabendazole. BZ-resistance in *N. filicollis* was found somewhat later. In a South Australian study that assessed resistance to oxfendazole using worm counts, there were eight cases of resistance in *N. spathiger* but also two in *N. filicollis* and five in *N. abnormalis* (Beveridge *et al.* 1990). A more recent British report has now also found a case of *N. battus* resistant to fenbendazole (Mitchell *et al.* 2011; Morrison *et al.* 2014).

There have been several reports on the prevalence of BZ-resistance in New Zealand (McKenna 1989, 1994, 2012, 2013; Waghorn *et al.* 2006). Over time the prevalence of BZ-resistance has increased. A New Zealand-wide survey of sheep farms in 2004/5, and a recent summary of laboratory data, reported that 87-89% of farms tested had *Nematodirus* spp. with less than 95% FECR to the BZ (Waghorn *et al.* 2006; McKenna 2013). In the 2004/5 national survey (Waghorn *et al.* 2006) the percent reduction in FEC for the genus, ranged from the severe (0%) to slight resistance (90%). Evidence for resistance in other classes has also been reported. For levamisole and ivermectin 11% and 7% respectively of farms had *Nematodirus* spp. resistance to BZs (Waghorn *et al.* 2006). Overall, this indicates that *Nematodirus* is resistant to the BZs on a large proportion of farms, with the other anthelmintic classes implicated in resistance to a lesser degree. While the initial report identified *N. spathiger* as the BZ-resistant species (Middelberg and McKenna 1983), several other studies have not differentiated the species (West *et al.* 1989; Macchi *et al.* 2001; Little *et al.* 2010; McKenna *et al.* 1995a; McKenna 1995b). In most of these studies the FECRT was used to identify resistance, the lack of species identification probably reflects the complexity in identifying species based on egg morphology (Onar 1975) or otherwise the difficulty in culturing this genus. The various studies in New Zealand commonly used larval culture techniques to help identify the various trichostrongyloid genera present but culture duration was commonly too short for many *Nematodirus* eggs to hatch (see section 2.6.1) and there are no reports of preconditioning the eggs with a period of chilling to stimulate *N. filicollis* to hatch. Therefore it remains to be known, if *N. filicollis* in New Zealand are resistant to the BZ class and if so the level of prevalence; this is also unknown for *N. spathiger*.

2.6.1 Faecal egg count reduction test

There are several methods for detecting anthelmintic resistance such as FECRT, egg hatch test, larval development assays, larval migration inhibition assays, and molecular based tests (Taylor *et al*. 2002; Coles *et al*. 2006). The standard method used is the FECRT as all anthelmintic classes can be tested (Coles *et al*. 2006). The test involves taking faecal samples prior to the animal being administered the anthelmintic to be tested and then counting the number of eggs present. Then usually seven to ten days later, re-sampling and counting any eggs that are present in the faeces (Coles *et al*. 1992), the delay in resampling is to avoid the suppression of egg-laying that the anthelmintic may cause (Taylor *et al*. 2002). The addition of an untreated control group allows for comparison, as egg numbers can increase or decrease between the pre and post sampling tests (Taylor *et al*. 2002). To determine the genera/species involved, faeces from the different animals (usually 10-12) of the same treatment are mixed together and then cultured to isolate the L3 so that species can be identified and then efficacies of the drench calculated for each species present.

There are a few factors that should be noted for *Nematodirus* spp. regarding this test. Firstly as *Nematodirus* produce few eggs, the egg counts in the test are often low. Therefore this genus may be present but not detected, this is especially a problem post treatment and could result in a false negative. Also as *Nematodirus* spp. takes longer to develop and hatch (section 2.2.3 and 2.2.4) they are often not found in standard cultures that are incubated for between 10 and 14 days. Often the efficacy of *Nematodirus* is reported from the egg counts alone, which as just mentioned is not ideal either. A modified egg hatch assay has successfully detected BZ-resistance in *N. spathiger* (Obendorf *et al*. 1986).

2.7 Molecular identification

In the past helminths were identified primarily on morphology or morphometry. This generally involved culturing faeces to isolate the L3 and then assessment of certain characters to make a determination. Occasionally eggs were useful for identification and adult worms could be extracted from the gut during post mortem examination and used for identification. While this is successful to some extent, there are also limitations with these methods. As such it can be inaccurate with too few characters to separate species, particularly the egg and larval stages, it is labour intensive and requires a skilled

diagnostician and the culturing technique that produces L3 can bias one species over another (Georgi and McCulloch 1989; Hoste *et al*. 1995; Silvestre and Humbert 2000).

There is a vast and growing amount of literature on the molecular tools used in helminth parasitology (for reviews see Gasser 1999, 2006; Gasser *et al*. 2008). Therefore, a brief overview of some aspects that relate to this thesis are presented. For example, the use of polymerase chain reaction (PCR) and a discussion of different target-regions of DNA, both with a focus on *Nematodirus*.

2.7.1 Polymerase chain reaction

Over the past 30 years an array of molecular techniques have been developed and improved to provide reliable, alternative methods for parasite identification, among other uses i.e. diagnoses of infection, parasite epidemiology and studies of anthelmintic resistance (Gasser 1999, 2006; Bisset *et al*. 2014). One of these techniques that has progressed molecular biology forward was the introduction of the PCR, which amplifies regions of DNA (Saiki *et al*. 1988). This only requires minute amounts of DNA for a successful outcome, which is valuable when assaying parasites, as their size and stage in their lifecycle are often not conducive to obtain large amounts of DNA (Gasser 1999, 2006). Since the introduction of PCR, it has been used widely to develop parasite diagnostic tests (Gasser *et al*. 2008).

The general principle of the PCR is to amplify selected regions of DNA using enzymes over numerous cycles. Each cycle has three steps: denaturation, annealing and extension that are produced using a programmable thermocycler (Singh 1997). Thus by heating the template of double stranded genomic DNA, it is denatured; then at a lower temperature, specific oligonucleotide primers anneal to their corresponding sequences, on the opposite strand of template. This is followed by extension in which the enzyme DNA polymerase catalyses the deoxynucleotide triphosphates present in solution to the primer sites (Singh 1997; Gasser 1999). The result is the formation of double stranded products that are used as the template in subsequent cycles along with the original DNA (Singh 1997; Gasser 1999). At the end of the PCR, millions of copies of the target sequence exist (Singh 1997; Gasser 1999).

These target sequences underpin reliable PCR-based identification of species, and identifying a suitable target region from a DNA sequence is crucial (Gasser 2006;

Gasser *et al*. 2008). As such the ribosomal DNA (rDNA) genes and associated spacer regions have provided suitable targets for developing diagnostic markers. In particular the internal transcribed spacer regions (ITS-1; ITS-2) have been used to separate both genera and species of the order Strongylida and other orders (Hoste *et al*. 1995; Gasser 1999, 2006; Bisset *et al*. 2014). In general both ITS-1 and ITS-2 have low levels of variability within a species (usually < 1.5%) but moderate levels between species (Gasser *et al*. 2008). Also because multiple copies of genomes are present in the rDNA of most nematodes, it lends itself to amplification by PCR (Bisset *et al*. 2014).

A number of studies have used the ITS-1 and/or ITS-2 regions in PCR-based assays to separate nematode species or genera of livestock (Bisset *et al*. 2014). For example: two species of *Haemonchus* were sequenced and recognised as different (Stevenson *et al*. 1995); five species of *Trichostrongylus* were characterised (Gasser and Hoste 1995); five genera *Ostertagia*, *Cooperia*, *Haemonchus*, *Trichostrongylus* and *Nematodirus* were separated but species-specific primers could not be developed (Schneider *et al*. 1999); differences in sequences of eight gastro-intestinal species were recognised, including *N. filicollis* and *N. helvetianus* (Heise *et al*. 1999); five species all from different genera, found in the bovine animal were distinguished using a single multiplex PCR assay (Zarlenga *et al*. 2001); nine parasite species (including *N. battus* and *N. filicollis*), representing six families found in feral sheep were identified using PCR of rDNA from eggs and cultured larvae (Wimmer *et al*. 2004); ten species of six genera (Bott *et al*. 2009) and five species all from different genera (Roeber *et al*. 2011) were identified and to some degree quantified from sheep; and most recently a multiplex PCR-based assay was used to recognise all common species from sheep in New Zealand including 15 species from eight genera, including *N. spathiger* and *N. filicollis* (Bisset *et al*. 2014). Of these examples several use the ITS-2 region including the last example described by Bisset *et al*. (2014). Specific studies recognising individual species of *Nematodirus* have also been reported in other studies. Examples include: the ITS-2 region of rDNA from *N. spathiger*, *N. filicollis*, *N. helvetianus* and *N. battus* were found to be sufficiently different to be used in identification (Newton *et al*. 1998). The ITS-1 region of these four species was also found to be sufficiently different to separate species (Audebert *et al*. 2000) and was then used to infer phylogenetic relationships. Gasser *et al*. (1999b), using the ITS-2 region, delineated four other *Nematodirus* species found in wild ruminants. Nadler *et al*. (2000) found differences in the sequences of *N.*

battus, *N. filicollis*, *N. spathiger* and *N. helvetianus* using several different regions of rDNA, including the ITS-1 and ITS-2 regions. Overall not only do these studies show the large impact PCR-based assays, using the ITS regions have had on diagnostics, but also that they can be applied to further studies with a degree of confidence.

Many of the early studies used these molecular technologies in a single genus or a single family of nematodes, and therefore were not necessarily species-specific (Wimmer *et al*. 2004; Bisset *et al*. 2014). While this was of use to certain areas of parasitology, such as phylogenetics, it was not so useful for diagnostic testing. This is because livestock are infected with a large number of mixed species, of which any number of these could be present when tested (Bisset *et al*. 2014). Thus a test for a single genus was of little use diagnostically. Either several assays were required to cover the potential options or a species could be missed. Additionally for some species the research had not been conducted, so had no corresponding DNA sequence to use. However it appears each study contributed new information that was able to be used as building blocks, so that more useful techniques could be developed, such as the multiplex assay described Bisset *et al*. (2014). Reliable diagnostic testing also became an issue with contamination of samples by the DNA of other organisms, causing inconclusive results in assays. Due to the magnitude of amplification in the PCR, only a tiny amount of contaminate is required for it to be amplified (Comes *et al*. 1996) and confuse the subsequent interpretation. This has generally been resolved through environmental considerations. Another limitation to this PCR-based technology is that it is considerably more expensive than traditional microscopy (Comes *et al*. 1996; Singh 1997; Gasser *et al*. 2008). Also the initial PCR-based assays were not quantitative (Comes *et al*. 1996; Singh 1997); which limited their use to identification only and did not give an idea of the intensity of an infection (Comes *et al*. 1996). This appears to be remedied through the use of a real-time PCR method (Bott *et al*. 2009; Roeber *et al*. 2011). Overall in comparison with traditional morphological identification, cost and practicability are probably the most prohibitive for the general uptake of molecular methods as a diagnostic tool; however the benefits of PCR-based techniques are that they are more sensitive and more specific (Singh 1997) which makes them an important tool to consider when undertaking research that involves diagnostics.

2.8 Conclusion

In conclusion there is a wealth of information on the epidemiology of *Nematodirus* spp. However, these studies have often not differentiated the species involved. Consequently a full understanding of the dynamics of *N. filicollis* and *N. spathiger* is lacking, this especially relates to *Nematodirus* in New Zealand. The advent of new techniques like PCR-based molecular identification now allows different approaches to be taken to resolve some of these issues.

Chapter 3

A survey of the prevalence of *Nematodirus spathiger* and *N. filicollis* on farms in the North and South Islands of New Zealand.

A-MB Oliver, DM Leathwick and WE Pomroy

3.1 Abstract

AIM: To compare the prevalence of *N. spathiger* and *N. filicollis* on a sample of farms in the lower South Island with the lower and central North Island of New Zealand by way of a presence/absence survey.

METHODS: A presence/absence non-random survey was conducted in which pooled faeces from lambs from 50 farms in the lower and central North Island (n=22) and lower South Island (n=28) were cultured, with and without a post-culture period of chilling, to produce third-stage parasitic nematode larvae (L3). After recovery using the baermann technique, individual L3 were identified to *Nematodirus* spp. using a PCR assay. This identified the species that were present on each farm that were then used to compare the regions.

RESULTS: *Nematodirus filicollis* was present in 38/50 (76%) samples from which *Nematodirus* spp. larvae were cultured, compared with 50/50 (100%) in the case of *N. spathiger*. No regional differences were observed in the prevalence of either species (p=0.74). Of the *Nematodirus* spp. L3 recovered from the unchilled samples, 415/428 (97%) were *N. spathiger* and 13/428 (3%) *N. filicollis*. After chilling 594/695 (85%) of the *Nematodirus* spp. L3 were *N. spathiger* and 101/695 (15%) were *N. filicollis*.

CONCLUSION: Despite the non-random nature of the study the evidence suggests that both these species are likely to occur sympatrically on most sheep farms throughout New Zealand. In general *N. filicollis* eggs did not hatch without a period of chilling and this has implications for the identification of these larvae using conventional culture methods.

KEY WORDS: *Nematodirus*, *spathiger*, *filicollis*, *distribution, New Zealand*

3.2 Introduction

Four species of the genus *Nematodirus* have been reported in sheep in New Zealand (McKenna 2009) although only three of these, *N. abnormalis, N. filicollis* and *N. spathiger*, were reported in a historical survey of post-mortem worm count samples collected from throughout the country (Brunsdon 1961). The latter two species are considered the most important, as they are more prevalent and dominate in infections (Brunsdon 1961; Vlassoff and McKenna 1994). The genus is reported frequently in faecal egg counts from lambs throughout New Zealand. On occasions an acute outbreak of disease can be attributed primarily to the genus *Nematodirus*, but it more usually occurs as one of several genera contributing to parasitism in the young lamb (Pomroy 1997; Vlassoff *et al*. 2001).

In an analysis of post-mortem worm counts, both *N. filicollis* and *N. spathiger* were present although *N. filicollis* tended to predominate in infections from the North Island (52% of infections compared to 26% for *N. spathiger*), while in the South Island the prevalence of both species was similar (44% and 48% for *N. filicollis* and *N. spathiger*, respectively; Brunsdon 1961). However, a subsequent study on the number of nematode larvae on pasture indicated regional differences in the distribution of the two species. When three North and two South Island sites were compared, *N. spathiger* was more common in the North and *N. filicollis* in the South Island (Vlassoff 1982). Over time this information has evolved to the extent that it is now a common perception that *N. filicollis* is more prevalent in the South Island and *N. spathiger* in the North Island, although this may have changed as a result of benzimidazole resistance (Rattray 2003; Pomroy 2010).

In a review of the relative abundance of common helminth genera in various regions in New Zealand, Vlassoff and McKenna (1994) concluded that the southern provinces showed higher percentages of *Nematodirus* spp. in infections than in the north. This difference in abundance may be linked to the differences in species distribution suggested by Vlassoff (1982). However without further studies that differentiates larvae to species few conclusions can be drawn. Thus, while infection with the *Nematodirus*

genus has been considered more important in the South than the North Island (Vlassoff and McKenna 1994), and outbreaks of disease have been largely restricted to Canterbury, Otago and Southland (Brunsdon 1967), it remains unclear whether this is linked to differences in species abundance or to other factors.

The current study was initiated as part of a wider investigation into the epidemiology of the two most prevalent species in the genus *Nematodirus* in New Zealand. The aim was to clarify this apparent disparity in the literature by investigating the distribution of *N. spathiger* and *N. filicollis* through a presence/absence survey in the lower South Island and lower and central North Island of New Zealand.

3.3 Materials and methods

3.3.1 Collection of eggs

Faecal samples from lambs were collected between 04 November 2010 and 05 April 2011. Samples were sent to the AgResearch parasitology laboratory (Palmerston North, NZ) from commercial farms that were involved in various extension projects, and from veterinarians who collected samples when the opportunity presented from commercial sheep or sheep and beef farms, with an average flock size of more than 2000 breeding ewes. On several farms faecal samples were collected from 10 lambs, while the majority of samples were pooled on collection from approximately 30 lambs into a 600 mL container. Upon arrival at the laboratory faecal nematode egg counts were conducted on all samples, with *Nematodirus* spp. eggs being identified on the basis of their size (Soulsby 1968). This was undertaken using the modified McMaster method adapted from the method described by Lyndal-Murphy (1993), where one egg counted was equivalent to 50 epg (see Appendix 1 for full SOP). Fifty farms provided samples that contained eggs of the *Nematodirus* genus. The majority of samples were collected from two regions, the southern North Island (n= 17) and southern South Island (n=28). This maximised the number of samples that could be processed with the limited resources available, with the aim of identifying any differences between the North and South Islands, should they exist. A small number of additional farms from the central North Island (n=5) were also used, to increase overall numbers. In the North Island, farms

were located from south of Rotorua to south of Eketahuna, while in the South Island they extended from west of Darfield to north of Otautau (Figure 3.1). (See Appendix 2 for collection details).

Figure 3.1. Approximate locations in New Zealand of 50 sheep farms that provided faecal samples from lambs containing eggs of the *Nematodirus* genus.

3.3.2 Egg culture

Samples containing *Nematodirus* spp. eggs were cultured to produce infective-stage larvae. Faeces from individual animals from each farm were pooled, and approximately 20g of faeces from each farm weighed and mixed with medium-sized vermiculite, before being incubated at 20°C for at least 42 days to allow maturation to L3 within the egg. Previously, large numbers of *Nematodirus* spp. eggs had failed to hatch after being cultured in this way and so, for this study, each faecal culture was weighed and halved after incubation. Larvae were recovered immediately from one half using a modified Baermann procedure, where faeces were placed on a sieve, in a 300 mL funnel and

covered with water, then larvae were recovered from the bottom of the funnel after 48 hours at room temperature (Hendrix 1998). The other half was chilled at 4°C for at least 6 months. After chilling, eggs were held at 13°C for 14 days to allowing hatching before larvae were recovered as above. Samples containing L3 were siphoned, after sedimentation overnight, and then stored at 4°C until they were processed for molecular analysis (see Appendix 1 for full SOP).

3.3.3 Molecular analysis

3.3.3.1 Template preparation

Individual L3 were removed from the sample and placed into 0.2 mL thin-wall strip tubes, which contained 70% ethanol. When available, 32 larvae were isolated per farm from the unchilled sample, while 48 larvae were isolated from the chilled culture. The full protocol for identification of L3 is described in Bisset *et al.* (2014). See Appendix 1 for the SOP. Briefly, prior to lysis, approximately 80% of the ethanol was removed by pipette and the remaining left to evaporate before 10 µL of lysis solution (1 mL Viagen DirectPCR (Tail); Viagen Biotech Inc, Los Angeles, CA, USA), and 25 µL of Proteinase K recombinant, (PCR grade; Roche, Basel, Switzerland) was added to each tube. The samples were then lysed in a thermal cycler, by heating to 55°C for 16 hours, followed by 1 hour at 90°C and then 10 minutes at 4°C. The strip tubes were then placed at -80°C for at least 30 minutes, to aid mechanical separation of larval DNA bound to any incompletely lysed protein. Samples were thawed and diluted with the addition of 20 µL of high-performance liquid chromatography water.

3.3.3.2 PCR assay

Larvae were identified to species using a PCR-based assay described by Bisset *et al.* (2014). See Appendix 1 for the SOP. The method utilises individual larval lysates as template for the assay reactions and identifies the presence of unique species-specific sequence markers located in the second internal transcribed spacer region of the ribosomal DNA of each of the larval species. Assays contained 1.5 mM $MgCl_2$, 1 µL 10x *Taq* buffer, 100 µM of each deoxyribonucleotide triphosphate, and 0.25 units Platinum *Taq* DNA polymerase (Life Technologies, Carlsbad, CA, USA) and high-performance liquid chromatography water. In addition, 3 pmol of two generic primers and the species-specific primers for *N. spathiger* and *Chabertia ovina*, 5 pmol of the

primer for *N. filicollis* and 2 pmol of the primer for *Oesophagostomum venulosum* were added. The primer details are given in Table 3.1. Lastly 1 μL of template DNA was added to give a total volume of 10 μL. The inclusion of primers for *O. venulosum* and *C. ovina* was to ensure that any L3 recovered with long filamentous tails were correctly identified to species.

The conditions for PCR were 95°C for 8 minutes, then 12 cycles of 94°C for 15 seconds, 60°C reducing 0.5°C/cycle for 15 seconds, 72°C for 30 seconds; followed by 25 cycles of 94°C for 15 seconds, 54°C for 15 seconds and 72°C for 30 seconds. To complete the PCR, there was an additional extension of 72°C for 7 minutes and finally cooling at 10°C for 10 minutes.

Using the electronic documentation program Gel.doc 2.6.4 (Bio-Rad, Hercules, CA, USA), PCR products were visualised after electrophoresis in a 2% agarose-TAE (0.04M Tris-acetate, 0.001M EDTA, pH8.0) gel, containing SYBR Safe DNA gel stain (Life Technologies). The resulting bands were assigned to species based on their size (see Appendix 1, page 95 for example gel).

Table 3.1. Species-specific primer sequences, and the two generic primers, used in multiplex PCR assays to differentiate between larvae of four strongylid target species, with their resulting product sizes in base pairs (bp).

Target species	Primer	Sequence (5'-3')	Product size (bp)
N. filicollis	NEFIRV1	GGGATTGACTGTTACGATGTAA	162
N. spathiger	NESPRV1	CATTCAGGAGCTTTGACACTAAT	213
O. venulosum	OEVERV1	CGACTACGGTTGTCTCATTTCA	323-7
C. ovina	CHOVFD2	CAGCGACTAAGAATGCTTTGG	115-7
Generic	ITS2GFnest	CACGAATTGCAGACGCTTAG	370-398
Generic	ITS2GRnest	GCTAAATGATATGCTTAAGTTCAGC	

3.3.4 Statistical analysis

The results from the two halves of each culture (i.e. chilled and unchilled) were combined to provide the proportion of farms that were either positive or negative for the

presence of *N. filicollis*. The prevalence of *N. filicollis* in North and South Island farms was compared using a two proportion t-test, using Fisher's exact test, as normal approximation may be inaccurate with the small sample size, and a value of $p<0.05$ as statistically significant. Analysis was undertaken using Minitab Statistical Software v15.1.0.0 (Minitab Inc, PA, USA).

3.4 Results

Pooled faecal samples were cultured and tested using PCR from 22 North Island and 28 South Island farms (Figure 3.1). From the L3 identified to species in faecal cultures, a small number were *O. venulosum* or *C. ovina*, but these were not part of the study and so were not considered further. Of the L3 identified as *Nematodirus* spp., *N. spathiger* was present on all 50 farms tested, while *N. filicollis* was present in 16/22 (73 (95% CI = 50-89)%) samples from the North Island and 22/28 (79 (95% CI = 59-92)%) of the South Island samples (raw data shown in Appendix 2). There was no difference in prevalence of *N. filicollis* in samples between regions (p=0.743).

Of the *Nematodirus* spp. L3 recovered from the unchilled samples, 415/428 (97%) were *N. spathiger* and 13/428 (3%) *N. filicollis*. After chilling 594/695 (85%) of the *Nematodirus* L3 were *N. spathiger* and 101/695 (15%) were *N. filicollis*.

3.5 Discussion

In this study there was no evidence for a difference in the prevalence of *N. spathiger* and *N. filicollis* on the farms sampled in the North and South Islands of New Zealand. Although the entire country was not surveyed, the areas chosen represent the major sheep farming areas and were selected to be sufficiently divergent as to detect a geographical difference, should one exist. Eggs were the life-stage chosen for collection, leading to the identification of L3, because samples were easy to obtain and therefore more farms could be tested, when compared with the collection of adult worms. The obvious weakness with this approach is that with only a single sample collected from each farm, a species may have been present on a farm but not recorded in the single sample. However, given that *N. spathiger* was identified from 100% of farms

and *N. filicollis* from 76%, this does not appear to have impeded the study to any great extent.

Nematodirus filicollis and *N. spathiger* were present in a similar percentage of the North and South Island farms tested. Although this was a non-random survey, farms were not selected for any reason likely to relate to the species of *Nematodirus* present, and so the results should provide a useful overview of the distribution of these two species. These results are generally consistent with those of Brunsdon (1961), who found the two species were widely distributed throughout New Zealand. However, the results of the present study are in contrast to the statement by Vlassoff (1982) that *N. spathiger* was more common in the North and *N. filicollis* in the South Island, based on larvae on pasture from a variety of farms throughout New Zealand. Overall, the concept that *N. filicollis* is found predominantly in the South Island is not supported by the present data.

The results were similar to that of an Icelandic study, where both *N. filicollis* and *N. spathiger* were prevalent in most parts of the country (Richter 2002). Whilst the climate in Iceland is more extreme and generally cooler than in most sheep farming areas of New Zealand it does indicate that both species can overlap. In contrast, differences in the regional distribution of these species were reported in South Australia, with *N. filicollis* found only in the southern wetter areas and *N. spathiger* predominated in low rainfall areas of the north and west (Beveridge and Ford 1982).

Limited information has been provided to date to explain these regional differences. It has been inferred by the authors that climate is a key factor determining regional differences in the distribution of *Nematodirus* spp. in South Australia (Beveridge and Ford 1982). Vlassoff and McKenna (1994) concluded that the relatively uniform climate of New Zealand allows most parasite species to develop throughout the country and our conclusions on the distribution of *N. spathiger* and *N. filicollis* support this.

The differing results and perceptions regarding the distribution of these two species within New Zealand could, in part, reflect differences in the development of the two species on pasture. Compared with *N. spathiger*, *N. filicollis* is generally much slower in completing the pre-parasitic phase of its lifecycle due to its requirement for chilling (Thomas and Stevens 1960; van Dijk and Morgan 2009). By measuring the numbers of larvae on pasture, the results of Vlassoff (1982) may have been influenced by a greater opportunity for chilling of developed eggs of *N. filicollis* in the south, due to the

generally lower temperatures, than in the north. Similarly, because *N. filicollis* will generally not hatch in a faecal culture without subsequent chilling (Thomas 1959a; Thomas and Stevens 1960), it is unlikely to be encountered in routine laboratory diagnostics, potentially reinforcing the perception that it is less prevalent in some parts of the country. This was the case in the current study and resulted in the addition of a chilling period for half of each sample, so that fair comparisons between species could be made.

The finding that both species were present together on 76% of farms is likely to be conservative, due to only a single sample being collected from each farm; i.e. if multiple samples had been collected from each farm, over a period of time, it is possible that some of the farms that had previously tested negative for *N. filicollis* would subsequently test positive, thereby increasing the overall number positive for this species. Given this, it is likely that *N. filicollis* and *N. spathiger* occur sympatrically on the majority of sheep farms in New Zealand, and it is clear from studies here (Tetley 1935; Brunsdon 1961) and overseas (Hoberg *et al*. 1986; Skirnisson 2011) that they frequently occur as concurrent infections in lambs. However, there are important differences between these two species in the requirements for development of the free-living stages on pasture and it remains to be determined what impacts this might have on the dynamics of each species.

3.6 Acknowledgements

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Chapter 4

Benzimidazole resistance in *Nematodirus spathiger* and *N. filicollis*

A-MB Oliver, WE Pomroy, DM Leathwick

4.1 Abstract

AIM: To determine the prevalence of benzimidazole (BZ) resistance in *Nematodirus spathiger* and *Nematodirus filicollis* from a sample of New Zealand farms.

METHODS: The efficacy of albendazole against *Nematodirus* spp. was assessed by faecal egg count reduction tests (FECRT) undertaken on 21 sheep farms throughout New Zealand, with the majority of these farms being selected as a result of samples sent to the laboratory for routine testing. On each farm, one group of 12 animals was treated with albendazole (ABZ) (4.75mg/kg) while another group of 12 remained as an untreated control. Faecal samples were collected from all animals at the time of treatment and 7-10 days later. Faecal egg counts were performed using a modified McMaster method. Larvae were cultured from pooled post-treatment faecal samples from each group by incubation at 20°C for 6 weeks, 4°C for 26 weeks then 13°C for 2 weeks. The resulting infective larvae were identified to species using PCR-based techniques with species specific primers for the ITS-2 region of rDNA. The proportion of the two species in culture was applied to the group mean faecal egg counts and used to calculate the efficacy for both species.

RESULTS: ABZ-resistance was found on 95% (20/21) of farms for *N. spathiger* which was a higher prevalence ($p<0.05$) than for *N. filicollis* with only 40% (4/10) of farms.

CONCLUSION: The results confirm the high level of resistance in this genus in New Zealand and that ABZ-resistance was more common in *N. spathiger* than *N. filicollis*. While resistance to benzimidazole anthelmintics has been reported previously in New Zealand, this is the first report of *N. filicollis* being resistant to BZ anthelmintics.

KEY WORDS: *Nematodirus*, *spathiger*, *filicollis*, *anthelmintic, resistance*

4.2 Introduction

Nematodes of the genus *Nematodirus* are common parasites of young lambs in New Zealand (Vlassoff and McKenna 1994) and in other parts of the world (Horak 2003; van Dijk and Morgan 2009; Domke *et al*. 2013). Infection is usually concurrent with other trichostrongylid species (Pomroy 1997), although on occasions a pure infection can occur. In acute cases of nematodirosis, the onset of disease occurs suddenly and death can follow within two days (Brunsdon 1967). Such cases have been intermittently reported since the 1950s, especially in the provinces of Southland, Otago and Canterbury (Brunsdon 1967; Chalmers 1985; Anonymous 2010).

Although four species of *Nematodirus* have been recorded from sheep in New Zealand (McKenna 2009), only *N. spathiger* and *N. filicollis* are common throughout the country (Oliver *et al*. 2014). *Nematodirus* species differ in their biology from most strongylid species in that the free-living stages develop on pasture within the egg, and for *N. filicollis* in particular, a period of chilling is generally required before eggs will hatch to release the infective-stage larva (L3) (van Dijk and Morgan 2009). The extent to which chilling is required appears to differ considerably between these two species (van Dijk and Morgan 2009; Oliver *et al*. 2014) which suggests that their epidemiology might be quite different. *Nematodirus* spp. infection is often evident only in young lambs, with some transmission being directly from young lambs in one season to young lambs in the following season, rather than a period of cycling through lambs over the course of a year (Vlassoff 1973).

Control of nematode infection in young lambs is largely based upon regular treatment with broad-spectrum anthelmintics (Lawrence *et al*. 2007), such as the benzimidazoles (BZ). At recommended dose rates, the efficacy of albendazole against adult *N. spathiger* and *N. filicollis* in sheep has been reported to be between 94-100% (Theodorides *et al*. 1976; Schalkwyk *et al*. 1979; Diez Banos *et al*. 1979). As with other nematode species, resistance to anthelmintics has developed in *Nematodirus* spp., with resistance in this genus to the BZ class of anthelmintics detected over 30 years ago (Middelberg and McKenna 1983; Vlassoff and Kettle 1985; Chalmers 1985). A New Zealand-wide survey of sheep farms in 2004/5, and a recent summary of laboratory data, reported that 87-89% of farms had *Nematodirus* spp. resistant to BZ (Waghorn *et al*. 2006; McKenna 2013). In addition, evidence of levamisole and ivermectin resistance in this genus was

indicated (Waghorn *et al*. 2006; McKenna 2013). While earlier reports of BZ-resistance identified *N. spathiger* as the resistant species (Middelburg and McKenna 1983; Vlassoff and Kettle 1985), more recent studies have not identified the species involved (West *et al*. 1989; Macchi *et al*. 2001; Little *et al*. 2010; McKenna *et al*. 1995a; McKenna 1995b). As most of these later cases have relied on the faecal egg count reduction test (FECRT) to identify resistance, the absence of a species identification presumably reflects the difficulty in identifying species based on morphology of their eggs or infective stage larvae (Onar 1975), or alternatively difficulty in culturing *Nematodirus* spp.

The current research aimed to determine the extent to which resistance to a BZ anthelmintic has developed in these two species.

4.3 Methods

4.3.1 Source of samples

Samples of faeces were collected from lambs on 27 commercial farms between October 2010 and March 2011. All samples were collected as part of FECRT being carried out on the farms and the majority were collected by veterinarians (see Appendix 1 for the SOP). Samples which did not contain at least 10 eggs of *Nematodirus* spp. were discarded, which left a total of 21 farms for this study. These farms were located throughout New Zealand (Table 4.2; see page 54). For the purpose of this study, samples from two groups of 10-15 lambs (an untreated control group and an ABZ-treated group) collected at the time of treatment and 7-10 days after treatment were utilized. The ABZ-treated animals were dosed orally by syringe with albendazole (Albendazole; Ancare, Auckland, New Zealand), at the manufacturer's recommended dose rate of 4.75mg/kg.

4.3.2 Parasitology

All samples were processed with a modified McMaster technique (modified from Lyndal-Murphy 1993) to determine the faecal nematode egg count (FEC) for *Nematodirus*, which is possible because *Nematodirus* eggs are distinguishable from

other strongyle eggs. In brief, a 2 g faecal sample per individual lamb was assessed, and each egg counted represents 50 eggs/g of faeces (see Appendix 1 for the SOP).

The samples collected 7-10 days after treatment were cultured from both groups of lambs. All remaining faeces after the FEC was conducted were combined within their treatment group by farm, mixed with vermiculite and incubated at 20°C for at least 42 days to allow for egg development. After this, cultures were transferred to 4°C to be chilled for at least 6 months. Post chilling, the cultures were incubated at 13°C for 14 days to induce hatching. Hatched L3 were isolated using a modified Baermann technique (Hendrix 1998; Oliver *et al*. 2014). Samples containing L3 were siphoned to 10 mL, after sedimentation overnight, and then stored at 4°C until they were processed for the identification of larvae.

4.3.3 Molecular diagnostics

Nematodirus larvae were identified using a PCR-based assay that identifies species-specific sequences in the ITS-2 region of rDNA (Bisset *et al*. 2014; Oliver *et al*. 2014). See Appendix 1 for the SOP. In brief, 32-48 individual larvae per treatment group were isolated into 0.2 mL strip tubes and lysed in a thermal cycler. Then a PCR assay was set up using 1µL of the lysed template DNA in a final volume of 10µL. Two generic (ITS2GF and ITS2GR) and four species specific primers (NEFIRV1, NESPRV1, OEVERV1 and CHOVFD2) were used (Table 4.1).

Table 4.1. Species specific and generic primers, with their associated sequences and product sizes used to identify larvae to species.

Target species	Primer	Sequence (5'-3')	Product size
N. filicollis	NEFIRV1	GGGATTGACTGTTACGATGTAA	162
N. spathiger	NESPRV1	CATTCAGGAGCTTTGACACTAAT	213
O. venulosum	OEVERV1	CGACTACGGTTGTCTCATTTCA	323-7
C. ovina	CHOVFD2	CAGCGACTAAGAATGCTTTGG	115-7
Generic	ITS2GFnest	CACGAATTGCAGACGCTTAG	370-398
Generic	ITS2GRnest	GCTAAATGATATGCTTAAGTTCAGC	

4.3.4 Calculations and statistical analyses

Estimation of resistance: An overall efficacy based on FEC for ABZ against *Nematodirus* spp. was calculated using Equation 4.1 (Presidente 1985):

$$FECRT\ (\%) = 100 \times (1 - (T_2/T_1) \times (C_1/C_2))$$

where T and C are the arithmetic means of the FEC for the treated and control groups and $_1$ and $_2$ denote pre- or post-treatment respectively. This overall efficacy was used as a covariate in the statistical analysis (see below).

After PCR, the proportion of larvae corresponding to each species was applied to the FEC to determine the number of eggs of each species present. The efficacy against each species was then calculated, again using Equation 4.1. Due to *Nematodirus* spp. producing few eggs (Crofton 1957), a threshold of a mean of 10 eggs/g for each species in the untreated samples was set, with those farms under this number not included for analysis. A reduction in eggs by less than 95% post treatment was considered resistant, as defined by Coles *et al*. (1992).

To determine if the proportion of *N. spathiger* and *N. filicollis* larvae changed as a result of treatment, a comparison of the proportion of larvae of each species recovered from the untreated and post-ABZ samples was undertaken. Initially, this was attempted using analysis of covariance and a model which included ABZ treatment as the predictor and the overall efficacy of treatment as a covariate. The latter was included to establish whether any changes in proportion were related to the level of resistance in the populations. However, despite a range of transformations the assumptions for ANOVA could not be met and so a permutation ANCOVA (Fisher 1935; Kabacoff 2011), which doesn't depend on these assumptions, was used utilizing the same model. Due to the two species comprising the proportion, the results show only *N. spathiger* as the analysis for *N. filicollis* is effectively the same.

In order to compare the proportion of farms with resistant *N. spathiger* and those farms with resistant *N. filicollis* a 2 proportion *t*-test was carried out using Minitab Statistical Software v 16.2.2.

4.4 Results

The larval cultures from all 21 farms (Appendix 2) were dominated by *N. spathiger*. On average 83% (range 46-100%) of the larvae recovered from the untreated group were *N. spathiger* and this increased significantly to 94% (range 45-100%) following treatment with ABZ ($p=0.03$). It follows then, that the proportion of *N. filicollis* declined significantly following treatment with ABZ, from 17% in the untreated to 6% in the treated ($p=0.03$). This implies the ABZ treatment was less effective against *N. spathiger* than against *N. filicollis*. This shift in proportions was not influenced by the efficacy of treatment based on the overall FECRT ($p=0.324$).

Based on a threshold criteria of 10 epg, 21 farms yielded a valid test for *N. spathiger*, of which 20 (95%) had an efficacy below 95% (Table 4.2; raw data shown in Appendix 2). Of these the efficacy was <50% for 35% (7/20) of tests and <10% for 15% of tests (i.e. 3 tests returned less than 10% efficacy. For *N. filicollis* there were 10 valid tests of which 4 (40%) had efficacies below 95% and of these the efficacy was <50% for only 1 of 4 farms. The lowest efficacy for *N. filicollis* was 41%. The proportion of farms on which resistance was detected in *N. spathiger* was significantly higher than the proportion showing resistance in *N. filicollis* ($p<0.05$).

4.5 Discussion

The aim of this study was to gauge the level of resistance to BZ anthelmintics in each of the two *Nematodirus* spp. studied. As expected, based on earlier surveys, *Nematodirus* species resistant to BZ anthelmintics were present on a high proportion of farms (Waghorn *et al*. 2006; Little *et al*. 2010; McKenna 2013). BZ-resistance in *N. spathiger* was almost universal, and was detected on all but 1 of the 21 farms tested. In contrast, resistance in *N. filicollis* was less common, being diagnosed on only 4 of 10 farms. As almost every farm had resistant *N. spathiger,* this presumably explains why the proportion of this species increased after treatment and also why the overall efficacy did not affect this shift.

Table 4.2. Efficacy of albendazole against *Nematodirus spathiger* and *Nematodirus filicollis* on 21 farms in New Zealand. FEC=faecal egg count; nq = not qualified.

Locality	Overall		*N. spathiger*		*N. filicollis*	
	Efficacy (%)	Proportion lambs positive post treatment (n/total treated)	FEC (eggs/g) in untreated lambs (post)	Efficacy (%)	FEC (eggs/g) in untreated lambs	Efficacy (%)
Ruatoria	0	0.6 (6/10)	40	0	0	nq
Pleasant Point [1]	0	0.4 (5/14)	36	0	0	nq
Owhango	10	0.7 (8/12)	58	12	0	nq
Tolaga Bay	26	0.4 (5/12)	25	26	0	nq
Gore	39	0.8 (9/12)	62	0	72	88
Pleasant Point [2]	48	0.4 (5/13)	113	45	7	nq
Rotorua	56	0.2 (2/12)	15	66	10	41
Pio Pio	61	0.4 (5/12)	36	45	35	77
Reporoa [1]	61	0.5 (6/12)	43	55	7	nq
Dannevirke	66	0.2 (3/15)	80	62	10	100
Reporoa [2]	75	0.2 (2/12)	29	75	0	nq
Eketahuna	75	0.1 (1/12)	22	74	1	nq
Te Anau [1]	75	0.3 (4/12)	25	54	25	97
Havelock North	81	0.1 (1/11)	164	79	14	100
Outram	85	0.3 (3/11)	150	85	0	nq
Palmerston North	88	0.4 (6/15)	153	88	153	89
Hastings	89	0.2 (2/12)	19	84	10	100
Culverden	93	0.3 (3/12)	29	86	29	100
Balclutha	93	0.1 (1/12)	69	93	3	nq
Te Anau [2]	93	0.2 (2/12)	105	93	13	100
Blenheim	100	0 (0/12)	69	100	2	nq

The severity of resistance (i.e. the percentage reduction in FEC) was broadly similar between the two species in the number of farms with an efficacy less than 50%. The lowest percent reduction for *N. filicollis* is similar to the reduction of 40.8% that was reported for the genus as a whole (Little *et al*. 2010) and in the national survey the genus had a range of efficacies between 0% and 90% (Waghorn *et al*. 2006), similar to that found in this study for *N. spathiger*. The large number (95%) of farms with BZ-resistant *N. spathiger* reported in the current study is similar to the levels of resistance found in earlier reports for the genus as a whole. This included a national survey which reported 89% prevalence (Waghorn *et al*. 2006) and a more recent report of 87% in consolidated diagnostic laboratory results (McKenna 2013). Earlier reports from diagnostic cases submitted to laboratories (McKenna 2010, 2012) reported a prevalence varying from 77-90% from the period 2004-2011. Taken together, these reports indicate a high prevalence of BZ-resistance in this genus. However, none of the reports differentiate between the two species studied here.

This is the first report in New Zealand that *N. filicollis* is resistant to the BZ class of anthelmintic, or indeed any anthelmintic class; in contrast, BZ-resistant *N. spathiger* was first reported in New Zealand over 30 years ago (Middelberg and McKenna 1983). Surprisingly, there are few reports of BZ-resistance in any species of *Nematodirus* elsewhere in the world. Those which do report resistance to the genus level only (Rosalinski-Moraes *et al*. 2007; Diez-Banos *et al*. 2008), although there are two reports of BZ-resistance in *N. spathiger* in Australia (Beveridge *et al*. 1990; Obendorf *et al*. 1991), and single reports from Algeria (Bentounsi *et al*. 2007) and Iraq (Mohamed and Al-Farwachi 2008). A search of the literature could only find one earlier report of BZ-resistance in *N. filicollis,* which was in South Australia (Beveridge *et al*. 1990). The related species, *N. battus,* which is not found in New Zealand, was only recently reported for the first time as being BZ-resistant in the United Kingdom (Mitchell *et al*. 2011). There is no obvious reason for the apparent difference between the high prevalence of BZ-resistant *Nematodirus* spp. in New Zealand compared to elsewhere in the world.

Nematodirus species have poor fecundity, with few eggs produced in relation to the number of adult worms present in the small intestine (Crofton 1957; Chalmers 1985). This has implications when using the FECRT to diagnose resistance, as the egg counts often do not reach a suitable threshold (minimum number) to allow interpretation.

Nevertheless, to date there has not been a more useful test to detect resistance in this genus with live animals. As there are difficulties in distinguishing the larval stages of the two species morphologically, most reports of resistance using the FECRT only report to the genus level.

Differences in the prevalence of BZ-resistance demonstrated between the two species in the present study might be related to differences in their epidemiology. Both species are widely distributed on farms throughout New Zealand, often occurring concurrently in mixed infections (Oliver *et al*. 2014) and so presumably they experience similar exposure to anthelmintic treatments. However, the development and hatching rates differ for *N. filicollis* and *N. spathiger* (Thomas 1959a) with the major difference being that the former species requires a period of chilling before hatching (van Dijk and Morgan 2009; Oliver *et al*. 2014). Because a high proportion of *N. spathiger* eggs hatch soon after they have completed development to L3 (Kates 1950; Kates and Turner 1955) the resulting larvae on pasture have the opportunity to be ingested by grazing lambs, and create a new generation of adult worms, within a few weeks of the eggs being shed. In contrast, the requirement for chilling means that *N. filicollis* eggs are likely to sit on pasture for long periods before hatching. Hence, while *N. spathiger* has the opportunity to complete multiple generations per year, this may not be the case with *N. filicollis*. In England, in a climate similar to New Zealand, it was concluded *N. filicollis* would have only one generation per year, occasionally two (Thomas 1959a), whereas *N. spathiger* may have five to six (Crofton 1963). It is clear from this work that both species are capable of developing resistance to BZ anthelmintics, and yet resistance is more common in *N. spathiger*. Although it cannot be determined without more work, it seems reasonable to speculate that resistance might be less common in *N. filicollis* because it has a much longer generation time (i.e. the build-up of resistant genes over generations of selection takes much longer) and/or the proportion of the population on pasture (i.e. in refugia) may be greater over much of the year.

Varying levels of resistance between different species within the same genus has been reported for other trichostrongylid genera. For example, it has been shown that within the genus *Trichostrongylus*, *T. colubriformis* exhibits a higher prevalence of resistance to BZ and levamisole anthelmintics than either *T. axei* or *T. vitrinus* (Waghorn *et al*. 2014). The results for *Nematodirus* and *Trichostrongylus* highlight the importance of studying individual species when assessing anthelmintic resistance. Moreover,

understanding the individual species biology and epidemiology and how this influences the development of anthelmintic resistance need to be considered. Barger (1999) believed that the most critical requirement for sustainable control of helminths is the comprehensive knowledge of their epidemiology. Therefore the ability to accurately identify a species is paramount, which recent use of molecular techniques has now made possible. With these methods, the resistance status of *N. spathiger* and *N. filicollis* should be investigated for the other anthelmintic classes.

4.6 Acknowledgements

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Chapter 5

Chilling requirements for hatching of a New Zealand isolate of *Nematodirus filicollis*

A-M.B. Oliver, W.E. Pomroy, S. Ganesh, D.M. Leathwick

5.1 Abstract

The eggs of some species of the parasitic nematode *Nematodirus* require a period of chilling before they can hatch; *N. filicollis* is one such species. This study investigated this requirement for chilling, in a New Zealand strain of this species. Eggs of *N. filicollis* were extracted from lambs faeces, incubated at 20°C to allow development to the third stage larvae within the egg. These eggs were then placed into tissue culture plates and incubated at: 2.7°C (±0.99), 3.6°C (±0.90), 4.7°C (±0.35), 6.4°C (±0.37), 8.0°C (±1.54) or 9.9°C (±0.14) for up to 224 days. At 14 day intervals until day 84, then every 28 days, one plate was removed per temperature and placed at 13.1°C (±0.44) for 14 days to allow hatching. Eggs were then assessed for hatching. From this data, chill units were calculated and the Gompertz model fitted. Even though hatching overall was low, a greater proportion of eggs hatched with chill accumulation. Eggs required 800-1000 chill units for maximum hatching. Consequently in the field, more than one season of chilling before hatching would be required. As such a generation time could take more than one year to complete. This is quite different to the hatching dynamics of *N. spathiger*, the other main species found in New Zealand sheep, which does not display this requirement for chilling and hatches immediately once the third stage larvae are developed.

KEYWORDS: *Nematodirus*; *filicollis*; *hatching*; *chill*

5.2 Introduction

Nematodes of the genus *Nematodirus* are important disease-causing parasites of lambs (Thomas 1959b), on occasions causing severe pathology and even deaths (Kates and Turner 1953; Thomas and Stevens 1956; Brunsdon 1961). A characteristic of this genus is that development to the infective third larval stage (L3) occurs within the egg, allowing them to persist on pasture for long periods (Thomas 1959b; Thomas and Stevens 1960). In addition, in some species, hatching occurs soon after the completion of development, while in others a period of chilling is required before the eggs will hatch (Thomas and Stevens 1960; van Dijk and Morgan 2008, 2009; Oliver *et al*. 2014).

New Zealand has two species of *Nematodirus*, *N. spathiger* and *N. filicollis*, which are commonly found infecting sheep. These species appear to have quite different population dynamics in that *N. spathiger* eggs hatch readily after the completion of development to the L3 (Kates 1950; Kates and Turner 1955), whereas *N. filicollis* eggs will not hatch, following the completion of development, without a period of chilling (Thomas and Stevens 1960; van Dijk and Morgan 2009). Better management of *Nematodirus* infection in lambs is likely to be assisted by a better understanding of their dynamics on pasture (Barger 1999) and yet little is known about the epidemiology of these parasites in New Zealand. To improve our understanding of the population dynamics of *N. filicollis* this study was conducted to define the chilling requirements necessary for eggs to hatch after the completion of development to the L3.

5.3 Materials and methods

5.3.1 Recovery of *Nematodirus* eggs

Faeces containing *N. filicollis* eggs were obtained from a pooled collection from approximately 150 naturally infected 4 month old lambs. Eggs were extracted from faeces within 6 hours of being collected using a tiered sieve method (Onyango-Abuje 1984). In brief, faeces were mixed into a slurry with tap water, and then passed through three sieves of decreasing pore size (150 μm, 100 μm and 64 μm). Eggs were retained on the 64 μm sieve. To obtain eggs with minimal faecal debris this was mixed with

saturated NaCl (sg 1.2) in a volumetric ratio of 1:14 to float the eggs. These were then recovered with a suspended 18.5 cm glass plate and were subsequently washed over a 64 µm sieve to remove the saline and stored in water.

5.3.2 Incubation of eggs

Eggs were incubated at 20°C, in a small amount of distilled water for 116 days to allow development to L3 and hatching of non *N. filicollis* eggs. The eggs in a suspension were then placed on a 40 µm sieve for 24 hours to remove any larvae that had hatched. Approximately 500 larvae were removed and 55 of these were identified by a PCR-based assay (see next page). These were predominantly other trichostrongyle species (69.1%) or *N. spathiger* (27.3%), with the residual 3.6% identified as *N. filicollis*. The remaining eggs were recovered into distilled water and 1 mL was dispensed into the middle four wells of a 24-well tissue culture plate (Falcon BD) delivering approximately 25 eggs into each well. Water was added to the unused wells of the plate to provide humidity and the lid placed on the culture plate.

5.3.3 Study design

The developed eggs were exposed to one of six constant temperatures i.e. 2.7°C (±0.99), 3.6°C (±0.90), 4.7°C (±0.35), 6.4°C (±0.37), 8.0°C (±1.54) and 9.9°C (±0.14). Temperatures were monitored (Squirrel 1000 data loggers) throughout the experiment. At every temperature and for each sampling interval, one plate was incubated with each well representing one replicate, giving four replicates per temperature. At intervals of 14 days up to day 84, then every 28 days until day 224 one randomly assigned plate was removed from each temperature and placed at 13.1°C (±0.44) for 14 days to allow hatching. The number of hatched L3 and the number of un-hatched eggs were then recorded for each well. At day 28, 100 µl of Amphotericin B was added to all the wells after fungi were found to be growing in some wells.

5.3.4 Identification of larvae

To confirm the hatched larvae were *N. filicollis* they were individually identified using a PCR-based assay that identifies species-specific sequences in the second internal transcribed spacer (ITS-2) of rDNA (Bisset *et al*. 2014; Oliver *et al*. 2014). See Appendix 1 for the SOP. In brief, larvae were lysed using 10 µL of lysis solution (1 mL Viagen Direct PCR (tail), Viagen Biotech Inc, Ca, USA and 0.25 mL of PCR grade Proteinase K recombinant, Roche, Basel, Switzerland) in a thermal cycler for 16 hours at 55°C, 1 hour at 90°C, and 10 minutes at 4°C, then placed at -80C for at least 30 minutes, followed by a 1:3 dilution with high performance liquid chromatography (HPLC) water.

The reaction mixture for the multiplex PCR assay consisted of four species specific primers and two generic primers (Table 5.1) that were added to: 1.5 mM $MgCl_2$, 1µL 10x *Taq* buffer, 100µM of each dNTP, and 0.25 units Platinum®*Taq* DNA polymerase (Life Technologies) and HPLC water. Lastly 1 µL of template DNA was added for a total volume of 10 µL. The thermal profile used for the PCR was: 95°C for 8 minutes, then 12 cycles of: 94°C for 15 seconds, 60°C reducing 0.5°C/cycle for 15 seconds, 72°C for 30 seconds; followed by 25 cycles of: 94°C for 15 seconds, 54°C for 15 seconds and 72°C for 30 seconds. There was an additional extension of 72°C for 7 minutes and cooling at 10°C for 10 minutes.

PCR products were electrophoresed through a 2% agarose-TAE (0.04M Tris-acetate, 0.001M EDTA, pH8.0) gel, containing SYBR Safe DNA gel stain (Life Technologies) and visualised using the electronic documentation program Gel.doc 2.6.4 (Bio-Rad) under ultraviolet trans-illumination conditions. The resulting bands (see Appendix 1, page 95 for example gel) were assigned to species based on their size (Table 5.1).

Table 5.1. Species specific and generic primers, with their associated sequences, the amount added to the reaction mixture and product sizes used to identify larvae to species.

Target species	Primer	Sequence (5'-3')	Amount added (pmol)	Product size (bp)
N. filicollis	NEFIRV1	GGGATTGACTGTTACGATGTAA	5	162
N. spathiger	NESPRV1	CATTCAGGAGCTTTGACACTAAT	3	213
O. venulosum	OEVERV1	CGACTACGGTTGTCTCATTTCA	2	323-7
C. ovina	CHOVFD2	CAGCGACTAAGAATGCTTTGG	3	115-7
Generic	ITS2GFnest	CACGAATTGCAGACGCTTAG	3	370-398
Generic	ITS2GRnest	GCTAAATGATATGCTTAAGTTCAGC	3	

5.3.5 Statistical analysis

In order to fully evaluate the effect of different times and temperatures on egg hatching, a variable designated as chill units (CU) was calculated, using

$$CU = (11°C - t)d,$$

where t is temperature and d is number of days of storage at that temperature. The constant 11°C was chosen as it approximates the threshold for development within the egg (van Dijk and Morgan 2009).

The proportion of eggs hatching was averaged over the four replicate wells of each plate and the natural log of this mean plotted against chill units. A series of weighted non-linear regression models were then evaluated for goodness of fit. The weights were defined as 1/standard deviation over the four replicates. Two values were removed from the analysis as the proportion values for all four replicates were zero. Of the models tested, the Gompertz model, defined as:

$$y = Ae^{Bx^C},$$

gave the best fit and so the asymptote and the pseudo R-squared (measure of goodness of fit) were calculated. All statistical analyses were carried out using the R software (R Core Team, 2014).

5.4 Results

The majority (approximately 75%) of *N. filicollis* eggs did not hatch, even after 224 days at temperatures between 2°C and 10°C (see Appendix 2 for raw data). At the end of the study the majority of the larvae within these unhatched eggs were motile and so must be considered viable.

There was a trend for eggs to hatch with longer exposure to all temperatures used and for a higher proportion of eggs to hatch after exposure to the lower temperatures (Figure 5.1). When temperature and time were combined into chill-units the relationship with hatching was curvilinear and asymptotic (Figure 5.2). The asymptote was 0.11 hatching (back-transformed from the Ln scale; 95% CI=0.0628 - 0.1929) with a pseudo r-squared value of 56.7%. This relationship indicates that eggs accumulated chill-units faster at lower temperatures, and that about 800-1000 CU were required before the maximum proportion of eggs hatching was achieved. The majority (> 99%) of larvae identified after hatching were *N. filicollis*.

5.5 Discussion

The aim of this study was to define the chilling requirements for hatching of *N. filicollis* eggs after they have completed development to L3. Hatching increased with the accumulation of chill units in a curvilinear relationship but with an asymptote at about 11% egg hatch i.e. the majority of eggs did not hatch regardless of the amount of chilling. Amalgamating time and temperature into chill units is a concept previously used in plant science (Alburquerque *et al*. 2008; Rose and Cameron 2009; Kuden *et al*. 2013) and is analogous to the degree-day concept often used in invertebrate phenology studies (Nealis *et al*. 1984; Lobinske *et al*. 2002; Lewis *et al*. 2003). Although the data is quite variable, fitting chill units to the data helped consolidate the results from different times and temperatures.

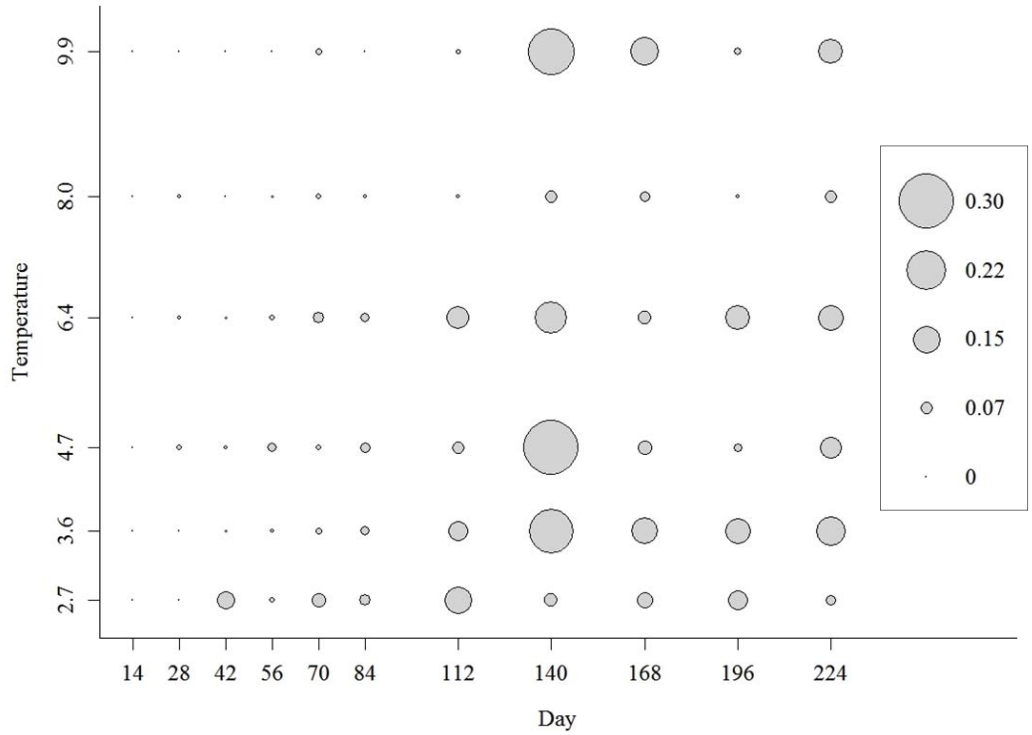

Figure 5.1. The proportion of *N. filicollis* eggs hatching (shown by size of circles) at 13.1°C (±0.44), after being incubated to the third stage larvae at 20°C and then exposed to 2.7°C (±0.99), 3.6°C (±0.90), 4.7°C (±0.35), 6.4°C (±0.37), 8.0°C (±1.54) or 9.9°C (±0.14) for various time periods between 14 and 224 days.

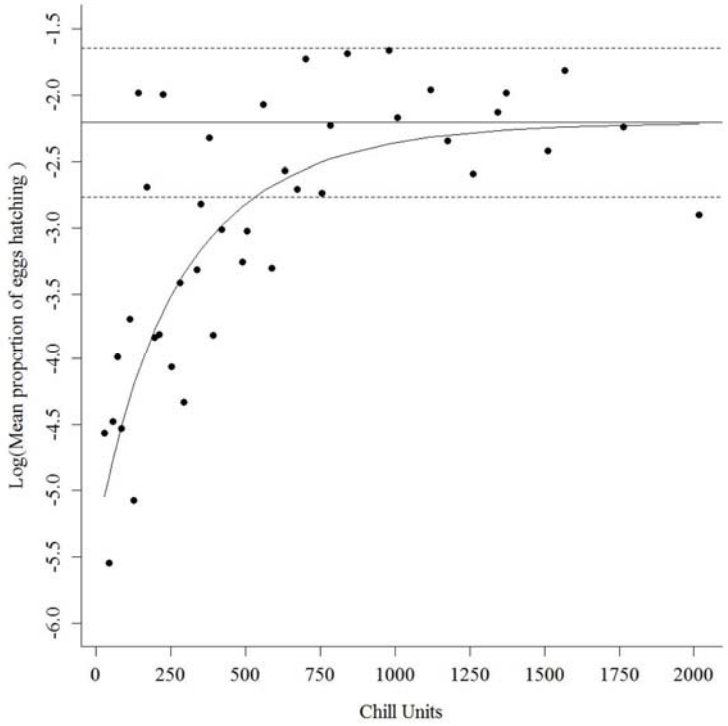

Figure 5.2. The log of mean proportion of eggs hatching as chill units increase, including the line of best fit from the Gompertz model.

Based on the fitted relationship, it would require about 1000 days at 10°C to reach maximum egg hatch whereas at 2°C this would be achieved in 111 days. An anomaly in the data is that while there was consistently low egg hatch after storage of eggs at 8°C, some of the samples stored at 10°C showed relatively high egg hatch (Figure 5.1). This is out of context and it is unclear why more eggs would hatch after storage at 10°C than at 8°C. It was, however, noted that there appeared to be more fungal contamination in some of the wells exposed to 8°C and this may have influenced the number of larvae hatching.

A similar study, using a UK isolate of *N. filicollis*, also found that eggs would not hatch without chilling, and documented that chilling only occurred below 10°C, with a stronger response towards 4°C (van Dijk and Morgan 2009). In this study nearly 75% of eggs hatched following only 33 days of chilling at 4°C (van Dijk and Morgan 2009), which equates to about 231 CU in our calculations. However, in another study, after chilling at 3°C for 6 months, only 35% of eggs hatched (Thomas and Stevens 1960). Also, Christie (1962) recorded only 10% of developed eggs hatching after 11 weeks at 9°C. However, it appears that these eggs were not incubated at a higher temperature to facilitate hatching. Therefore, there appears to be considerable variation in hatching of chilled eggs in different studies, and it is unclear whether this reflects differences in the experimental conditions or differences between isolates.

Extrapolating our results into the field suggests that this New Zealand isolate of *N. filicollis* requires a large number of chill units in order to release the eggs for hatching. In some years this could mean that more than one season of chilling would be required to accumulate enough chill units for the eggs to hatch, particularly given the relatively short and mild winter climate that New Zealand can have in some regions. This suggests that *N. filicollis* from different regions of New Zealand could have quite different hatching patterns. In the warmer north hatching of a single cohort of eggs may be more protracted, occurring over two years or more, while in the colder south and/or at higher altitudes hatching may take less time and be more compressed. Consequently the generation time of this species could be longer than one year, at least for a portion of the population.

However, this suggestion does not concur with earlier New Zealand studies of larvae on pasture which concluded that one or more generations could occur each year (Brunsdon 1963; Vlassoff 1973). Both these studies found peaks of *N. filicollis* L3 in spring and autumn. In Brunsdon's (1963) study, which utilized pasture that had not been grazed for two years prior to the experiment, a substantial number of *N. filicollis* larvae were recovered at all times of the year, with large peaks in autumn. It was concluded these larvae predominantly came from the spring/summer deposition, with the small spring peak a result of larvae surviving over winter from the previous autumn, with only a small number of these larvae hatching from eggs that had overwintered. While, this picture would be consistent with the development of *N. spathiger*, which can hatch immediately after development is completed within the egg, it does not fit with the need for *N. filicollis* eggs to experience chilling before they will hatch (Thomas and Stevens 1960; van Dijk and Morgan 2009; Oliver *et al*. 2014).

Based on this and other studies the dynamics of *N. filicollis* and *N. spathiger* are likely to be quite different. Because *N. spathiger* does not require chilling for the eggs to hatch (Thomas and Stevens 1960; Brunsdon 1963; Crofton 1963) generation times can be short and, under suitable conditions, reinfection can occur soon after eggs are deposited on pasture (Kates 1950). In contrast, because *N. filicollis* has a requirement for chilling before eggs will hatch (Thomas and Stevens 1960; van Dijk and Morgan 2009; Oliver *et al*. 2014), eggs deposited on pasture require both temperatures suitable for development to L3 (>11°C, van Dijk and Morgan 2009) and for the accumulation of chill units, before reinfection can occur. Under conditions typical of some regions in New Zealand this might require more than one year to achieve. This could have consequences for the evolution of anthelmintic resistance. Resistance to the benzimidazole class of anthelmintics in New Zealand is more common in *N. spathiger* than in *N. filicollis* (Oliver *et al*. unpub. see Chapter 4) which would be consistent with a longer generation time of the latter species, and also the possibility of a larger proportion of the population being on pasture i.e. unexposed to treatment.

In summary this New Zealand isolate of *N. filicollis* required a long period of chilling for eggs to hatch and even then the level of hatching was low. This has implications in the field, where it is possible one generation could take two years or more depending on

the exposure to chilling. This is different to the immediate hatching of *N. spathiger* eggs, which consequently has the potential for several generations per year.

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Chapter 6

General Discussion

The overarching aim of this thesis was to study the dynamics of *N. spathiger* and *N. filicollis,* the two commonly found *Nematodirus* species in New Zealand lambs.

Nematodes of the genus *Nematodirus* are an important component of the parasite burden of sheep in New Zealand, being both common (Pomroy 1997; Vlassoff and McKenna 1994) and pathogenic at relatively low numbers (Brunsdon 1967; Vlassoff and McKenna 1994; Anonymous 2010). In addition, the genus occurs frequently in reports of resistance to one or more classes of anthelmintic (Waghorn *et al*. 2006; McKenna 2013), with the prevalence of resistance reported as being between 87-89% to BZ, 11-25% to levamisole and 7-9% to the macrocyclic lactones. Recent research on the epidemiology of gastro-intestinal parasites in New Zealand has focused on other species such as *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* (Leathwick *et al*. 2011; Waghorn *et al*. 2011; Reynecke *et al*. 2011). The little research that has been conducted on the dynamics of *Nematodirus* spp. in New Zealand was undertaken approximately 40-50 years ago (Brunsdon 1960, 1961, 1962, 1963; Vlassoff 1973). Given the potential pathogenicity of the genus, its frequent occurrence in infections and its obvious ability to develop resistance to anthelmintics, then it is surprising that so little is known of its dynamics in this country.

The early studies recorded that infective larvae of *Nematodirus* spp. were largely present on pasture throughout the year but that the two species differed somewhat in their seasonal patterns. *N. filicollis* tended to have two seasonal peaks, a small spring rise and a more definitive one in autumn, while *N. spathiger* peaked from mid-summer to autumn with few larvae present through winter and spring. Also both species were widely distributed throughout both the North and South Islands, with *N. filicollis* dominating infections more often in the North Island than *N. spathiger*; while in the South Island the two species had similar levels of dominance in infections (Brunsdon 1961).

Despite these findings, over the last decade or more, there has been a consistent perception that *Nematodirus* infection in lambs is predominantly a South Island problem, and further, that *N. filicollis* only occurs in the South Island (Pomroy 2010).

The results of this study (Chapter 3) clearly demonstrate that this is not the case with both species being present in the majority of samples irrespective of which part of the country they came from. It seems likely, given the findings of Chapter 5, that this perception may have been reinforced because *N. filicollis* eggs do not hatch without chilling and so are seldom seen in routine laboratory faecal cultures. Hence, even if present, *N. filicollis* larvae would not be seen under routine diagnostic tests. Further, very few diagnostic worm counts are undertaken when investigating clinical problems.

The approach used to conduct the prevalence and anthelmintic resistance studies (Chapters 3 and 4) utilized the recent developments in DNA technology which enabled the accurate identification of L3 from faecal cultures (Bisset *et al*. 2014). This enabled a sampling programme which involved more farms than would have been possible using identification of adult worms recovered from slaughtered lambs. The use of the PCR-based assay to determine the presence of *Nematodirus* on farms for either survey or resistance purposes had not been utilized previously in New Zealand. The assay was also used in identifying *Trichostrongylus* spp. for the same purposes (Waghorn *et al*. 2014). These studies highlight the usefulness that molecular methods bring to the research of nematodes.

Surveys indicate that BZ-resistance is common in *Nematodirus* in New Zealand but no studies conducted in the last 15 years have determined the species involved and so we had no real idea whether this is restricted to one species or a species complex. Therefore, in Chapter 4 the BZ-resistance status of *N. filicollis* and *N. spathiger* was measured. Results showed that both species had efficacies below 95% for albendazole in some tests and were therefore considered resistant on those farms, but this occurred more often in *N. spathiger* than *N. filicollis*. The confirmation that *N. filicollis* is resistant was not unexpected, given the length of time since BZ-resistance in *N. spathiger* was first reported and that numerous other helminth species also show resistance to the BZ class (Waghorn *et al*. 2006). What was perhaps unexpected was that *N. filicollis* did not have the same prevalence of BZ-resistance as *N. spathiger*. Given that both species were similarly distributed on farms throughout the country, they are presumably given a similar level of exposure to the anthelmintic and it might therefore be expected they would have a similar prevalence of resistance.

One possible reason for differences in levels of resistance would be if there were large differences in their population dynamics of the species. This was indicated by the finding (in Chapters 3 and 5) that *N. filicollis* has an almost obligate requirement for chilling before eggs will hatch whereas *N. spathiger* does not. If *N. filicollis* had fewer generations per year than *N. spathiger* then this could result in less exposure to anthelmintics and less opportunity for the reassortment of resistance genes through mating, thereby at least partially explaining the observed differences. Overall it is obvious BZ-resistance is common in the genus, with almost all farms testing positive for resistant *N. spathiger*. Also given that 40% of farms tested positive for resistant *N. filicollis*, clearly indicating the potential of the species to become resistant, then it would seem reasonable to expect that given more time BZ-resistance in *N. filicollis* will eventually become ubiquitous on farms in New Zealand. With the additional reports of levamisole and ML resistance in *Nematodirus* – though at a reduced level at this point, (Waghorn *et al*. 2006; McKenna 2013) the anthelmintic resistance status of these parasite should be of concern.

The requirement for chilling before eggs of *N. filicollis* will hatch was known in the scientific literature but details of how long eggs need to be held at different temperatures was not. In Chapter 5 we applied a novel technique (seldom applied to parasites) of calculating the accumulated 'chill-units' necessary for eggs to hatch. This is equivalent to the technique of accumulation of degree-days, often used in insect and plant studies, but it is in reverse. This showed that the hatching of *N. filicollis* eggs increased with the number of chill units to which they had been exposed, even though a large number of eggs remained unhatched. Rather unexpected was the large number of chill units required for hatching, with 800-1000 CU needed for maximum hatching. Given this result it would seem likely that eggs of *N. filicollis* would require up to one or even more seasons of chilling in some regions before eggs will hatch on pasture and this is likely to mean fewer generations per year than for *N. spathiger*. This result supports the supposition outlined above that a possible reason for a lower incidence of BZ-resistance in *N. filicollis* is linked to its epidemiology through a longer generation time than *N. spathiger*.

In summary, this study has advanced our knowledge of the biology of these important parasites in New Zealand. The widely-held perception that *N. filicollis* is only found in the South Island of New Zealand was not supported by the results, which showed

instead that both species were present sympatrically throughout most of the country, as described previously (Brunsdon 1961). Despite this, the prevalence of BZ-resistance in *N. filicollis* was lower than the prevalence in *N. spathiger*. A possible explanation for this is the longer generation time that *N. filicollis* has, which directly reflects the large amount of chilling required prior to hatching. This research into the two *Nematodirus* species has shown the importance of studying individual species as opposed to just the genera. It is essential to understand the individual species biology and epidemiology as these can affect other areas of parasitological interest, such as the development of anthelmintic resistance.

Chapter 7

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Chapter 8

Appendices

Appendix 1 Standard Operating Procedures

SOP 1: Modified McMaster faecal egg counting method

Purpose: This method is used to determine the number of gastro-intestinal parasite eggs in sheep faeces.

Materials and equipment:
2g sample measurer/dispenser
Trays and Vials (60 mL)
28 mL plastic vial
Electric Mixer with interlacing mixing head
Saturated saline solution
Hydrometer
Fine strainer (a tea strainer has fine mesh)
Small bowl (approximately 200 mL)
Teaspoon
Transfer pipette (3 mL)
3 chamber counting slide (McMaster)
Tray to hold slides (clean meat tray)
Microscope
Mechanical counter
Pen
Recording sheet
Rubber Gloves
Tissues

Procedure:
Wear gloves throughout this process.
Sort the samples into numerical order (if applicable) and record the numbers on the record sheet.
Set out the trays and vials on the bench depending on how many samples there are. Then measure out two grams of each sample and place into the 60 mL vial.
Test saturated saline with hydrometer and adjust to 1.2 sg.
Fill the 28 mL vial with saturated saline solution (1.2 sg) and add to the vial containing the faeces.
Using the electric mixer, mix thoroughly and pour the slurry over the fine sieve so the liquid containing the nematode eggs is caught in the small bowl and the faecal debris is caught in the sieve. Crush any lumps through the sieve using a teaspoon and rinse teaspoon.
Discard the debris in the sieve. Place required number of counting slides onto a tray.
Stir the solution in the bowl from side to side, not round and round with a clean teaspoon and remove a transfer pipette full of solution whilst still mixing.

Hold pipette in a near horizontal position and place the opening of the pipette against the opening of the counting slide and fill the slide in one smooth flow, avoiding air bubbles. Repeat these steps until required number of samples are completed.
Allow slide to stand for two minutes before counting so eggs can float to the top surface.
Using a compound microscope, count either, top or bottom edge, and one side of the slide as well as the middle including any eggs visible under the lines.
Multiply the number of eggs by 50 to get the total number of eggs per gram (epg).
Record the result on the appropriate sheet.
Clean all utensils with water.

Notes:

This multiplication factor is obtained in the following manner

2g faeces + 28 mL saline = 30 mL

30 mL / 0.3 mL size of slide chamber = 100 eggs per 2 grams

100 eggs per 2 grams = 50 epg

Hence multiply the number of eggs seen by 50 to get the number of epg

The saline solution should be made from common salt, not iodised or table salt as these have additives. The density or specific gravity of the saline should be 1.2.

Do not stand the samples in saline overnight, as we don't know what effect this has on the eggs. Store all samples at 4°C as this stops eggs hatching.

SOP 2: Gastro-intestinal nematode larval culture and identification method

Purpose: This method is used to prepare larval cultures to obtain gastro-intestinal parasite larvae from ruminant faeces and identify the resultant larvae.

Materials and equipment:

Larval culture

Faecal sample
Plastic container with lids
Medium grade vermiculite
Spatula/spoon
Control temperature room or incubator at 22°C*[1]
Deionised water
Gloves
Marker pen

Baermann technique

Glass Funnel with rubber tube attached
Clamp
Sieve (approximately 2 mm mesh)
Stand for funnel
Tissue*[2]
Water (tap is suitable)
Beaker
50L Centrifuge tube and lid
Stand for centrifuge tube
Siphon
Adhesive labels
Pen

Identifying larvae

Microscope
Eyepiece micrometre
Glass chamber
Pipette
Lugol's iodine

Procedure:

Larval culture

To avoid cross-contamination isolates should be cultured separately and cleanliness should be a high priority.
Wear gloves.
Take faeces and place in a plastic container.
Add vermiculite to the faeces. Mix thoroughly using the spoon/spatula
Add water if culture is particularly dry. The mixture should be crumbly and when squeezed firmly a small amount of excess moisture should be present.

Place the lid on top of the container but do not make the container airtight.
Label the outside of the plastic container with the isolate, date cultured, date due out of culture, using an indelible marker pen.
Place in control temperature room at 22°C for 10 days*[1]
Stir culture every 2-3 days to allow aeration. Add water when necessary to maintain the desired consistency and humidity, faeces should be kept moist but not soggy.

Baermann technique
After 10 days in the controlled temperature room, larvae should be present.
A glass funnel with attached rubber tubing should be placed in a stand so that the bottom of the rubber tubing is about 10-15 cm from the bench top.
Close off the rubber tubing with the clamp.
Place the coarse sieve inside funnel and add tap water using a beaker so that the bottom of the sieve is just submerged.
Split leaves of tissue and line the inside of the sieve with one leaf of tissue to act as a filter.*[2]
Gently place cultured faeces on the tissue-lined sieve, no deeper than 2 cm.
Add more tap water until the faeces is just covered.
The sample is left in the funnel for at least 12 hours, (usually overnight).
The clamp is released slightly to allow the liquid containing the larvae to flow into the labelled 50 mL centrifuge tube and is closed once the centrifuge tube is full. The tube is capped with the lid, and placed in the tube rack at 4°C overnight. This allows larvae to settle to the bottom of the tube before the liquid is siphoned to 10 mL.

Identifying larvae morphologically
Place 1 mL of the resulting sample, into a glass chamber slide, using a pipette. Add a drop of Lugol's iodine to kill and stain the larvae. Place the chamber slide on the microscope and examine under 100 x magnifications. An eyepiece micrometre is used to measure the length of the larvae and/or features of the larvae that can distinguish it to genus and sometimes species.

*Modifications for culturing *Nematodirus* larvae
1. *Nematodirus* spp. were cultured at the lower temperature of 20°C as this had been shown to be optimal.
2. The tissue lining was not used when extracting *Nematodirus* larvae, as their long sheath tails can get caught in the tissue.

SOP 3: Method for preparation of larval lysates

Purpose: To prepare third stage larvae for identification by PCR.

Materials:
Small Petri dish with sample of larvae in
Hypodermic needle with eyelash attached to pick out worms
70% Ethanol
0.2 mL thin wall strip tubes and lids
Dissecting Microscope
Viagen DirectPCR (Tail)
Roche Proteinase K (recombinant)
MQ sterile water
Pipettes (1 mL and 100 µL)
Mastercycler PCR machine
Freezer -80°C

Method:
Add one small drop of 70% Ethanol to each of the 0.2 mL thin wall tubes.
Transfer individual larvae using eyelash 'worm picker' to a tube, ensuring there is one larva in each.
Store at 4°C until the 96 well tray is full.
Once tray is complete remove lids from tubes allowing ethanol to evaporate.
Prepare lysis solution (for 96 larvae) in a 1.5 mL centrifuge tube:
Add 500ul Viagen DirectPCR (Tail) (AB-102-T)
to 500ul sterile MQ water
and 25ul Roche Proteinase K (recombinant).
Mix thoroughly then spin down in a benchtop centrifuge for 20 seconds.

Dispense 10ul aliquots of lysis solution into 0.2 mL thin-wall strip tubes.
Replace tube lids.
Incubate tubes in PCR cycler at 55^{o}C for 16 hours, 90^{o}C 1 hour, 4^{o}C 1 minute.
Freeze lysates (-80^{o}C) until required. (Freezing seems to improve PCR of template by disrupting cellular structures).
Dilute lysates 1 in 3 (add 20ul of PCR water and vortex).
Store at -20^{o}C.

SOP 4: Protocol for PCR identification of parasitic larvae

Purpose: To identify gastro-intestinal larvae to species using a molecular approach that is PCR-based.

Materials and method:

Prepare 1.5 mM $MgCl_2$ Master-mix for PCR assay (enough for 200x 10 µl reactions) (mix thoroughly) (can be frozen for several weeks if necessary):

Mastermix
1030 µl HPLC water
200 µl 10x Buffer
60µl $MgCl_2$
100 µl dNTPs (4 mM dNTP stock: 40ul of each 100 mM stock plus 840 µl HPLC water (store at -20°C)
10 µl Platinum Taq

Set up multiplex PCR assay mix (96x 10 µl reactions):

700 µl 1.5 mM Master-mix
30 µl ITS2GFnest
30 µl ITS2GRnest
20 µl OEVERV1 [= *Oesophagostomum* specific primer]
50 µl NEFIRV1 [= *Nematodirus filicollis* specific primer]
30 µl NESPRV1 [= *Nematodirus spathiger* specific primer]
30 µl CHOVFD2 [= *Chabertia ovina* specific primer]
10 µl HPLC water

Dispense 9 µl aliquots into 0.2 thin-wall strip tubes and add 1 µl template to each (i.e. diluted larval lysate); mix; spin down; place strip tubes in Mastercycler PCR machine.

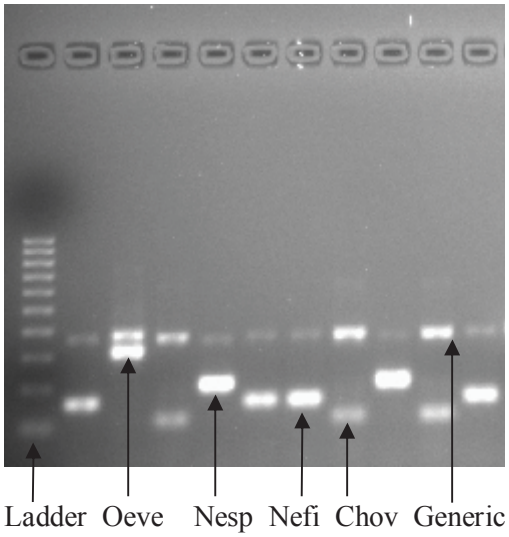
PCR Program

Hot-start Taq activation – 95°C for 8 minutes
Denature – 94°C for 15 seconds
Anneal – 60°C for 15 seconds
Extend – 72°C for 30 seconds
No of cycles – 12 (annealing temp reducing 0.5°C/cycle)
Denature – 94°C for 15 seconds
Anneal – 54°C for 15 seconds
Extend – 72°C for 30 seconds
No of cycles – 25
Additional extension – 72°C for 7 minutes
Cool – 10°C for 1 minute
Note: Higher annealing temperature to start is to increase specificity, then the lower temperature to greatly increase "copy number" (Run time 1.5 hours)

Prepare a 2% agarose gel to visualise PCR products [180 mL TAE /3.6g agar/18ul SYBR green] with 4 x 26 tooth combs.

Load reactions into wells 2-25 for each comb; Add DNA ladder in wells 1 and 26
Run gel for 1.2 hours at 130V and then scan gel in transilluminator, under UV light.

Examples of Assays



Abbreviations are: Oeve - *O. venulosum*, Nesp - *N. spathiger*, Nefi - *N. filicollis*, Chov - *C. ovina*

Primer Sequences

Target species	Primer	Sequence (5'-3')	Product size
N. filicollis	NEFIRV1	GGGATTGACTGTTACGATGTAA	162
N. spathiger	NESPRV1	CATTCAGGAGCTTTGACACTAAT	213
O. venulosum	OEVERV1	CGACTACGGTTGTCTCATTTCA	323-7
C. ovina	CHOVFD2	CAGCGACTAAGAATGCTTTGG	115-7
Generic	ITS2GFnest	CACGAATTGCAGACGCTTAG	370-398
Generic	ITS2GRnest	GCTAAATGATATGCTTAAGTTCAGC	

SOP 5: Faecal nematode egg count reduction test

Purpose: This method is used to determine the efficacy of the anthelmintics in reducing the worm burdens in lambs.

Materials:

30 mL plastic vial with lid
60 mL vials with lid
Gloves
Anthelmintics
Syringes
Marker pen
Field record sheet
Dose rate sheet

Method:

At weaning select 60-70 well grown lambs of even body weight that have not previously been drenched.
Run as one mob close to the yards for easy availability.
Collect 10 fresh faecal samples, off the ground will do, and submit to laboratory for analysis to determine levels of infection by way of faecal egg count.
When the average egg count reaches at least 700 eggs per gram and there are no zero samples, testing can proceed.
Bring lambs in fresh off pasture and draft randomly into 5 groups of 12 animals. For example the first 5 lambs off the race allocated to 5 groups, the next 5 animals are similarly allocated until each group contains 12 lambs. DO NOT allocate the first 12 lambs to one group.
All lambs need to be individually identified, and a record kept of the tag, liveweight, treatment and dose volume for each lamb on the supplied form headed Drench Test Field Sheet. This form must be returned with the pre samples.
Work on one group at a time: tag lambs, rectal faecal sample, weigh and record weight, check chart for dose volume and record this, administer correct dose volume carefully.
Rectal faecal samples are taken from each lamb. Each container needs to be uniquely identified. Label both vial and lid with tag number and treatment group. Samples must be obtained from all lambs. Re-sample non givers at the end, or after lunch. This is extremely important, as missed animals will downgrade the value of the test.
A faecal sample must be 5 grams for Pre samples and as much as possible for the post drench sample to allow for egg count and culture. The quantity is important as it becomes a major problem if there are insufficient faeces to enable cultures to be completed.
Treat each lamb at the dose rate recommended for its individual liveweight from the dose rate chart included. Use the syringe provided and a clean syringe for each drench type.

Treatment groups

GROUP	TREATMENT	No. of LAMBS	DOSE RATE
1	Untreated	12	
2 (Albendazole)	Albendazole	12	1 mL/5kg
3 (Nilverm)	Levamisole	12	1 mL/5kg
4 (Arrest)	Alb/Lev combination	12	1 mL/5kg
5 (Ivomec)	Ivermectin	12	1 mL/4kg

Lambs will be run together for a further 7-10 days, before post drenching rectal faecal samples will be taken. Remember, as much faeces as possible for this sample. Remember to label lids and vials.

For post drench sampling, draft lambs into the treatment groups. This enables the collection of samples from only one group at a time and thus minimises the risk of cross-contaminating faecal material. Where this is not practical, endeavour to keep your gloves as clean as possible (wipe on sheep or rinse in water) between animals. This minimises the risk of cross-contaminating eggs in faecal material.

After post drench sampling, all lambs should be drenched with an effective product and returned to the flock.

All samples must be kept cool, but not frozen, and couriered overnight to the laboratory.

Appendix 2 Supplementary Data

Chapter 3 Supplementary data:

Date faeces collected from lambs on the 50 North and South Island farms for the *Nematodirus* distribution study and the number of larvae identified to *N. spathiger* and *N. filicollis* using the PCR assay in the unchilled and chilled samples of the faecal cultures and the total for each species.

Island[1]	Farm Locality Date	Data[2]	*N. spath*[3] unchilled	*N. filicollis* unchilled	Total unchilled	*N. spath* chilled	*N. filicollis* chilled	Total chilled	Total *N. spath* chilled + unchilled	Total *N. filicollis* chilled + unchilled	Total chilled + unchilled	*N. spath* total present (1)/ absent (0)	*N. filicollis* total present (1)/ absent (0)
N	Tararua 4/11/2010	a	1	3	4	2	61	63	3	64	67	1	1
S	Darfield 9/11/2010	a	61	0	61	83	0	83	144	0	144	1	0
S	Pleasant Point 12/11/2010	a	28	0	28	30	14	44	58	14	72	1	1
S	Otautau 19/11/2010	a	18	12	30	17	35	52	35	47	82	1	1
N	Palmerston North 19/11/2010	a	10	9	19				10	9	19	1	1
S	West Tuapeka 19/11/2010	a	31	0	31	31	16	47	62	16	78	1	1
S	Sth Otago 19/11/2010	a	21	7	28				21	7	28	1	1
S	Oamaru 24/11/2010	a	20	0	20	30	0	30	50	0	50	1	0
S	Gore 24/11/2010	a	19	0	19	20	20	40	39	20	59	1	1
N	Rotorua 26/11/2010	a	16	1	17	19	25	44	35	26	61	1	1
N	Manawatu 2/12/2010	a	1	3	4	1	17	18	2	20	22	1	1
S	Ashburton 2/12/2010	a	0	2	2	12	2	14	12	4	16	1	1
S	Te Anau 22/12/2010	a	11	0	11	8	32	40	19	32	51	1	1
S	Te Anau 22/12/2010	a	9	5	14				9	5	14	1	1
S	Oamaru 22/12/2010	a	29	1	30	42	6	48	71	7	78	1	1
S	Balclutha 22/12/2010	a	4	0	4	17	7	24	21	7	28	1	1
S	Te Anau 22/12/2010	a	2	2	4	2	3	5	4	5	9	1	1
S	Te Anau 22/12/2010	a	0	0	0	2	1	3	2	1	3	1	1
S	Te Anau 22/12/2010	a	34	0	34	35	11	46	69	11	80	1	1
S	Te Anau 23/12/2010	a	21	2	23	34	14	48	55	16	71	1	1
N	Reporoa 23/12/2010	a	24	2	26	36	9	45	60	11	71	1	1
N	Ongaonga 29/12/2010	a	2	28	30	9	19	28	11	47	58	1	1
N	Dannevirke 8/01/2011	b	6	0	6	25	3	28	31	3	34	1	1
S	Balclutha 17/01/2011	a	13	13	26				13	13	26	1	1
S	Balclutha 18/01/2011	b	28	0	28	45	2	47	73	2	75	1	1
N	Marton 21/01/2011	a	1	0	1	5	4	9	6	4	10	1	1
N	Rotorua 25/01/2011	b	8	3	11	2	44	46	10	47	57	1	1
N	Marton 25/01/2011	b	31	0	31	47	0	47	78	0	78	1	0
N	Owhango 25/01/2011	b	18	1	19	32	16	48	50	17	67	1	1
N	Marton 28/01/2011	b	30	0	30	14	0	14	44	0	44	1	0
S	Palmerston 4/02/2011	b	19	0	19	45	0	45	64	0	64	1	0
S	Palmerston 4/02/2011	b	21	8	29				21	8	29	1	1
N	Waipukurau 16/02/2011	b	29	0	29	22	2	24	51	2	53	1	1
N	Reporoa 16/02/2011	b	26	0	26	22	0	22	48	0	48	1	0
S	Te Anau 16/02/2011	b	27	0	27	41	4	45	68	4	72	1	1

Island[1]	Farm Locality Date	Data[2]	*N. spath*[3] unchilled	*N. filicollis* unchilled	Total unchilled	*N. spath* chilled	*N. filicollis* chilled	Total chilled	Total *N. spath* chilled + unchilled	Total *N. filicollis* chilled + unchilled	Total chilled + unchilled	*N. spath* total present (1)/ absent (0)	*N. filicollis* total present (1)/ absent (0)
S	Omarama 18/2/2011	b	21	0	21	39	2	41	60	2	62	1	1
S	Oamaru 18/02/2011	b	27	0	27	26	7	33	53	7	60	1	1
N	Hastings 23/02/2011	b	14	1	15	32	0	32	46	1	47	1	1
S	Hyde 2/03/2011	b	27	0	27	33	8	41	60	8	68	1	1
S	Omarama 2/03/2011	b	2	0	2	37	2	39	39	2	41	1	1
S	Moeraki 2/03/2011	b	3	0	3	4	0	4	7	0	7	1	0
N	Rangitikei 8/03/2011	b	27	0	27	35	4	39	62	4	66	1	1
S	Oamaru 10/03/2011	b	3	0	3	10	0	10	13	0	13	1	0
N	Alfredton 10/03/2011	b	20	0	20	34	4	38	54	4	58	1	1
N	Alfredton 10/03/2011	b	21	0	21	45	0	45	66	0	66	1	0
N	Alfredton 10/03/2011	b	0	0	0	2	0	2	2	0	2	1	0
N	Eketahuna 21/03/2011	b				21	1	22	21	1	22	1	1
N	Mt Bruce 22/03/2011	b	7	0	7	2	3	5	9	3	12	1	1
N	Alfredton 5/04/2011	b	1	0	1				1	0	1	1	0
S	Outram 21/03/2011	b				47	0	47	47	0	47	1	0

[1.] N= North Island S= South Island, New Zealand.

[2.] Samples labelled 'a' and 'unchilled' had actually had short periods of chilling – they had been kept at 20°C then 4°C for varying periods of time while it was determined what conditions *N. filicollis* required to hatch. Samples labelled 'b' are as described. Data from 'a+b' were used for the 50 farms tested for presence/absence of *N. spathiger* and *N. filicollis* on New Zealand farms. Only data from samples labelled 'b' were used for the numbers of L3 recovered from unchilled and chilled cultures (Chapter 3 results, paragraph 2).

[3.] *N. spath* = *Nematodirus spathiger*.

Chapter 4 Supplementary data:

Individual *Nematodirus* spp. faecal egg counts for the pre and post BZ treated and untreated groups including the undifferentiated overall efficacy with 95% confidence intervals for the 21 farms used in the study of BZ-resistance in *Nematodirus* spp.

Farm locality Pre date Post date	Overall efficacy (95% CI)	Group	Pre/ Post	Individual FEC														
Ruatoria	0	BZ	Pre	0	0	50	0	0	100	0	50	50	0					
24/02/2011	(0-85)		Post	50	200	0	100	0	100	0	0	50	50					
10/03/2011		Untr	Pre	0	50	0	0	0	100	0	0	0	50					
			Post	50	50	0	50	0	0	0	100	50	100					
Pleasant Point [1]	0	BZ	Pre	0	150	50	0	100	50	50	0	0	0	150	500	50	100	
14/03/2011	(0-71)		Post	0	0	0	100	0	0	50	50	0	0	0	500	0	50	
22/03/2011		Untr	Pre	0	0	0	100	0	350	100	50	0		0	450			
			Post	50	0	50	100	0	0	50	50	100		0	0			
Owhango	10	BZ	Pre	150	200	100	50	300	50	100	300	150	50	0	50			
7/03/2011	(0-73)		Post	150	0	0	0	0	50	50	100	50	150	50	100			
14/03/2011		Untr	Pre	200	0	100	0	0	300	150	50	350	50	150	0			
			Post	50	0	150	0	0	0	50	50	100	0	200	100			
Tolaga Bay	26	BZ	Pre	50	0	0	100	100	0	0	100	0	200	0	0			
5/01/2011	(0-86)		Post	50	0	0	50	50	0	0	0	0	0	100	100			
14/01/11 [*]		Untr	Pre	0	0	50	0	50	0	50	0	50	100	50	0			
			Post	0	0	0	0	100	50	50	0	50	0	0	50			
Gore	39	BZ	Pre	0	150	150	50	150	50	600	0	100	50	500	200			
28/02/2011	(0-81)		Post	50	100	300	0	50	200	350	0	50	0	100	150			
9/03/2011		Untr	Pre	200	0	100	50	0	0	50	250	150	500	50	100			
			Post	450	0	450	100	0	50	0	100	250	100	100	0			
Pleasant Point [2]	48	BZ	Pre	150	0	50	0	50	200	50	0	150	100	400	0	0		
14/02/2011	(0-86)		Post	0	250	100	0	0	0	150	0	100	0	200	0	0		
23/02/2011		Untr	Pre	200	100	0	0	50	250	0	100	100	100					
			Post	250	0	0	350	150	150	0	100	150	50					
Rotorua	56	BZ	Pre	50	0	100	0	50	50	0	0	0	100	0	100			
13/12/2010	(0-93)		Post	0	50	100	0	0	0	0	0	0	0	0	0			
22/12/2010		Untr	Pre	0	50	0	0	100	50	50	0	50	50	0	50			
			Post	50	0	0	0	100	50	0	0	0	0	100	0			
Pio Pio	61	BZ	Pre	100	100	0	0	100	50	0	50	50	0	150	0			
26/01/2011	(0-93)		Post	50	0	100	0	0	100	0	50	0	0	0	200			
07/02/2011 [*]		Untr	Pre	50	100	0	50	0	50	0	0	100	50	0	0			
			Post	50	0	0	150	150	100	100	50	0	100	50	100			
Reporoa [1]	61	BZ	Pre	150	50	0	0	50	0	0	0	100	0	50	0			
29/03/2011 [*]	(0-94)		Post	50	0	0	100	250	0	0	50	300	0	100	0			
05/04/2011 [*]		Untr	Pre	0	50	0	0	0		0	0	50	0	0	0			
			Post	100	0	100	0	0		0	50	100	50	100	50			
Dannevirke	66	BZ	Pre	100	0	250	0	150	0	0	0	0	0	0	0	50	50	0	50
30/12/2010	(0-91)		Post	0	50	300	0	0	0	0	0	0	50	0	0	0	0	0	0
6/1/2011		Untr	Pre	50	100	0	0	0	150	150	0	0	50	0	50	50	50	50	100
			Post	200	0	0	0		50	400	150	0	100	100	50	50	0	200	50

Farm locality Pre date Post date	Overall efficacy (95% CI)	Group	Pre/ Post	Individual FEC														
Reporoa [2]	75	BZ	Pre	50	0	0	0	0	0	0	0	150	200	0	0			
1/02/2011	(0-96)		Post	0	0	0	0	0	0	0	0	50	0	0	50			
8/02/2011		Untr	Pre	50	0	0	0	50	150	50	0	0	50	0	0			
			Post	0	0	0	50	0	200	0	50	0	50	0	0			
Eketahuna	75	BZ	Pre	50	50	100	0	50	0	100	100	0	50	50	50			
7/03/2011	(0-97)		Post	0	0	50	0	0	0	0	0	0	0	0	0			
16/03/2011		Untr	Pre	0	50	100	100	100	50	50	100	100	50	50				
			Post	0	100	50	0	50	0	0	0	50	0	0				
Te Anau [1]	75	BZ	Pre	100	150	0	0	0	100	50	50	50	150	0	300			
4/04/2011	(31-91)		Post	0	50	0	0	0	50	0	0	0	50	0	200			
13/04/2011		Untr	Pre	50	100	100	0	50	0	50	0	0	50	0	0			
			Post	50	150	200	0	0	0	50	100	0	0	50	0			
Havelock North	81	BZ	Pre	0	50	0	0	250	0	0		250	250	50	150			
22/03/2011	(0-99)		Post	0	0	0	0	200	0	0		0	0	0	0			
30/03/2011		Untr	Pre	0		100	50	400	50	100	650	200	0	150	200			
			Post	50		150	50	0	100	0	0	50	50	0	1500			
Outram	85	BZ	Pre		0	100	50	500	150	450	0	0	700	0	400			
9/03/2011	(52-95)		Post		0	0	0	0	50	0	0	0	100	0	50			
16/03/2011		Untr	Pre	150	300		750	650	500	250	0	100	100	100	0			
			Post	0	200		250	200	450	0	50	150	100	200	50			
Palmerston North	88	BZ	Pre	100	1050	150	150	850	0	0	0	0	300	350	100	250	950	50
24/11/2010	(64-96)		Post	0	0	50	0	50	0	50	0	100	50	100	0	0	0	0
3/12/2010		Untr	Pre	600	100	50	650	1300	300	550	200	250	200	650	300	150	350	150
			Post	450	100	100	400	950	350	600	200	250	150	500	0	250	100	200
Hastings	89	BZ	Pre	50	100	50	0	0	0	100	100	50	50	100	50			
21/03/2011	(12-99)		Post	0	0	0	50	50	0	0	0	0	0	0	0			
28/03/2011		Untr	Pre	0	50	0	0	0	0	50	50	0	0	100	0			
			Post	0	0	0	100	0	0	0	50	0	50	150	0			
Culverden	93	BZ	Pre	0	0	0	50	50	0	0	100	0	50	50	300			
11/04/2011	(0-100)		Post	0	100	0	50	0	0	0	50	0	0	0	0			
19/04/2011		Untr	Pre	0	0	0	0	0	150	0	0	0	0	0	0			
			Post	100	0	100	100	0	100	200	0	0	100	0	0			
Balclutha	93	BZ	Pre	0	0	0	150	0	0	50	400	0	0	0	0			
7/03/2011	(0-100)		Post	0	0	0	0	0	0	0	0	0	0	0	50			
16/03/2011		Untr	Pre	250	100	0	200	0	100	50	0	0	0	0	0			
			Post		50	0	150	0	0	100	0	100	400	0	0			
Te Anau [2]	93	BZ	Pre	250	100	0	150	150	150	150	0	200	50	200	50			
21/12/2010	(61-99)		Post	0	0	0	0	0	0	0	0	50	0	0	50			
29/12/2010		Untr	Pre	100	50	100	150	50	250	0	50	300	100	100				
			Post	0	0	300	350	200	150	50	0	0	50	200				
Blenheim	100	BZ	Pre	100	250	200	150	150	0	250	100	0	50	100	450			
9/02/2011*	(nc)		Post	0	0	0	0	0	0	0	0	0	0	0	0			
16/02/2011		Untr	Pre	150	0	50	50	0	100	0	100	100	50	0	100			
			Post	250	200	50	0	0	250	0	0	100	0	0	0			

* date the samples arrived at laboratory, not necessarily the day when they were collected, paper work was incomplete for the collection date.
CI= Confidence interval, (nc) = not calculated, BZ =Animals treated with Benzimidazole, Untr= Animals untreated.

Chapter 4 Supplementary data continued:

The number of larvae identified to *Nematodirus* species using a PCR assay in the BZ treated and untreated groups post treatment after culturing the pooled faeces.

Farm locality	*N. spathiger* in the untreated group	*N. filicollis* in the untreated group	*N. spathiger* in the treated group	*N. filicollis* in the treated group
Ruatoria	32	0	58	0
Pleasant Point [1]	35	0	48	0
Owhango	39	0	46	1
Tolaga Bay	64	1	49	1
Gore	12	14	26	3
Pleasant Point [2]	49	3	53	0
Rotorua	27	19	14	17
Pio Pio	24	23	45	18
Reporoa [1]	6	1	37	0
Dannevirke	25	3	60	0
Reporoa [2]	26	0	46	0
Eketahuna	21	1	7	0
Te Anau [1]	1	1	18	1
Havelock North	36	3	48	0
Outram	47	0	48	0
Palmerston North	14	14	10	9
Hastings	2	1	5	0
Culverden	19	19	44	0
Balclutha	21	1	61	0
Te Anau [2]	49	6	1	0
Blenheim	46	1	0	0

Chapter 5 Supplementary data:

Raw data from the chilling experiment showing the actual numbers of hatched and unhatched *N. filicollis* L3s, at the various temperatures (°C) and time (days) that the eggs were exposed to. After this time all samples were subsequently held at 13°C for 14 days for hatching to occur. Note: the eggs were previously incubated at 20°C to allow for L3 development.

Day	Temp	Well	Hatched	Non-hatched	Total
14	2	1	0	21	21
14	2	2	1	20	21
14	2	3	0	20	20
14	2	4	0	21	21
14	4	1	0	38	38
14	4	2	0	26	26
14	4	3	0	20	20
14	4	4	0	33	33
14	5	1	0	26	26
14	5	2	0	18	18
14	5	3	0	24	24
14	5	4	0	30	30
14	6	1	0	30	30
14	6	2	0	28	28
14	6	3	0	26	26
14	6	4	0	34	34
14	8	1	0	24	24
14	8	2	0	12	12
14	8	3	0	24	24
14	8	4	0	20	20
14	10	1	0	27	27
14	10	2	0	29	29
14	10	3	0	20	20
14	10	4	0	24	24
28	2	1	0	24	24
28	2	2	0	32	32
28	2	3	1	25	26
28	2	4	0	27	27
28	4	1	0	42	42
28	4	2	0	18	18
28	4	3	0	22	22
28	4	4	0	18	18
28	5	1	1	18	19
28	5	2	0	35	35
28	5	3	2	26	28
28	5	4	0	12	12
28	6	1	1	26	27
28	6	2	1	22	23
28	6	3	0	26	26
28	6	4	0	30	30
28	8	1	1	24	25
28	8	2	1	25	26
28	8	3	0	23	23
28	8	4	0	23	23
28	10	1	1	24	25
28	10	2	0	34	34
28	10	3	0	25	25
28	10	4	0	31	31
42	2	1	3	19	22
42	2	2	1	19	20
42	2	3	2	23	25
42	2	4	2	21	23
42	4	1	1	19	20
42	4	2	0	26	26
42	4	3	0	22	22
42	4	4	0	22	22
42	5	1	0	25	25
42	5	2	1	24	25
42	5	3	0	28	28
42	5	4	1	25	26
42	6	1	1	36	37
42	6	2	0	29	29
42	6	3	0	25	25
42	6	4	1	32	33
42	8	1	0	25	25
42	8	2	0	27	27
42	8	3	0	33	33
42	8	4	0	15	15
42	10	1	1	32	33
42	10	2	0	26	26
42	10	3	0	28	28
42	10	4	0	24	24
56	2	1	0	23	23
56	2	2	2	26	28
56	2	3	0	24	24
56	2	4	1	19	20
56	4	1	1	26	27
56	4	2	0	26	26

Day	Temp	Well	Hatched	Non-hatched	Total
56	4	3	0	28	28
56	4	4	1	20	21
56	5	1	0	38	38
56	5	2	3	22	25
56	5	3	2	25	27
56	5	4	0	26	26
56	6	1	0	21	21
56	6	2	1	21	22
56	6	3	1	32	33
56	6	4	1	19	20
56	8	1	0	26	26
56	8	2	1	15	16
56	8	3	0	19	19
56	8	4	0	25	25
56	10	1	0	27	27
56	10	2	0	30	30
56	10	3	1	22	23
56	10	4	0	29	29
70	2	1	3	18	21
70	2	2	1	15	16
70	2	3	1	29	30
70	2	4	1	25	26
70	4	1	3	26	29
70	4	2	1	26	27
70	4	3	0	23	23
70	4	4	0	28	28
70	5	1	0	29	29
70	5	2	0	31	31
70	5	3	1	28	29
70	5	4	3	35	38
70	6	1	0	20	20
70	6	2	3	28	31
70	6	3	3	29	32
70	6	4	1	35	36
70	8	1	1	20	21
70	8	2	0	17	17
70	8	3	1	28	29
70	8	4	1	30	31
70	10	1	0	30	30
70	10	2	1	14	15
70	10	3	2	26	28
70	10	4	0	25	25
84	2	1	3	28	31
84	2	2	2	27	29
84	2	3	1	31	32
84	2	4	1	22	23
84	4	1	1	25	26
84	4	2	0	24	24
84	4	3	1	35	36
84	4	4	4	27	31
84	5	1	1	22	23
84	5	2	1	20	21
84	5	3	2	19	21
84	5	4	1	29	30
84	6	1	0	23	23
84	6	2	0	21	21
84	6	3	3	23	26
84	6	4	2	30	32
84	8	1	1	27	28
84	8	2	1	21	22
84	8	3	0	27	27
84	8	4	0	18	18
84	10	1	0	32	32
84	10	2	0	19	19
84	10	3	0	27	27
84	10	4	1	21	22
112	2	1	4	15	19
112	2	2	3	24	27
112	2	3	3	20	23
112	2	4	1	19	20
112	4	1	2	23	25
112	4	2	2	20	22
112	4	3	3	18	21
112	4	4	1	13	14
112	5	1	2	23	25
112	5	2	1	18	19
112	5	3	2	27	29
112	5	4	2	36	38
112	6	1	3	22	25
112	6	2	1	31	32
112	6	3	4	19	23
112	6	4	3	24	27
112	8	1	1	28	29
112	8	2	0	27	27
112	8	3	1	26	27
112	8	4	0	26	26
112	10	1	0	29	29
112	10	2	1	19	20
112	10	3	1	21	22
112	10	4	0	20	20
140	2	1	1	23	24
140	2	2	2	15	17
140	2	3	2	24	26
140	2	4	1	25	26

Day	Temp	Well	Hatched	Non-hatched	Total
140	4	1	4	25	29
140	4	2	9	22	31
140	4	3	5	15	20
140	4	4	1	15	16
140	5	1	7	25	32
140	5	2	9	23	32
140	5	3	4	19	23
140	5	4	5	16	21
140	6	1	2	15	17
140	6	2	6	16	22
140	6	3	2	20	22
140	6	4	2	19	21
140	8	1	0	16	16
140	8	2	3	21	24
140	8	3	1	15	16
140	8	4	2	32	34
140	10	1	4	17	21
140	10	2	3	16	19
140	10	3	6	20	26
140	10	4	5	17	22
168	2	1	1	28	29
168	2	2	2	13	15
168	2	3	3	18	21
168	2	4	0	24	24
168	4	1	1	34	35
168	4	2	5	23	28
168	4	3	6	25	31
168	4	4	2	20	22
168	5	1	2	23	25
168	5	2	2	27	29
168	5	3	2	16	18
168	5	4	1	32	33
168	6	1	0	17	17
168	6	2	2	20	22
168	6	3	0	25	25
168	6	4	4	21	25
168	8	1	0	23	23
168	8	2	4	27	31
168	8	3	0	18	18
168	8	4	1	14	15
168	10	1	4	25	29
168	10	2	4	23	27
168	10	3	3	17	20
168	10	4	2	19	21
196	2	1	2	17	19
196	2	2	0	27	27
196	2	3	2	34	36
196	2	4	5	20	25
196	4	1	3	21	24
196	4	2	4	31	35
196	4	3	5	30	35
196	4	4	2	18	20
196	5	1	1	24	25
196	5	2	1	28	29
196	5	3	3	29	32
196	5	4	0	16	16
196	6	1	4	18	22
196	6	2	2	23	25
196	6	3	1	16	17
196	6	4	4	23	27
196	8	1	0	20	20
196	8	2	1	21	22
196	8	3	0	25	25
196	8	4	1	34	35
196	10	1	1	20	21
196	10	2	0	20	20
196	10	3	2	28	30
196	10	4	1	19	20
224	2	1	1	23	24
224	2	2	0	16	16
224	2	3	3	30	33
224	2	4	2	26	28
224	4	1	3	27	30
224	4	2	4	17	21
224	4	3	2	21	23
224	4	4	4	19	23
224	5	1	2	24	26
224	5	2	4	31	35
224	5	3	3	19	22
224	5	4	2	19	21
224	6	1	3	24	27
224	6	2	1	17	18
224	6	3	5	33	38
224	6	4	5	22	27
224	8	1	2	22	24
224	8	2	0	32	32
224	8	3	4	31	35
224	8	4	1	24	25
224	10	1	3	22	25
224	10	2	2	17	19
224	10	3	3	18	21
224	10	4	3	24	27